Development of marine fish larval diets to replace *Artemia*

PART 1

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Development of marine fish larval diets to replace *Artemia – Part 1*

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NON-TECHNICAL SUMMARY

Outcomes Achieved to Date

The project benefits and outcomes can be divided into four sections (as per objectives): 1) System development, 2) Microdiet development, 3) Feeding protocols, and 4) *Artemia* production and enhancement. In general, outcomes and benefits will (and in fact already) flow to marine hatcheries (for finfish and other marine organisms), commercial and R&D aquaculture centres as well as other industry sectors.

System development

The development of the larvae rearing system, and the live food enrichment system adjoined with it, involved innovative solutions in terms of automated hatchery systems. Some parts of the systems have already been adopted by industry. It is envisaged that commercialisation of these systems will be progressed depending on demand.

The outcome of this activity is a better controlled environment for marine larvae and reduced maintenance time and manpower, resulting in more efficient commercial and research hatcheries.

Microdiet development

Several different microdiet preparation methods were tested during the project as well as different nutritional parameters. Some of the experimental diets match or even outperform commercially available diets and, therefore, present a commercial opportunity for microdiets that can be tailor-made to specific species and/or conditions. Moreover, characterising the chemical and physical parameters such as leaching and sinking rates enables recommendations to be made to hatcheries. Comparing commercial diets also resulted in specific recommendations to hatcheries such as mixing two different diets, one with higher attractability and the other with a better nutritional profile.

These recommendations resulted in optimising food and feeding practices for commercial hatcheries, leading to better growth and survival of larvae and reducing tank fouling by inert diets.

Feeding protocols

The development of co-feeding and weaning protocols was done in close collaboration with industry (WA). Strong links were developed with the Darwin Aquaculture Centre (DAC), NT (the largest barramundi hatchery in Australia) and Skretting Australia, TAS (the largest fish feed manufacturer in Australia). Feeding protocols were developed, validated and adopted with strong input from DAC. Based on this collaborative work, co-feeding protocols allow a reduction of *Artemia* use by 90% through to its complete elimination from the protocol.

The outcome of this activity is optimised utilisation of microdiets by the larvae and reduced reliance on live food with minimal or no effect on larval performances.

Artemia production and enhancement

Strong links were established with Cognis Australia Pty Ltd in relation to *Artemia* production. Pilot-scale grow-out trials were promising. Based on these results Cognis, together with FRDC and Department of Fisheries, Western Australia, are now investing in a new project aimed at commercialisation of *Artemia* production (FRDC 2004/238). Several products were developed, some of which are new, and are already commercially available in Australia.

The outcome of this activity is local *Artemia* (cysts and biomass) becoming available in Australia so that imported *Artemia* can be replaced thereby reducing the risk of supply shortage and introduction of unknown pathogens.

Commercially available live food enrichments were surveyed in terms of nutritional value as well as bacterial loads. Tailor-made enrichments as well as broodstock additives were manufactured for specific sectors and found to be effective.

The outcomes of this activity have been (1): A change in manufacturing processes for one major international commercial enrichment product, and (2): The local availability of specific products designed for specific fish species and/or addressing specific nutritional and/or physiological issues, rather than use of off-the-shelf general products. Commercial production has been established to support this service.

This project was initiated based on recommendations and R&D priorities as identified at the First Hatchery Feeds Workshop (Cairns, QLD 1999). Several aspects were found to have high priority, such as: systems, *Artemia* availability and its nutritional quality, microdiets to reduce and/or replace reliance on *Artemia*, and feeding and weaning protocols. It was recognised that these areas were 'bottle necks' in the further development of marine aquaculture, especially with new species. These high priority aspects were addressed in this project, which exceeded its objectives. Its outcomes include, among others, products and systems that are already commercially available, and laying the foundation to commercialisation of at least two new products / organisms, i.e. brine shrimp *Artemia* culture in Australia and larvae microdiets.

The development of the larvae rearing system and the associated live food enrichment system involved innovative solutions in terms of automated systems, dosing and feed delivery systems, and filtration. These systems allow better control and save time and money. Some parts of the systems have already been adopted by industry. For example, the tank design is currently being evaluated at the M.G. Kailis, Exmouth hatchery. The innovative microdiet feeding system is currently being installed at the Tasmanian Aquaculture and Fisheries Institute in their larvae tanks. The larvae rearing system and/or other specific systems can benefit any R&D centre involved in marine larvae rearing as well as commercial hatcheries. It is proposed that these systems will be progressed as a commercial product, depending on demand.

Brine shrimp *Artemia* is one of the most commonly used and suitable live feeds and is considered to be obligatory for the early life stages in fish and crustacean aquaculture. Fluctuations in quantities and qualities of *Artemia* cysts make its supply and nutritional quality unpredictable and, at a time of crisis (every few years due to El Nino), have placed considerable pressure on the aquaculture industry and could limit future prospects for expansion and development. The current project inspected potential production sites in Western Australia and found that Hutt Lagoon, a hyper saline lagoon was suitable in terms of site and cysts quality. Strong links were established with Cognis Australia Pty Ltd, (the company that harvests *Dunaliella salina,* red micro algae from Hutt Lagoon, for extraction of beta-carotene), in relation to *Artemia* production. Pilot-scale grow-out trials proved to be promising and led to the development of a new FRDC project, in which the company and the Department of Fisheries, Western Australia (DoFWA) are also now investing in commercialisation of *Artemia* production. Several products were developed, some of which are new, and are now commercially available in Australia.

Commercially-available live food enrichments were surveyed in terms of nutritional value and bacterial loads. Tailor-made enrichments as well as broodstock additives were manufactured for specific fish species and other marine organisms (including striped trumpeter, mahi-mahi, rock

lobster phyllosoma, and other aquaculture species) and these contributed to the understanding of the nutritional requirements and physiology of these species.

Several microbiology studies were carried out addressing some of the major problems with larvae – diseases and pathogens. The bacterial loads in *Artemia*, enrichments, rearing medium, and larvae were assessed and the role of enriched *Artemia* as a vertical carrier was investigated. The results contributed to optimised live food and larvae rearing protocols in commercial hatcheries as well as improved quality control with some commercial products.

Successful use of microdiets depends on their physical and nutritional characteristics and the way they are used e.g. feeding strategies and whether they are used in combination with *Artemia*. Several different microdiet preparation methods as well as different nutritional parameters were evaluated during the project. Chemical and physical parameters were identified for different diet particles, both commercial and experimental. Some of these diets match or outperform commercially available diets and therefore present a commercial opportunity for microdiets that can be tailor-made to specific species and/or conditions. Moreover, characterising the chemical and physical parameters such as leaching and sinking rates enable recommendations to be made to commercial hatcheries for specific feeding methods and rates, e.g. small amounts in short intervals for fast sinking diets. Comparing commercial diets also resulted in specific recommendations to hatcheries such as mixing two different diets, one with higher attractability and the other with a better nutritional profile. These recommendations resulted in optimising food and feeding practices in commercial hatcheries leading to better larvae growth and a reduction in tank fouling caused by inert diets. Microdiet development is an international priority and considerable practical international collaboration occurred as originally proposed for this project.

The development of co-feeding and weaning protocols was done in close collaboration with industry (Western Australia). Strong links were developed with the Darwin Aquaculture Centre (DAC), Northern Territory (the largest barramundi hatchery in Australia) and Skretting Australia, Tasmania (the largest fish feed manufacturer in Australia). Feeding protocols were developed, validated and adopted with strong input from DAC. Based on this collaborative work, co-feeding and weaning protocols were developed with a reduction of *Artemia* use by 90% through to its complete elimination from protocols.

As part of the project the PI organised a national workshop titled 'The Second Hatchery Feed and Technology Workshop'. The workshop was held in Sydney on 30 September and 1 October 2004, in conjunction with the Australasian Aquaculture Symposium and focused on recent developments in both industry and research as related to new species, feeds and technologies. The proceedings from the workshop give a current snapshot on hatchery technology and R&D across species in Australia. An R&D plan for 2005-2010 was drafted and is being finalised after consulting with stakeholders. It is envisaged that this R&D plan will help research investment agencies in prioritising R&D needs in this important area. Numerous international presentations were given and many scientific publications were prepared although the publication of some will be deferred due to commercial confidentiality.

Throughout the project life, the PI established close links with many industry partners, stakeholders and companies within WA as well as around Australia. A 'service centre' was established through the project where the Department of Fisheries, Western Australia supplied, free-of-charge, special live food enrichments and broodstock additives to private and government hatcheries. Subsequently, this service was commercialised, where appropriate.

Keywords: Fish larvae, *Artemia*, brine shrimp, microdiet, systems, protein, lipids, enrichments, feeding.

1.0 ACKNOWLEDGEMENTS

Challenger TAFE, Aquaculture Development Unit staff are thanked for their on-going help and assistance during the project with special thanks to Mr Bruce Gibney on his advice during the development of the larvae system. Darwin Aquaculture Centre (DAC) provided barramundi eggs and concentrated chlorella free of charge. Special thanks to Mr Jerome Bosmans from DAC for his assistance and advice in developing the co-feeding protocols and Skretting Australia for their assistance with microdiets. Dr Greg Maguire, Department of Fisheries, WA provided support, valuable comments and reviews throughout the project. Special thanks to John Heine for editing and proofing and Sandy Clarke for formatting and putting together this publication.

2.0 BACKGROUND

Marine finfish farming in Australia has been dominated by salmonids and wild caught southern bluefin tuna. However, barramundi farming is growing rapidly and should experience quantum increases of production, as very large NT and WA projects come on line. Snapper and yellowtail kingfish are established or emerging species. Researchers and industry are developing technology for a range of other species including groupers, lutjanids, whiting, striped trumpeter, dhufish and a variety of ornamental fish. Aquaculture or fisheries enhancement projects with these species are threatened if larval rearing costs are excessive.

One major problem in intensive culture of fish is nutrition during the early life stages. A major cause of mortality is inadequate food supply either by quantity or quality. Live food has been employed for culturing the early life stages of marine fish and is currently obligatory for successful culture past metamorphosis when the fish are weaned onto dry formulated diets. The main organism used as ‘live food’ in the aquaculture industry is the brine shrimp *Artemia* sp. On October 27th 1997, the Utah Division of Wildlife Resources (U.S.A) ordered an emergency closure of the Great Salt Lake (GSL) brine shrimp egg harvest, due to the threat of over-harvesting and the poor commercial quality of brine shrimp eggs. Since 80% of the world brine shrimp production originates from GSL, this sent a shock-wave through world aquaculture and resulted in increased production costs for both fish and shrimp post-larvae. Since 1997, GSL *Artemia* harvests have not recovered and have dropped dramatically. The 1999-00 harvest season supplied only 20% of world demand, leading to a world-wide shortage. In 2002, 1 kg of *Artemia* cysts cost $AU400-500, however then, even at these high prices, it was almost impossible to obtain them in Australia.

On this basis, any decrease in the use of imported *Artemia* in finfish hatcheries, would be economically advantageous. Three potential alternatives to the provision of imported *Artemia* are: (1) **microdiets** (i.e. dry, semi-dry or suspended micro-particles) which can represent a considerable saving in production costs and infrastructure as well as offering nutritional consistency and off-the-shelf convenience; (2) **‘local’ *Artemia*** that may present an Australian source of *Artemia* cysts; and (3) **alternative live food** such as copepods, that still require production facilities but represent better nutritional value (Stottrup 1999), reduced operational costs and global abundance.

2.1 MICRODIETS

Currently, microdiets cannot usually produce the growth and survival shown by fish fed live feeds such as rotifers and *Artemia* nauplii. It has become increasingly clear that the dietary needs, both quantitatively and qualitatively, of growing larvae are distinctly different from those of adult fish. In general, this stems from the fact that during larval development, the digestive tract is still forming

and is not totally functional. This leads to insufficient digestion and assimilation of dry microdiets. Recently, encouraging results for the use of microdiets as complete replacements of *Artemia* with clownfish were found by the PI (Kolkovski pers. com.)

The processes used to produce artificial larval diets were reviewed by Langdon et al. (1985). They can be divided into three major categories: microbound and microcoated diets (MBD), and microencapsulated diets (MED). The three forms have been used with varying degrees of success in supporting growth and survival of marine fish larvae. Artificial food particles must meet certain criteria: (1) **Acceptability** - particles must be of the correct size for ingestion, available in the water at a similar density to live feed and ingested at a similar rate; (2) **Stability** – formulated diets must remain stable with minimal leaching loss and particle breakdown until ingested; (3) **Digestibility** - diets must be as digestible as live prey and readily assimilated; (4) **Nutritional content** - must satisfy the nutritional requirements of the larvae (availability of essential nutrients must be similar to that of live prey organisms).

MBD include finely ground particles which are set into a gelled matrix. Although MBD have frequently been inadequate to support growth to metamorphosis in larval fish (Garatun-Tjeldsto et al. 1989), there have been studies where MBD yielded better survival than live feeds (Kanazawa et al. 1989). More recently, promising results were reported by Kolkovski et al. (1997) using co-feeding of *Artemia* and MBD for seabass larvae. Growth of larvae fed dry and live diets was significantly higher than larvae fed each diet alone.

Microencapsulated diets are composed of a core material (usually a mixture of nutrients) encapsulated with a shell or capsule wall. The capsule wall, in principle, is able to provide a barrier that prevents or at least minimises leaching of active compounds when microcapsules are immersed in water. Microcapsules with a hydrophobic lipid or water-insoluble polymer shell can retain water-soluble components of a diet when the capsules are immersed in water provided the shell has mechanical properties able to resist the osmotic pressure gradient that forms. The problem of producing suitable microcapsules is confounded by the need to have a capsule shell that changes its barrier properties when a target organism takes up the capsules so that the capsule contents are released. This can occur if the capsule shell biodegrades to some degree in the gut of the fish due to enzymatic attack. Diverse ranges of encapsulation technologies and shell materials have been investigated (Thies 1994, 1997). In many cases, the material encapsulated is a complex mixture of water-soluble and water-insoluble nutritional ingredients.

Microcapsules offer the advantages of controlling the leaching of attractant molecules (such as amino acids) while preventing leaching of important soluble nutrients (Jones et al. 1987). Microcapsules can be used as delivery vessels that cannot only carry nutrients but antibiotics, hormones, enzymes and other substances. To obtain sufficient incorporation of these substances into the larval body, larvae are usually incubated in solutions containing these substances. The use of microcapsules that are consumed by fish larvae can significantly reduce the amount of these substances given to the larvae and released to the environment.

Few species of fish have been successfully reared from first feeding exclusively on artificial diets and in most cases, success at the experimental level has yet to be reproduced on a commercial scale. In general, fresh water fish larvae are fairly large (4-10 mm) at hatching and can adapt to dry feeds relatively easily. This is particularly true for most of the salmonids, which possess a functional stomach at first feeding. Among other freshwater species, the most promising results have been achieved with coregonid larvae (Dabrowski et al. 1991). Other freshwater species, which have been reared on artificial diets, include: the common carp *Cyprinus carpio* (Charlon et al. 1986), the ayu, *Plecoglossus altivelis* (Kanazawa et al. 1985) and smallmouth bass *Micropterus dolomieu* (Ehrlich et al. 1989). However, for marine fish, many of which hatch with small yolk reserves and feed at an early stage (2-3 mm length), success with total replacement of conventional live feeds

has been extremely limited (Tandler and Kolkovski 1992, Kolkovski and Tandler 1997, Southgate & Partridge, 1998).

2.2 ALTERNATIVE FEEDS

Different alternative live feed organisms have been explored over past years. These include, wild zooplankton, *Moina* sp., *Parartemia* and copepods. Currently, none of these organisms are commercially available. Copepods are considered to be the best live food in terms of nutritional value, to the fish larvae. They have been used to complement rotifers prior to the use of *Artemia*, in the culture of specific fish species such as dhufish (WA), lutjanids (NT), cods and groupers (Qld), however, mass culture of copepods, at this stage, is not commercially feasible.

2.3 LOCAL *ARTEMIA*

Western Australia has significant salt and saline algae industries and some of these have the potential for *Artemia* cysts harvesting and *Artemia* rearing. The Port Gregory lagoon has a natural bloom of *Dunaliella salina* and *Artemia* sp. with an estimate of potential production of 500-1000 kg cysts per annum. Dampier Salt is the largest salt producer in the southern hemisphere. The production in the salt fields relies on introduced strain of *Artemia* from San Francisco Bay (*Artemia fransiscana*) to maintain high water clarity and reduce algae contamination. The production technology for this strain has been developed as a pilot scale at Dampier (Tyler 1996).

Lake Macleod (Dampier Salt) has large seasonal blooms of native *Parartemia* sp. that may be harvested as live or frozen larval food.

Cargill Salt (the world's largest salt producing company) WA field, in the Pilbara Region also has incidental production of *Artemia* and dedicated ponds that may be used as *Artemia* production ponds.

The suitability of these *Artemia* strains has not been adequately evaluated for marine finfish. In the salt fields, cyst production typically fluctuates greatly through time. Also, low hatching rates have been a problem with some batches from salt and algae fields, possibly due to harvesting methods. The harvesting of the salt fields *Artemia* cysts may provide a shorter-term solution to the current shortage of cysts and needs to be investigated, especially given high current market prices and the global shortage. Department of Fisheries, Western Australia will continue efforts to facilitate harvesting of cysts at these locations (and at Shark Bay), and where possible commercial trials with specialised farming of *Artemia* will be fostered.

3.0 NEEDS

The major problem area in the cultivation of marine fish is the culture of the early life stages, and control of larval nutrition is a key element. The use of live food for hatchery culture of marine finfish larvae is currently considered obligatory for successful culture. However, their use is costly, especially during recent years where global harvests of *Artemia* cysts have decreased sharply leading to a global shortage. To produce 10,000 snapper or barramundi juveniles (50 days old) past metamorphosis, 1.5 kg of *Artemia* cysts is needed. The production cost of *Artemia* nauplii is 10 cents / juvenile (Frankish, pers. com.), which includes *Artemia* cysts, enrichments, labor and running costs (e.g. heaters, air etc.). In 2000, 1 kg of *Artemia* cysts cost $AU 400-500, however, then, they were almost impossible to obtain in Australia. Replacing even 50% of imported *Artemia*

cysts may result in substantial cost savings leading to more efficient hatchery production and facilitating industry expansion.

The FRDC R&D plan for hatchery feeds (the outcome from the 1999 FRDC hatchery feeds workshop, Cairns, Qld.) put a high priority on R&D projects to find local solutions to overcome the '*Artemia* crisis' and reduce dependence on imported *Artemia* cysts. More specifically, it emphasised three particular research needs: (1) to assess the potential of Australian *Artemia* strains; (2) to determine the effectiveness of currently available artificial diets for finfish larvae; and (3) to develop 'local' artificial diets and protocols for weaning and co-feeding of live and dry diets. The R&D priorities (FRDC plan) for *Artemia* and artificial diets are shown in Appendix 3.

4.0 OBJECTIVES

i) To develop a standard testing system for evaluating live and artificial feeds for finfish larvae.

ii) To test currently available artificial (commercial) larval diets.

iii) To formulate artificial larvae diets.

iv) To assess the use of 'local' *Artemia* and improve their nutrition value.

v) To develop the use of co-feeding live and dry diets for partial or full replacement of *Artemia* nauplii.

All of these objectives were met and indeed the outcomes in most cases substantially exceeded the original objectives.

A flow diagram summarising the project activities (as submitted in the proposal) and outcomes is attached as Appendix 4.

5.0 DEPARTMENT OF FISHERIES, WESTERN AUSTRALIA (and collaborative partners)

5.1 SYSTEMS DEVELOPMENT

5.1.1 Intensive rearing system for fish larvae research – I. marine fish larval rearing system

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5.1.1.1 Abstract

Larvae nutrition and in general larvae culture are considered to be the 'bottle neck' for marine finfish culture. Fish larvae rearing experiments investigating nutritional factors or rearing protocols are carried out in various systems, from small beakers to very large commercial tanks, making it difficult to compare data across systems.

A continuous supply of live or dry feeds and a controlled environment i.e. temperature, filtration, photoperiod, oxygen and pH, are essential for any experimental or commercial system. These environmental factors are best controlled automatically in order to minimize variations between tanks. However, only a few automatic systems have been developed for marine finfish hatcheries.

An experimental larval rearing system was developed to reduce variability amongst tanks (due to manual feeding and other parameters) and enhance control of environmental parameters while reducing the workload. The system includes twenty-four conical tanks with the option of either an upwelling or bottom draining flow through water delivery system. The inlet water passes through a gas exchange column that saturates the water with dissolved oxygen and stabilizes the pH. The system was originally designed for nutritional experiments using formulated feeds. The use of an upwelling water inlet method extends the suspension time of inert particles in the water column and also helps to suspend very small or passive swimming larvae. However, when the system is used to grow-on larvae or juvenile fish it can easily be switched to bottom draining to provide self-cleaning water dynamics for high organic loads.

A unique outlet filter was developed that eases the daily routine of replacing screens when enriched live food is used. This filter can be exchanged with a screened standpipe and outlet surface skimmer when the bottom draining flow characteristics are engaged.

The system is fully controlled by a single programmable logic controller (PLC). The PLC controls the light intensity, photoperiod, dimming time, live food and algae pumping intervals, substantially reducing labor requirements.

Keywords: Fish larvae, rearing system, diet, experimental system

5.1.1.2 Introduction

It is generally accepted that the larvae stage of marine fish production is the major 'bottle neck' in the development of mariculture (Tucker, 1998). More specifically, nutrition is considered to be one of the most important issues in marine larvae research (Cahu and Zambonino, 2001; Koven et al. 2001). Considerable research has been carried out during the last few decades to identify nutrient requirements for fish larvae (Kolkovski and Dabrowski, 1999). Microdiets and live food

replacements are being trialled with many fish species (Kolkovski et al. 1997; Rosenlund et. al. 1997; Baskerville-Bridges and Kling, 2000). This type of work is carried out in many different systems from small beakers to very large commercial tanks, however, data generated from different systems is sometimes hard to compare. Moreover, in many cases, variation among the tanks within a given system is high. This is due to factors such as differences in flow rates, amounts of live and dry food administered, concentration of algae (in 'green-water' systems), temperature variation, and lighting. In many experimental systems manually feeding the fish larvae is not only labor intensive and time consuming (Papandroulakis et al. 2002), but also contributes to variation due to uneven food rationing between the tanks, for example the last tank usually gets the food from the bottom of the bucket.

In order to achieve relevant research, one of the factors to consider when designing an experimental system is the tank volume (Tucker, 1998). A balance is needed between more economical 'laboratory' size tanks and aquaria (low volume), that may permit a large number of replicates with a small number of larvae and low amounts of food that give more powerful statistical analysis, and commercial size tanks that give the advantage of 'real time' relevant and field constraint experiments. However, commercial large-volume tanks usually need high numbers of larvae and large amounts of live food, algae and diets. These resources are sometimes restricted, especially in institutes that do not have access to brood stock and/or large live food production facilities.

A solution to achieving more precise monitoring and reducing the variation among systems and tanks within experimental systems is the automation of control over environmental parameters and feeding. However, only a few automatic systems have been developed and described for marine hatcheries (Papandroulakis et al. 2002).

An experimental larvae culture system was developed to account for the factors mentioned above and to provide a system that is balanced between large commercial tanks and laboratory size tanks. The system was designed to be flexible and provide a reliable platform for a variety of experiments ranging from nutrition trials, rearing and weaning protocols using live and/or dry diets, to manipulating environmental factors such as light and temperature regimes. However, it is not restricted to this area of research but is suitable for a wide range of other experiments concerning any aspect of larvae culture.

5.1.1.3 Methods

General Layout

The fish larvae rearing system consists of twenty-four 270 l, temperature controlled, upwelling or bottom draining, flow through conical tanks with automated lighting, and algae and live feeds delivery systems. The tanks are arranged in three rows of eight. A Fiber Reinforced Plastic (FRP) grate walkway, 700 mm above the floor, between the rows allows the general maintenance of the tanks. The walkway sections are removable for easier access at the time of harvesting or tank maintenance.

Four 1,000 L conical tanks are located near the larvae rearing system to supply algae and live food. This tank placement was designed according to the availability of space. However, different arrangements of live food tanks are possible based on space, needs and finance.

5.1.1.4 Component design

Inlet water system

The inlet water system is based on a non-pressurized gravity fed ring system. The marine bore water (34‰) is pumped into a 3000 L sump from the main hatchery supply line (Fig. 1 & 2). The

water from the sump is then pumped through a set of disk filters (Arkal® filtration system) with nominal filtration of 25 µm and then a heater / chiller unit (Aquahort, 7 kW) able to adjust the water temperature to between 16°C and 28°C at a flow rate of 2 L min^{-1} $tank^{-1}$. The water then flows via a 50 mm PVC pipe ring for even distribution. The main ring is situated above the system. A sub-manifold ring of 25 mm PVC pipe is located between the main 50 mm ring and the tanks, which then supplies the water to each tank (Fig. 1 & 2). The sub-manifold ring allows easier maintenance and water flow control during the day-to-day work over a direct connection to the main 50 mm ring. It also presents substantial cost savings due to 50 mm PVC pipe and fittings being more expensive than 25 mm. The excess water from the sub-manifold and main ring then overflows back to the sump via an 80 mm PVC return pipe thus allowing water to be continually passed through the heater - chiller creating a greater thermal mass heated to the required temperature and saving water and energy when the water is heated. The return pipe is at the highest point of the system, which prevents air locks.

Since the water system was designed as a non-pressurized system with an open-end return, the flow rates for individual taps are not affected by changes in flow rate of other taps.

Currently only one temperature regime is possible for all the tanks due to the use of only one heater – chiller (or ambient water). However, with the use of multiple outlet heater – chillers connected to several water lines it is possible to have the advantage of several temperature regimes in the system.

Tanks

The tanks are conical and made from FRP with a smooth isothalic gel-coat inside to prevent osmosis. The inside wall is dark ('royal') blue with white conical bottom. The combination of blue and white allows the observation of larvae and sediment accumulation on the bottom, while preventing stress and clinging behavior when a clear water method is being used (Bristow et al. 1996, Naas et al. 1996). Each tank is self-supporting on three legs made from FRP reinforced 90 mm PVC pipe. The height of the tank is such that a 10 L bucket can be comfortably placed under the bottom valve; therefore the ball valve is 250 mm above ground level (Fig. 2 & 3).

A clear Perspex® inlet gas exchange column is situated on the side of each tank, hanging on the rim, with a 25 mm tube connecting the bottom of the column to a valve and then to a tee piece directly below the bottom tank fitting. A branch is taken from above the valve up to the lip of the tank for surface inlet water. The valve is turned off for bottom draining and on for upwelling water flow. Another 25 mm tube connects the tee piece at the bottom of the tank via a valve to the outlet at the top of the tank 50 mm below the rim. This valve is open for bottom draining and closed for upwelling water flow. Below the tee at the bottom of the tank is a ball valve, which allows for water and sediment accumulating on the tank cone to be flushed from the tank.

When the tank is in upwelling mode the flow-through characteristics involve water flowing into the gas exchange column at a nominal rate via a 12 mm flexible pipe supplied from the 25 mm pipe ring. It exits the column through the base via a connecting tube to the base of the larvae tank thereby creating an upwelling effect. Overflow drainage from the tank is through a removable outlet filter box with interchangeable screens fitted into a 32 mm socket on the inside of the tank 50 mm below the rim (Fig. 2 & 4). Flexible 25 mm tubing is connected to a 32 mm outlet on the outside of the tank by a tee piece, in order to direct overflow water into a floor drain channel. The upwelling water system causes water movement without 'dead-spots' in the tank and ensures oxygenation and dispersion of live and dry food (Backhurst et al. 1989) with minimum turbulence in the water column.

When the tank is in bottom draining mode the inlet valve at the bottom of the tank is closed and the water is directed to the surface via the branched tube (Fig. 3). The wastewater can either flow entirely through a 1mm mesh screened stand pipe to the bottom outlet or can be partially directed to the top outlet for surface skimming by adjusting the outlet valve at the bottom of the tank. In this case the filter box is exchanged for an overflow skimmer, which is made from an end capped PVC pipe with a 3mm slot cut at the water surface. The slot has 1mm mesh in order to prevent smaller larvae from being washed from the tank. On the opposite side of the skimmer pipe to the slot is a piece of PVC sheet attached diagonally to the pipe with perforated airline at the bottom of the plate. When air is passed through the airline, air bubbles move up and under the plate which creates a current directing surface water around the tank and towards the overflow outlet.

Gas Exchange Columns

The gas exchange columns are 900 mm long and are made from 80 mm diameter clear acrylic tubing. An upper Perspex bracket is welded onto each column 300 mm from the top, in order to hang it on the lip of the tanks, and a lower bracket, 500 mm below the first, holds the columns in a vertical position away from the side of the tank. The base of the column has a flat PVC bottom plate to which a PVC faucet adaptor is joined. A polypropylene barbed director is then screwed into the PVC faucet adaptor to enable a 25 mm clear tube to be connected to the column. This tube is then connected to a surface inlet tube and a threaded branch tee for bottom inlet, which is screwed into a 32 mm PVC faucet tee at the base of the tank.

In order for gas exchange to take place efficiently, bio-balls are packed loosely into the column with a fine air diffuser (20-40 µm pore size) below them (Colt and Bouck, 1984; Colt and Tomasso, 2001). The water flow is in the opposing direction to the rising air bubbles and therefore allows maximum contact between the water and the air bubbles.

The gas exchange column is extremely efficient in saturating the inlet water with oxygen (from 43% ± 2% to 103% ± 2% at 20°C). It also removes other gases such as carbon dioxide (Piedrahita and Grace, 1994) which increases the water pH (pH 7.6 to 7.95) (marine bore water used for the system has low dissolved oxygen and contains high levels of CO_2 resulting in low pH) and/or nitrogen to prevent super saturation and gas bubble disease in the larvae (Colt, 1986; Cornacchia and Colt, 1984) prior to reaching the tank. This eliminates the need for vigorous aeration inside the tank, which may stress fragile fish larvae.

When ocean water or recirculating system water with a high dissolved organic load is used, the column can operate as a protein skimmer. In this instance, vigorous aeration is used within the column and the overflow bubbles/water/organics are drained off the top of the column and removed from the system.

Outlet Screen Filter

The outlet filter boxes are constructed using PVC Foamex™, which is a lightweight, easily worked material, and will not leach any harmful solvents into the water (Figure 4).

The shape of the box is square when viewed from the sides and wedge shaped when viewed from the ends. The upper surface has a center brace rather than a full lid to allow the screens to be removed.

The sides of the box are open in order to allow the screens to slide into place using grooves cut into the Foamex on the inside of the box. There are two grooves for each side to allow the new screen to be located before removing the old one (preventing escape of larvae), when changing screens for cleaning.

There are several mesh size screens ranging from 49 µm to 500 µm for 'day' and 'night' use as well as for different larvae stages. At the bottom of each side of the filter box there is a porous tube, which provides an 'air curtain', in order to prevent the screens from becoming blocked. The air bubbles create a current across the screen, which are angled outwards at the top preventing the larvae being trapped on the screen. Inside the box there is a 4 mm outlet providing additional aeration, in the form of large air bubbles, which stirs the water and prevents accumulation of fine organic matter on the lower inner surface of the filter box.

The outlet of the box is a 32 mm adaptor situated 50 mm below the top of the box at one end. The outlet level is below the top of the screens in order to prevent the screens from overflowing and allowing larvae or live feeds to flow out. Screens of different mesh size are easily exchanged to allow flushing of live food at night and the complete renewal of enriched live feed the following morning before full light, via the automated delivery system. The box adaptor fits into the inside of the tank outlet, which helps hold the box in position.

This filtration design not only acts as a filter, preventing larvae and live and/or dry food to be flushed from the tank, it also acts as a surface skimmer, preventing an oil film on the water surface. A clean water surface facilitates swim bladder inflation and as a result promotes related factors such as growth and survival, and reduces deformities (Chatain, 1986; Chatain and Ounais-Guschemann, 1990; Summerfelt, 1996).

Lighting

The lighting system is designed to replicate the natural day/night cycle and is comprised of two separate systems in order to reduce costs. The first system, flood (spot) lights, is designed to ramp up and down in intensity to imitate the sun rising and setting. This helps to prevent larvae stress caused by sudden changes in the light intensity. The second, a fluorescent system, holds daylight for the period in between. The flood-lights are less costly to set up with a dimmer while the fluorescent lights are cheaper to operate for long periods. It is possible to get fluorescent lights with digital dimmers/ballast that will eliminate the use of two systems, however the cost of these special fluorescent lights is far greater than installing and operating two separate light systems. Moreover, although fluorescent lights can be dimmed, they will flash at start up and cannot dim down under 10% without flickering.

Both parts of the lighting system operate using a single programmable logic controller (PLC, Alpha series, Mitsubishi). This enables them to run on the same clock and therefore delivers accurate synchronisation. The PLC interface is supplied with the PLC and is easy to use, with pro-logic language software that is PC-Microsoft compatible.

The first system uses an analogue dimmer, controlling twelve 150 W incandescent light globes (flood-lights) to ramp up and down. The dimmer is controlled by the PLC which uses a 3 sec pulse via a relay to initiate the ramping up process, and then another to ramp down. The dimmer sequence period is 20 min for both increasing and decreasing ramp. Less expensive and more reliable digital dimmers are currently available, enabling the programming process to be faster and easier.

Once the flood-lights are at full intensity in the morning, a bank of twelve double four- foot daylight fluorescent lights (one for two tanks) is switched on and the flood-lights are switched off. At the end of the day the floodlights are switched on before switching off the fluorescent lights. The final dimming sequence is initiated immediately after the fluorescent lights are turned off. Currently, there is only one photoperiod regime possible, however, up to twelve photoperiod regimes are possible when each fluorescent light is connected directly to the PLC (by using PLC with enough outlets).

The whole system is covered with heavy-duty black PVC plastic sheets in order to exclude any natural light, which gives complete control over the photoperiod. The system has a red light to allow technicians to operate during periods of darkness, if needed.

The flood-lights are fixed in position above and between each pair of tanks providing 550 ± 50 lux at surface level, while the fluorescent system provides day light intensity between 300 ± 20 and 4,000 ± 60 lux depending on the height above the tanks.

The fluorescent lights are suspended above paired tanks using an adjustable bracket. Each bracket holds four lights and is made from galvanized angle iron, which is supported by a truck and rail assembly, allowing the bracket to move forwards and backwards above the tanks. The lights are then suspended from the bracket using chain pulled through keyhole slots. The chain locks into the slot at the height required. This allows the daylight intensity to be adjusted to suit the species of fish, and also enables the lights to be raised for ease of system cleaning.

5.1.1.5 Automated algae and live feeds distribution system

The larvae rearing system is built as a flow-through green-water system, meaning that a constant supply of algae is needed (Moretti et al. 1999). Coupled with the need for constant supply of live feeds (rotifers and *Artemia*), it is time consuming and usually demands a full time technician to supply the feeds in specific intervals during the day (Rabe and Brown 2000; Papandroulakis et al. 2002). Therefore an automatic distribution system was developed. Two separate identical feeding systems were installed, each is comprised of two 1000 L conical FRP tanks connected together by a 40 mm PVC pipe with individual ball valves allowing the use of both of the tanks or only one as required (Fig. 5). A submersible pump in each tank is connected in parallel to one distribution line (20 mm PVC pipe).

The distribution line is built as a ring surrounding the larvae rearing tanks and via an overflow, the line ends above the feeding system tanks for the return of algae and live food. The system is not pressurized due to the open end and vertical outlet pipes. This prevents any air locks and allows algae and live food left in the pipes between the feeding intervals to return by gravity to their corresponding tanks (Fig. 2).

Initial attempts at designing a distribution system resulted in an uneven flow of algae to each tank due to the higher pressure at the beginning of the line and initial flow time differences between the tanks at the beginning and the end of the line (it takes up to 30 seconds for the algae to arrive at the last tank). To compensate for the pressure gradient generated by the pump and backpressure within the manifold, a ‘relative outlet height gradient’ strategy was adopted. This was done by using vertical 20 mm pipes connected to the main (algae and live food supply) line above individual tanks, each with a 4 mm outlet at specific heights above the pump tank. During the process of filling the distribution line, the algae is at low pressure. This pressure is not enough to raise the algae in the vertical columns to overflow levels, until the algae in the pipe reaches the main return overflow, which creates additional back pressure in the line. This gives an equal increase in pressure across all the outlets, simultaneously raising the algae in each vertical pipe to their respective delivery level. The outlets are constructed from PVC tee connectors with a 90° elbow connected to a 1-metre 20 mm vertical PVC pipe and a 4 mm elbow fitted to it at the required level. Clear tubing directs the algae / live food from the elbow outlet to the water surface in order to prevent splashing. The first and closest outlet to the pump is also at the highest point while the last and the farthest outlet (where the pressure is the lowest) is at the correspondingly lowest overflow level. The height gradient (500 mm over 31m of feeding line) compensates for the pressure gradient and creates a constant and even flow throughout the system. This set up resulted in less than 5% variation (between the tanks) of algae and live food delivered to the tank.

Since the system is not pressurized, live food is not damaged passing through the open impeller pump and the distribution lines. At the end of the feeding interval, all the algae and live food is drained from the feeding line to the tanks and back to the live feed tanks, through the pump. Each of the two lines is independent which enables the system to have two algae or live food treatments of the same culture history or the use of the feeding tanks on a day on – day off basis. All the pumps (for individual lines) are controlled by the same PLC as the lighting system. Any pumping intervals can be programmed and changed at any given time based on the larvae species, age and feed needed. The algae and/or live food amounts are regulated by a combination of the concentration in the food tanks, pumping time and intervals (that can be updated during the run according to the larvae growth). Each distribution line is independent and can be programmed to deliver different amounts at different time intervals. A manual over-ride switch allows the technician to add or stop algae / live food to the tanks if needed during the day.

The number of food tanks and distribution lines is only determined by space, needs and budget. Currently, another two distribution lines can be added to the system (one for each food tank), increasing the flexibility of the system in terms of experiment design and treatments.

5.1.1.6 Operational procedures

The system was designed as a flow-through, green-water system, although, if needed, it can be changed to a recirculating system (by connecting all the tanks outlets to a water treatment system and then to the main sump). The design of the system is focused on efficiency of time and labor which leads to cost savings, and therefore includes automatic feeding and lighting systems.

A daily routine is started 20 min before the flood-lights are ramped up. Algae from one of the feeding systems (2 x 1000 L tanks) that was filled the previous day is pumped to the larvae tanks providing algae at the required density. The lights (flood-lights and fluorescents) are ramped up and switched on automatically as described above.

The morning duties include the following:

1. Replacing the screens in the box filters from 500 µm (or 250 µm) to 80 µm or 125 µm depending on the live food and larvae age. The large mesh filters allow flushing all the ‘old nutrient-depleted’ live food from the tanks during the night. The small mesh is utilized during the day and maintains the fresh enriched live food in the tank.
2. Harvesting the enriched live food (rotifers and *Artemia*) for the current day.
3. Initial feeding of larvae.
4. Preparing the live food for the next day (hatching, enriching).
5. Cleaning and filling the empty algae system (used the previous day) with fresh algae for the following day.
6. Feeding and monitoring the food concentration in the larval tanks.

Usually, the rotifers are divided into two portions; one is placed in high-density algae for enriching for the following days initial feed, immediately after harvesting. The density is usually kept between 10-30 rotifers ml^{-1} and 1-5 nauplii ml^{-1} (Moretti et al. 1999). The rest of the daily ration is placed in the algae tanks and distributed automatically at set intervals during the day. The intervals depend on the number and age of larvae and are programmed to maintain a set live food density in the larvae tanks. This set up is used when rotifers are given as a standard food. However, when testing a specific factor with rotifers (i.e. enrichment, probiotics etc.), the rotifers are given manually

through the day and not placed in the food tanks. Following harvest, *Artemia* is kept at 4°C and fed manually to the larvae several times daily. *Artemia* are best fed in batch additions, to allow equal opportunity for all larvae to access them. Feeding events are reduced in number when the larvae are being weaned onto dry microdiet, rather than reduced numbers of *Artemia* per feed.

The larvae tanks are cleaned by siphoning the walls with a 'broom siphon' every few days, depending on the larvae stage and feed type. During the cleaning process, the inlet water is stopped, preventing re-suspension of the dirt.

Harvesting the larvae from the tank is done by first reducing the water level in the tank to 10-15 L by inserting a screened standpipe with a solid 32 mm bottom section fitted into the tank water inlet. A screened bucket is suspended in a water bath, as this reduces water pressure on the internal side of the screen and prevents larvae from being forced onto the screen, and is positioned under the bottom ball valve. The larvae are then drained into the bucket through the fully opened ball valve by removing the standpipe. Due to the low volume of water left in the tank and the double collector buckets, no pressure is put on the larvae resulting in low stress harvesting.

5.1.1.7 Conclusion

The experimental tank system is simple and easy to operate. It provides a strong tool to execute most larval experimental designs while minimising unintended variation within the system. It allows operation with minimal manpower due to the automation of the feeding system and the ease of the daily routine i.e. filter exchange. Several fish species ranging from temperate to tropical (pink snapper *Pagrus auratus*, yellowtail kingfish *Seriola lalandi* and barramundi *Lates calcarifer* were reared successfully in the system, testing a variety of factors including different live food enrichments, microdiets, and environmental settings.

5.1.1.8 Acknowledgments

This research was supported by the Australian Fisheries Research and Development Corporation (FRDC), project 2001/220. The system was built at the Aquaculture Development Unit, Challenger TAFE, Fremantle, Western Australia as part of collaboration with the Department of Fisheries, Western Australia (DoFWA). The authors acknowledge the contribution of Mr. B. Ginbey (Challenger TAFE) to the design of the water system, Mr. S. Ben-Atia (National Centre for Mariculture, Israel) for his contribution in the initial design of the filter box, Mr. I. Lightbody (DoFWA) for construction assistance and Dr. G. Maguire and J. Heine (DOFWA) for their useful comments on the manuscript.

5.1.1.9 Figures

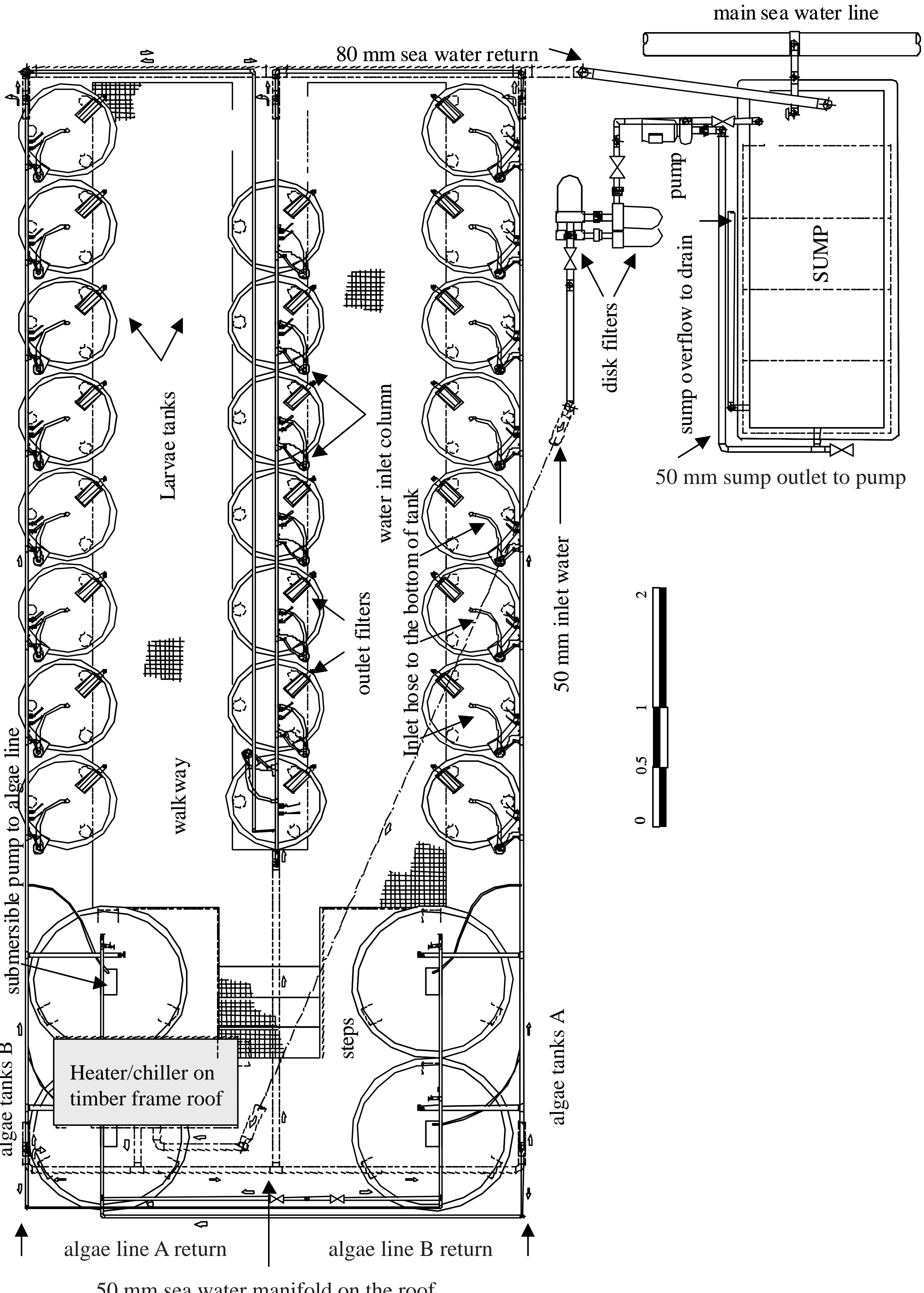


Figure 1a. Experimental system, plan view.

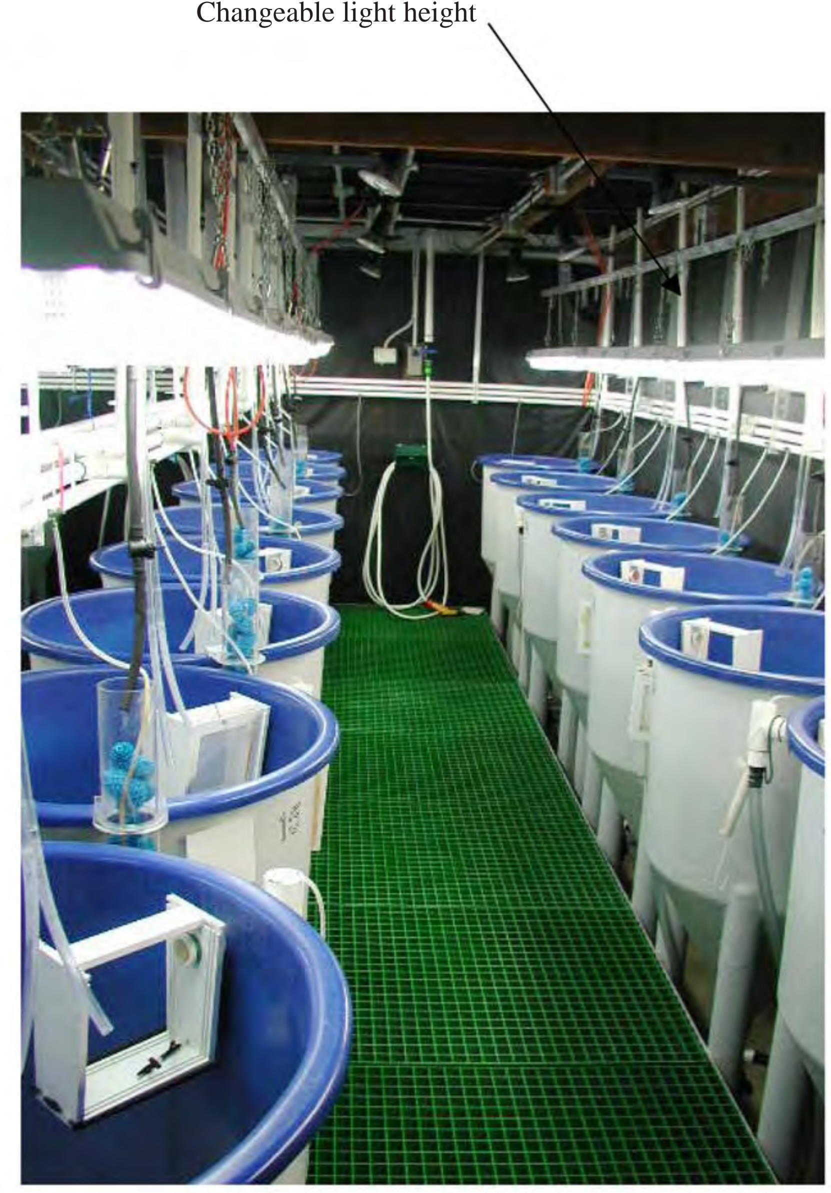


Figure 1b. Experimental system, perspective view.

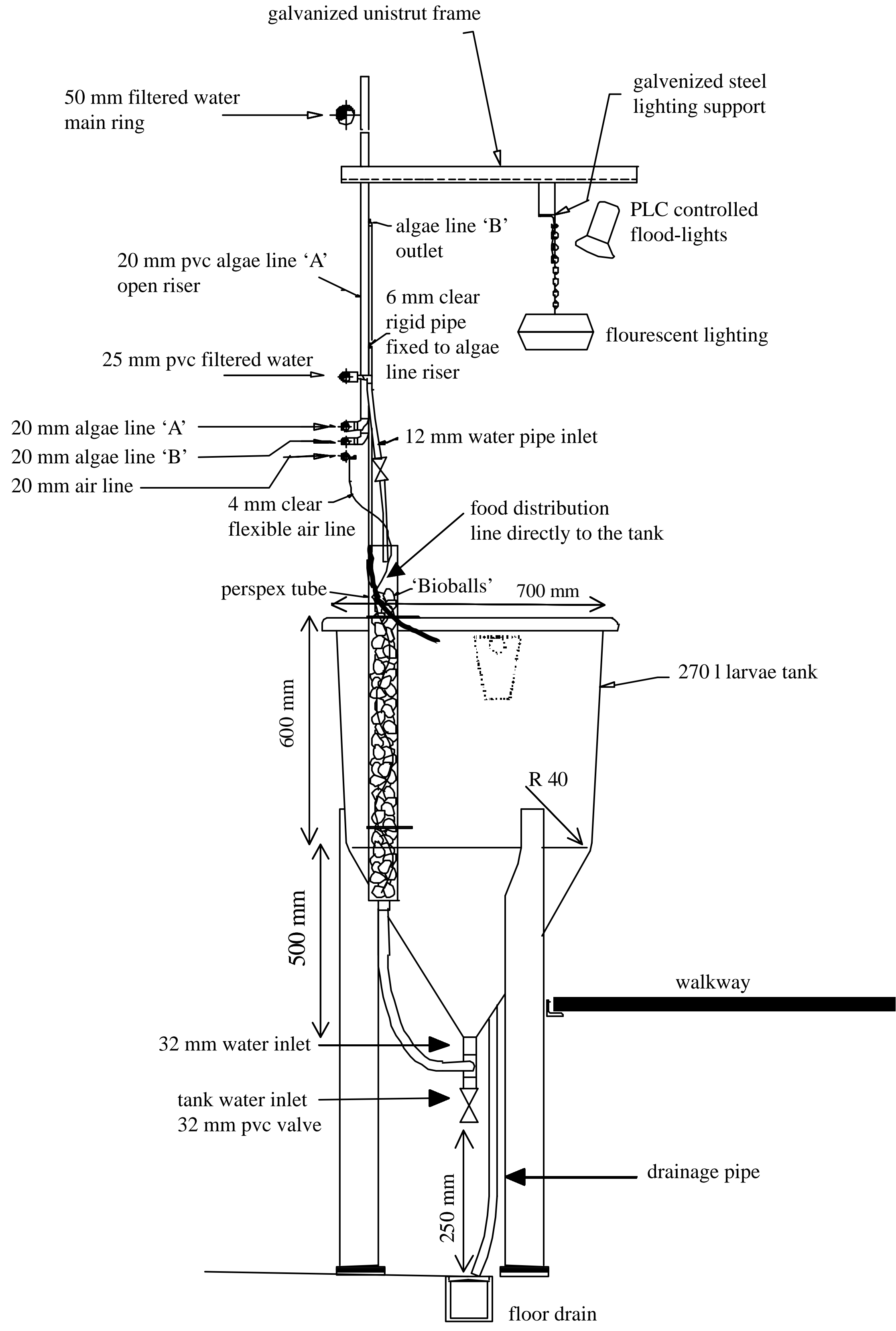


Figure 2a. Experimental larvae tank and supporting systems (elevation); 2c, Inlet water column.

Figure 2b. Experimental larvae tank and supporting systems (elevation); 2c, Inlet water column.

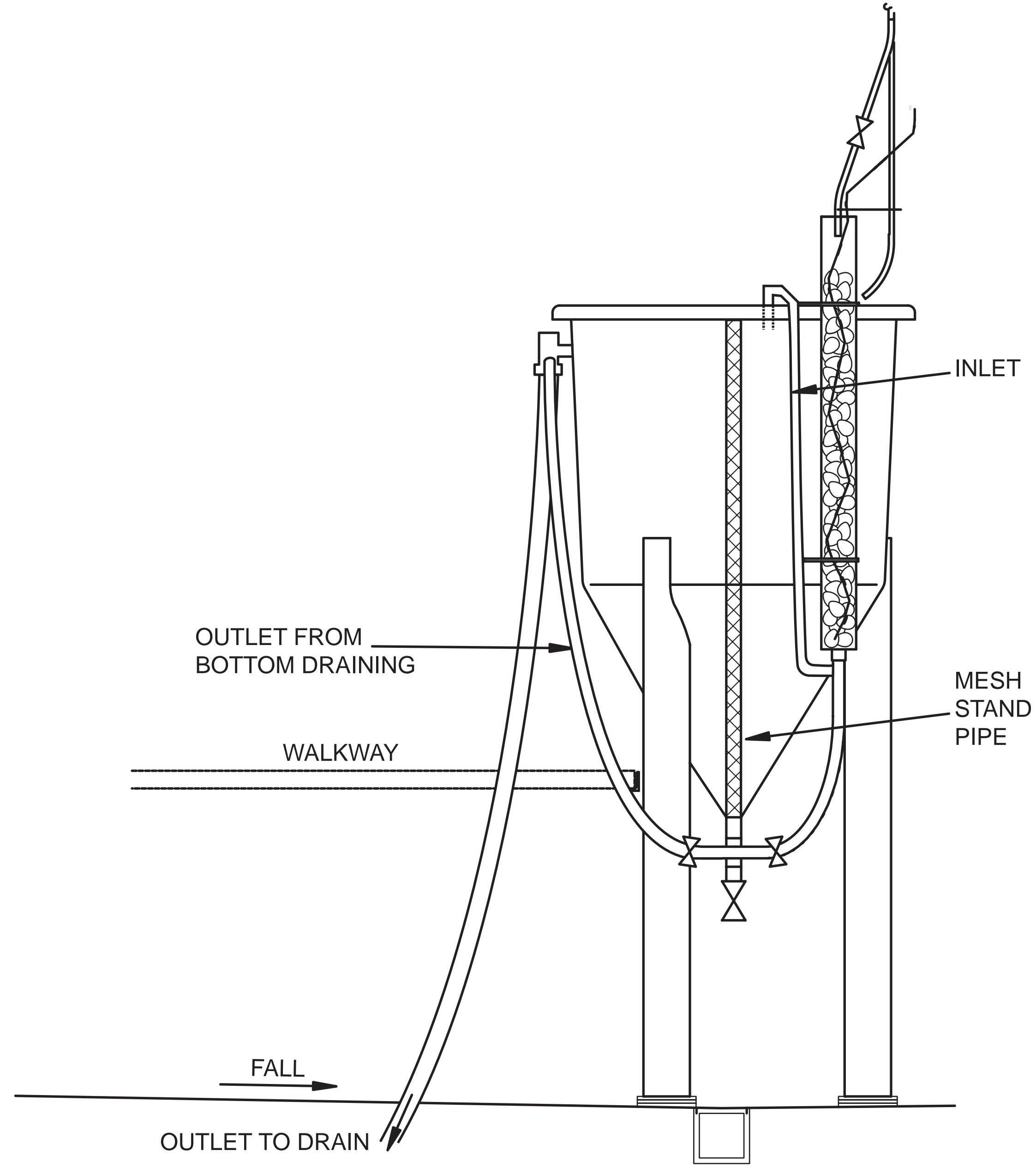
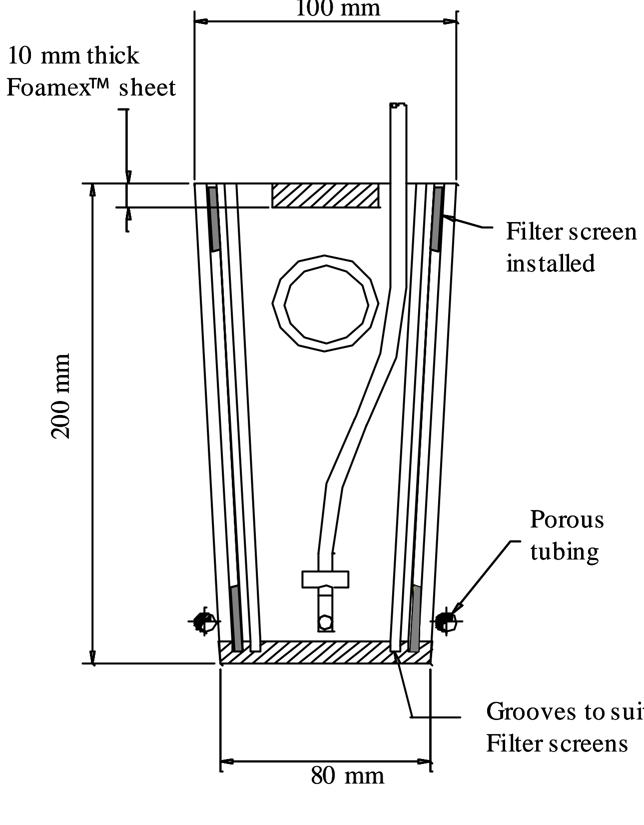


Figure 3. Experimental larvae tank - bottom drain.



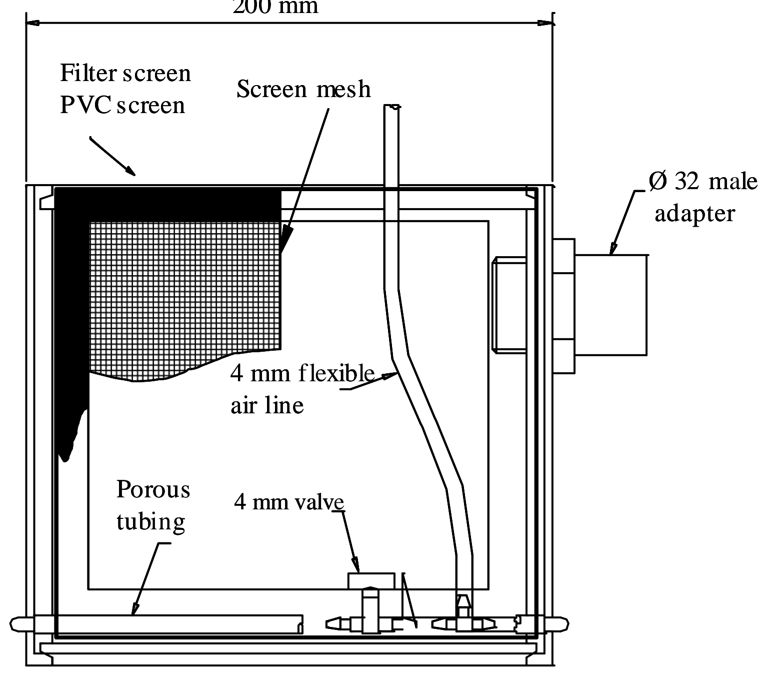


Figure 4a. Outlet filter box (A end elevation, B. side elevation).

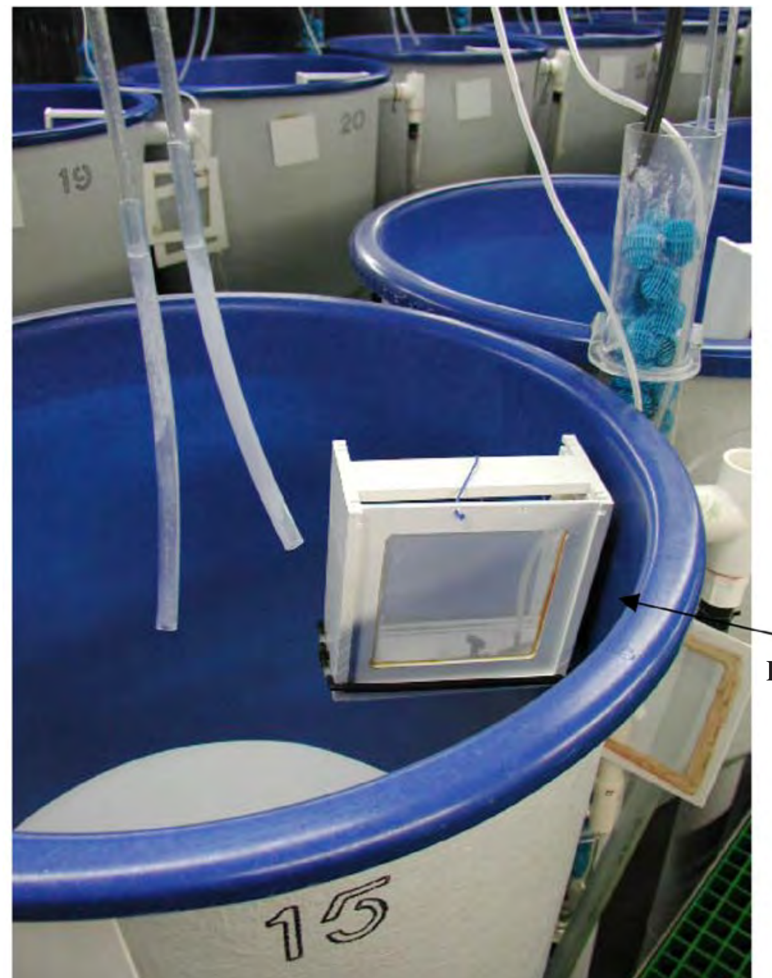


Figure 4b. Outlet filter box (A end elevation, B. side elevation).

Figure 5. Food distribution system.

5.1.1.10 References

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5.1.2 Intensive rearing system for fish larvae research – II. *Artemia* hatching and enriching system

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5.1.2.1 Abstract

Live food such as *Artemia* is considered to be an essential part of any marine finfish hatchery. Standard methods were developed for hatching and enhancing the nutritional value of the *Artemia* nauplii by using different enrichment products. Although there is a variety of commercially available products in the market, further research on more specific enrichments for solving specific nutritional deficiencies in fish larvae is still required. A simple, compact and reliable experimental system was developed for these nutritional experiments. The system was built as a compact, all-in-one system with eight 50 L conical tanks in a water bath. The system reduces variation between the replicates (tanks) otherwise resulting from individual heaters and aeration. It reduces the manpower and time needed for harvesting and to re-establish the system on a daily basis by the ability to harvest, wash and refill all of the tanks at the same time with the same volume of water. The system has been used for a variety of experiments, such as comparing commercial and experimental enrichments, bacterial monitoring and evaluation of different *Artemia* procedures.

Keywords: system, enrichment, *Artemia*, hatching, live-food, fish, larvae

5.1.2.2 Introduction

Currently, marine finfish larvae culture still relies heavily on the use of live food organisms such as rotifers *Brachionus plicatilis* and *Artemia* during their early life phase. During the last two decades, much research effort was given to the development of formulated diets that can completely replace the use of live food. However, the use of live food still remains an integral part of any marine finfish hatchery (Kolkovski and Dabrowski, 1999).

Therefore, better and more efficient methods for hatching, rearing and enhancing the nutritional profile (enrichment) of *Artemia* and rotifers have been developed by private companies and scientific research facilities (INVE Aquaculture, Belgium; Sorgeloos et al. 2001). The variety of commercially available enrichment products enables the grower to choose the most appropriate product for the specific needs of particular fish species. Usually these enrichments are based on oil emulsions or dry powder with different levels and ratios of fatty acids and lipid groups, vitamins and other ingredients such as anti-oxidants (Sorgeloos et al. 2001).

The techniques for hatching, enriching and harvesting *Artemia* nauplii are standardized and the effects of environmental factors such as temperature, light and aeration are well described (Lavens and Sorgeloos, 1996). Developing and/or testing live food rearing methods and/or enrichment products requires a system that can accurately control environmental variables and which has enough replicates with a size and volume that can imitate a commercial scale operation. Since cysts hatching percentage of a particular batch (hatching percentage depends largely on strain and batch (season, harvest, processing etc.) Vanhaecke and Sorgeloos, 1983, 1989) depends on the aeration (Babu et al. 2001) and temperature (Lavens and Sorgeloos, 1996), these variables, along with lighting and harvesting systems, need to be standardized across the entire experimental system in order to achieve a high degree of consistency among replicates.

Taking these factors into account, an experimental live food system was designed and developed to test different aspects of live food culture. In the design, consideration was given to minimizing daily labor inputs.

5.1.2.3 System Description

The compact system was designed to be relocated as a single unit. It incorporates 8 x 50 L black conical fiber reinforced plastic (FRP) tanks in a black high-density polyethylene (HDPE) rectangular holding tank (Fig. 1).

The apex of the bottom cone of each tank is translucent to allow light to pass through the tank walls. A threaded outlet on each tank (PVC ∅ ¾″ BSP threaded connector embedded in the FRP) was connected to a PVC elbow through the polypropylene holding tank wall by a tank valve adaptor (Fig. 2). PVC pipe then connects the elbow to an outlet ball valve. All eight ball valves connected to the tanks face the front of the holding tank. This valve arrangement allows the (manual) harvesting of all eight tanks simultaneously, by allowing the technician to simultaneously oversee the process.

The inlet water system is comprised of a ring sub-manifold supplied by a main 2″ (50 mm) PVC ball valve connected to the hatchery water supply (marine bore water, 35 ppt). Individual ¾″ ball valves supply water to each tank from an overhead position. All the individual valves are set to the same synchronous flow-rate ensuring an equal volume and current of water when the main valve is fully open. This insures that after harvesting, *Artemia* are washed with the same volume of water in all the tanks and for the same period of time. This is especially important when using the system for bacterial studies since variation in the water volume, current and time period will cause variation in the bacteria levels.

5.1.2.4 Aeration System

To obtain an optimum level of incorporation of enrichment into *Artemia*, oxygen levels must not drop below 4 ppm (Lavens and Sorgeloos, 1996). Due to the nature of most of the commercial enrichment products i.e. oil emulsion, combined with the temperature recommended for enriching (26°-28°C) and the high *Artemia* densities recommended, the ability of the water to retain sufficient oxygen after adding the enrichment is limited, resulting in sub-optimal conditions. Different aeration systems have been developed through the years (Babu et al. 2001, Lavens and Sorgeloos, 1996, Sorgeloos et al. 1986), however, the current system includes dual aeration systems, one supplying air and the other supplying oxygen (Fig. 1- 2).

Air is supplied through a PVC pipe ring connected to the main hatchery air supply (from a central air blower). A brass needle and seat valve supplies air to each tank. A flexible 4 mm air hose is used to connect the valve to an end-cap glued to a PVC standpipe. Ensuring that the tank outlet valve is closed, the air is bubbled through six 1 mm holes at the bottom of the standpipe. This creates large bubbles that vigorously aerate and circulate the hatching or enrichment media, ensuring the movement of the cysts and *Artemia* in the tank without any ‘dead spots’ in the base of the cone, where cysts can collect and remain unhatched. The standpipe therefore functions as a standard standpipe, preventing cysts sinking into the outlet pipe, and also as an aerator.

The second aeration system is based on oxygen supply using a compressed oxygen cylinder. The same basic ring structure as the aeration system is used, with a flexible hose connected to a flow meter on the oxygen bottle which controls the supply of oxygen to the manifold. The oxygen is bubbled into the water via fine porous teflon diffusers, which minimize the bubble size and maximize bubble surface area for gas exchange. The combination of air and oxygen allows the retention of 80-100% oxygen saturation in the system with a relatively low amount of oxygen being used (1-3 L min^{-1} for the whole system) for a maximum density of *Artemia* (GSL strain INVE Aquaculture, 1 x 10^6 nauplii l^{-1}).

5.1.2.5 Heating system

The optimum temperature for commonly used *Artemia* hatching and enriching is 26°C - 28°C (Lavens and Sorgeloos, 1996), although there are *Artemia* strains with different temperature optima (Vanhaeke et al. 1984). Therefore, submerged heaters are typically used in other *Artemia* systems. These heaters are made of glass (aquarium heaters, 150-300 W) for small tanks and titanium or stainless steel for larger tanks. Individual submersible heaters inside a tank present a number of problems:

1. Variation between tanks temperatures due to inconsistent individual heaters;
2. Electrocution hazard if the heater is not properly connected or isolated;
3. Fire hazard when the heater doesn't have an automatic on/off switch and it is left on during harvesting;
4. Breakages when glass heaters are handled.

To avoid these problems, the individual tanks were placed in a 1,000 L high-density polyethylene (HDPE) holding tank that is kept continually full of fresh water. Two 1 kW titanium heaters are submerged, one at each end of the tank maintaining a constant temperature of 28 ± 0.1°C. Two airlifts ensure even water circulation around the tank. Since the heaters are constantly submerged in water there is no need to switch them off when harvesting, which minimizes human error (Fig. 1). Due to the large thermal mass of the water bath, the speed at which new tanks of water can be brought up to the required hatching temperature is accelerated. This enables greater efficiency when conducting treatments on a daily basis.

In order to demonstrate the advantage of temperature control and the low variation between tanks that the system has over stand-alone tanks, a comparison between 6 tanks (50 l) in the system and 6 identical stand-alone tanks was carried out. The tanks were filled with ambient marine bore water (20°C) and left to heat over night in the system and stand-alone with aquarium heaters (300W) set to 28°C. In an attempt to mimic normal harvesting protocol, the next morning, the tanks were emptied and filled again with marine bore water. Temperature loggers (eTemperature, OnSolution Australia) were placed in each of the tanks. The time taken for the system and stand-alone tanks to reach a set temperature was recorded, as well as the temperature variation between the tanks (Fig. 3). At an ambient air temperature of 20°C ± 0.5°C, the system tanks reached a temperature of 27.5°C after 216 min., while the stand-alone tanks reached the same temperature after 320 min. Clearly, the lower the air temperature is, the longer it will take the stand-alone tanks to reach the desired temperature. However, time taken to reach a set temperature in the system tanks does not change with ambient air temperature.

Reducing the temperature variation between the tanks also reduces the oxygen variation due to the relationship between temperature and oxygen saturation in water. For example, dissolved-oxygen concentration in 30‰ salt water at 27°C is 6.72 mg/l and 6.61 mg/l at 28°C (Creswell, 1993). Although not a large difference, it would contribute to variation of the results when conducting experiments.

5.1.2.6 Enrichment administration

According to most of the manufacturer's instructions, commercial *Artemia* enrichments are given in two equal portions (12 h and 6 h prior to harvest). This ensures that the enrichment taken in by the *Artemia* is fresh and keeps the dissolved oxygen levels to a maximum. The first half is usually poured directly into the tank. An automatic distribution mechanism ensures the delivery of the second portion of the enrichment to each *Artemia* tank during the night.

The distribution system is comprised of a 1 L glass bottle containing the enrichment, a timer-controlled air-pump system and an insulated holding container. The bottles containing the enrichments are set in the insulated container between ice blocks (important in summer when the air temperature can exceed 40°C), which keeps the enrichments chilled until they are auto-administered. Each bottle cap has two flexible hoses passing through it, one connected to the timer-controlled air system and the other one set as the delivery line from the bottom of the bottle through the cap to the individual *Artemia* tanks. At the set time the air pump is switched on by the timer, pushing air into the bottle, causing the enrichment to flow through the second hose and siphon to the tank. This simple and cost effective system enables the distribution of eight separate enrichments to individual tanks. Since mechanical pumps or expensive peristaltic pumps are not used there is no limit to the number of enrichments and tanks used (Fig. 1- 2).

5.1.2.7 Harvesting

Merchie (1996) described a simple, widely used method, for harvesting newly hatched nauplii using a light source. In the current system, two light sources are installed one above each tank and the other submerged in the water bath near the bottom cone of each tank. Each set of lights can be turned on/off individually. The harvesting process is commenced by removing the aeration lines (oxygen and stand pipe / aerator) and turning on the upper lights. The nauplii are then attracted to the surface, which enables the separation of the unhatched cysts and debris that sink to the bottom by draining it through the outlet valve. The tank is then remixed and oxygenated by replacing the aeration in the tank for a few seconds and then removing it. The nauplii are then concentrated around the clear cone bottom by changing the light source to illuminate beneath the tanks and covering the top of the tank with a light proof lid. The empty cyst shells can then be scooped from the surface. Alternatively, the last few litres that contain the floating shells can be discarded.

After separating the unhatched cysts and shells, the newly hatched nauplii are harvested into 15 L harvesting buckets with internal filters (Sorgeloos and Léger, 1992). The internal filter (125 µm) preventing escapes of nauplii during the washing stage. Overflow due to filter blockage is prevented by an air curtain across the filter mesh. After concentrating the nauplii, the main water valve is turned on and the nauplii are washed. Since all the individual valves (to each tank) are set to have the same flow rate, all the eight tanks can be harvested and washed at the same time with the same amount and flow of water, effectively reducing variation between tanks (Fig. 2).

After harvesting the culture tanks, the internal standpipes are washed thoroughly, disinfected with oxalic acid and than rinsed again with seawater. This process is similar to any tank system, however, it has the advantage of flushing the acid from all the tanks at the same time therefore optimizing use of labor. The tanks are then ready for re-filling with water for the enrichment stage. In the case that only some of the tanks were used for hatching, the nauplii can be immediately put into the others after rinsing. The system allows hatching and enriching stages to run side-by-side when enough tanks are available.

The same method of harvesting is carried out with enriched *Artemia*. While there is no need to use the lighting as the shells have already been removed, it is still necessary to wash the enriched *Artemia*, which helps to reduce bacterial levels introduced into the fish larvae culture tanks (Dehasque et al. 1993; Verdonck et al. 1994). The washing procedure is done in the harvesting buckets using the water supply through each tank.

5.1.2.8 System advantages

The *Artemia* system is compact and simple to operate. The system minimizes variation in environmental conditions (temperature, aeration, light) and handling (water flow during rinsing) between the tanks.

The system design promotes operator safety when compared to any stand-alone tank with an individual (usually glass) heater due to the fact that there is no need to switch on and off any heaters and remove them from the tank (no breakage risks).

Components such as the enrichment automatic-doser present a viable and (very) inexpensive alternative to peristaltic or mechanical pumps usually used for the delivery of the enrichments.

The system design reduces the time and effort usually involved with *Artemia* hatching and enriching procedures. Factors such as simultaneous harvesting and harvest bucket reliability (preventing overflow) allow the operator flexibility and the freedom to complete other duties during the harvesting and rinsing of the nauplii. This can be very useful for students or researchers running their experiments with minimal help.

The system is a useful tool for any research institute involved in larvae nutrition. Although small, the system can be duplicated or the principles can be applied to larger volume tanks.

5.1.2.9 Acknowledgments

This research was supported by the Fisheries Research and Development Corporation (FRDC), project 2001/220. The system was built at the Aquaculture Development Unit, Challenger TAFE, Fremantle, Western Australia as part of collaboration with the Department of Fisheries, Western Australia. The authors thank Dr G. Maguire and J. Heine for their review and useful comments.

5.1.2.10 Figures

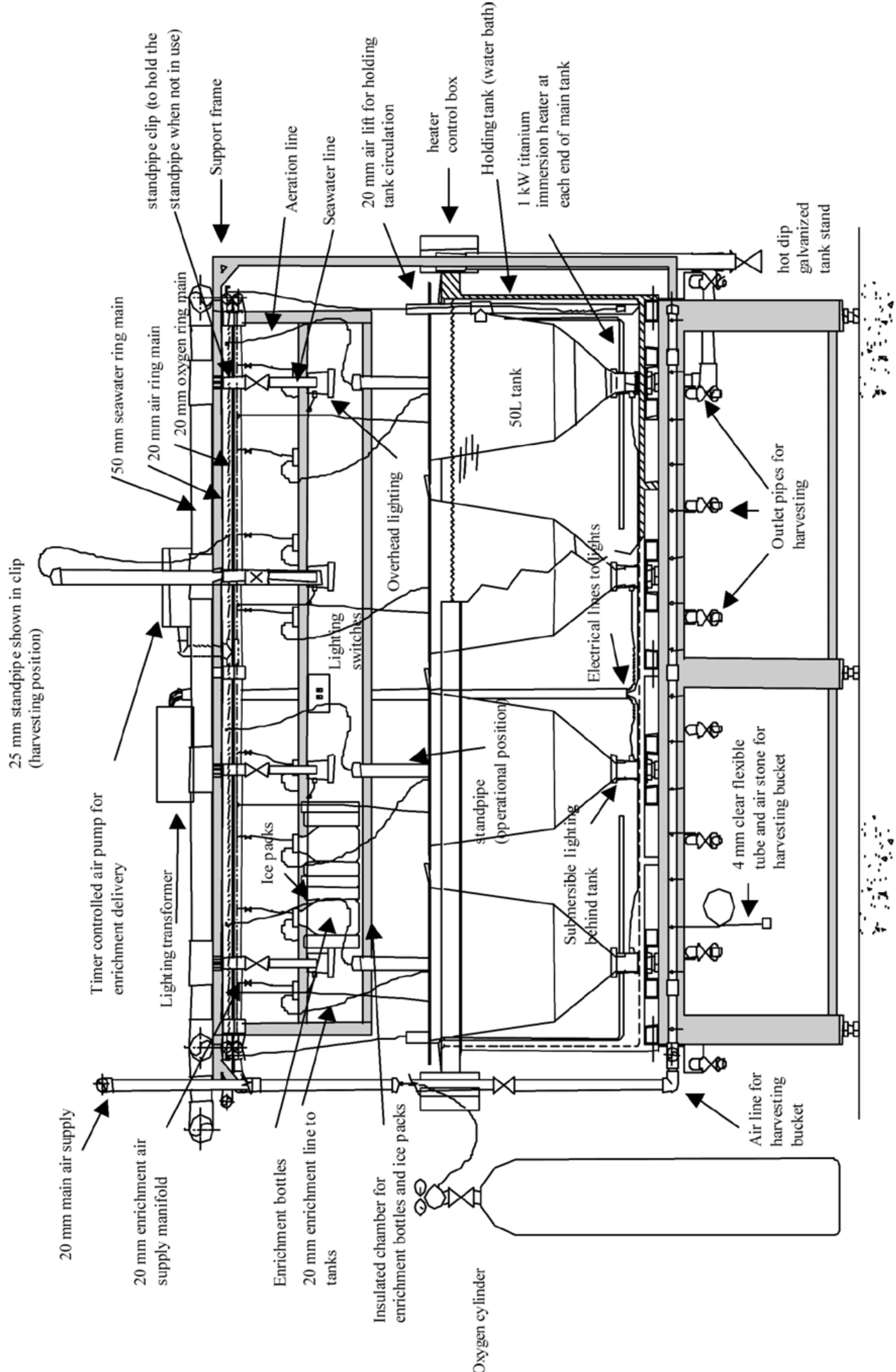
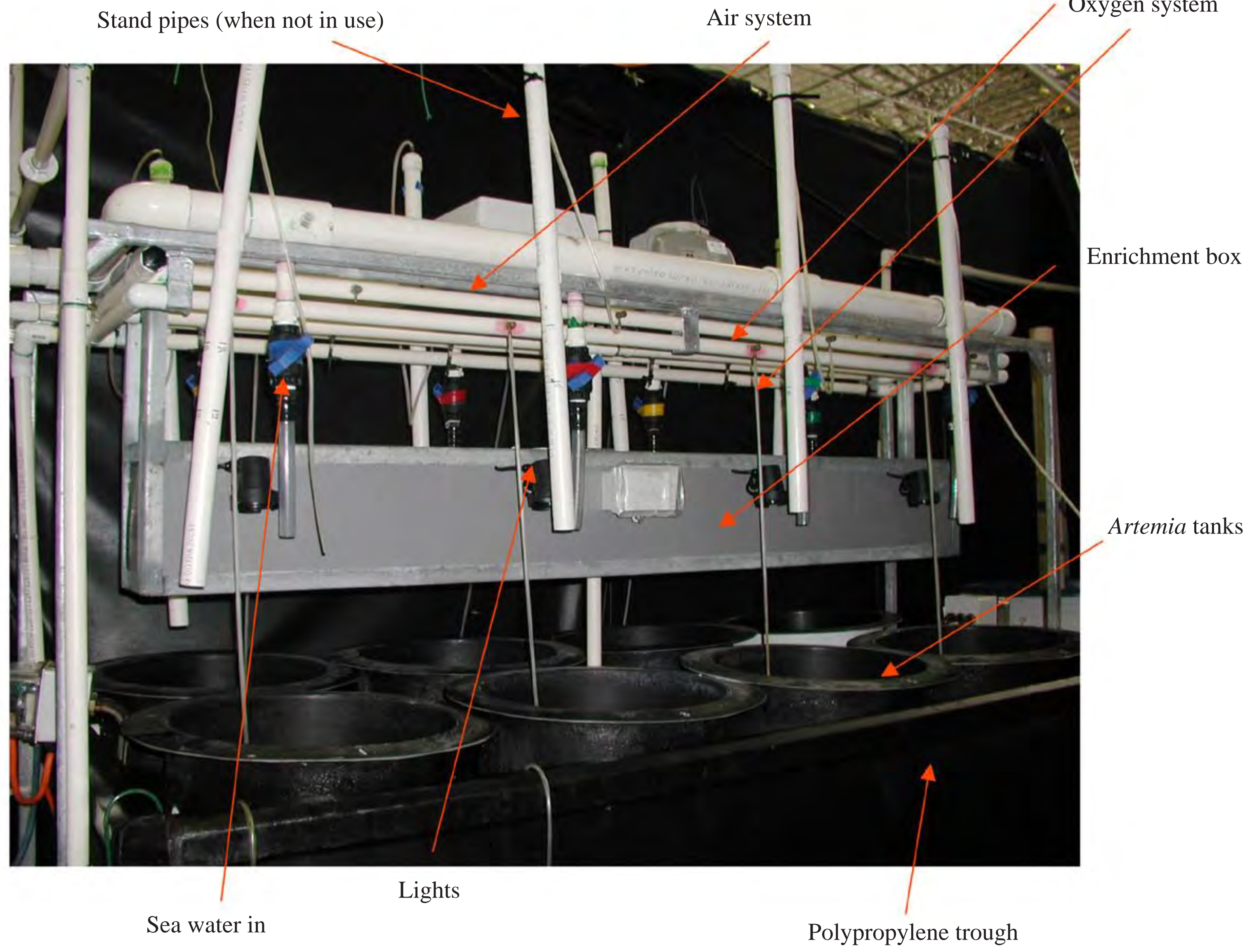


Figure 1a-h. *Artemia* enrichment system, front elevation and components.

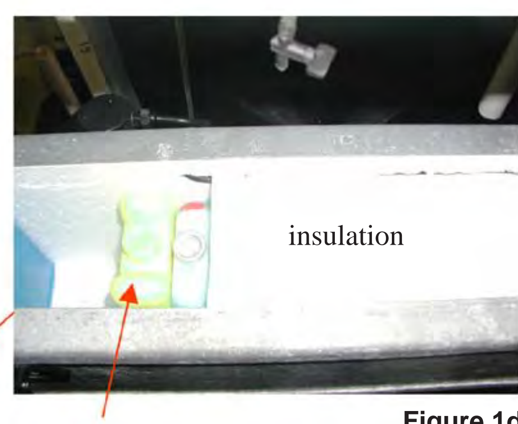
Figure 1b.



Air pump for the automatic enrichment distributors

Figure 1c.

Enrichment box



Ice blocks

Figure 1d.

Underwater light on

Figure 1e.

Light switch

Above light on

Figure 1f.

Figure 1g.

Figure 1h.

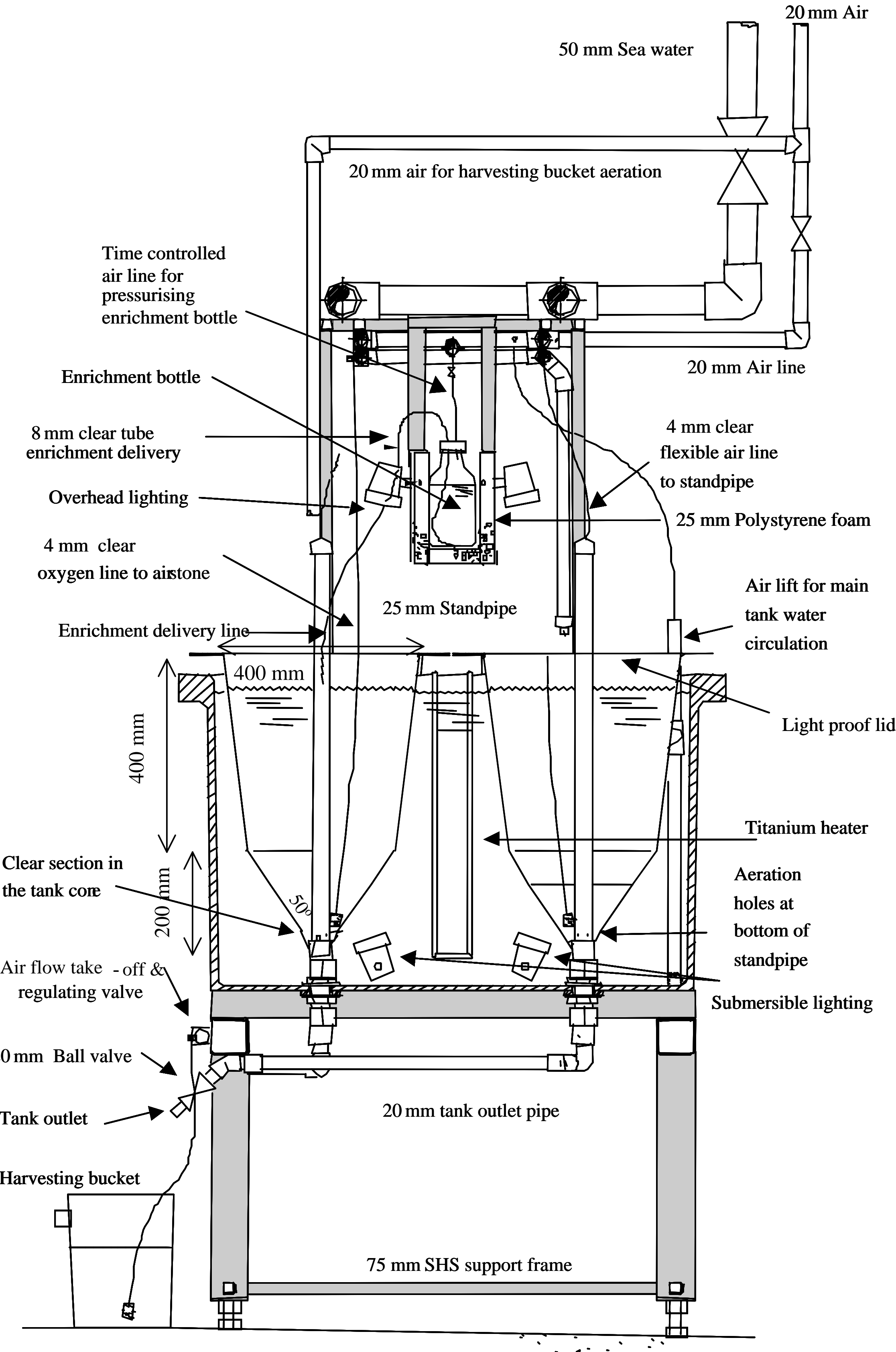


Figure 2. *Artemia* enrichment system and harvesting bucket, side elevation.

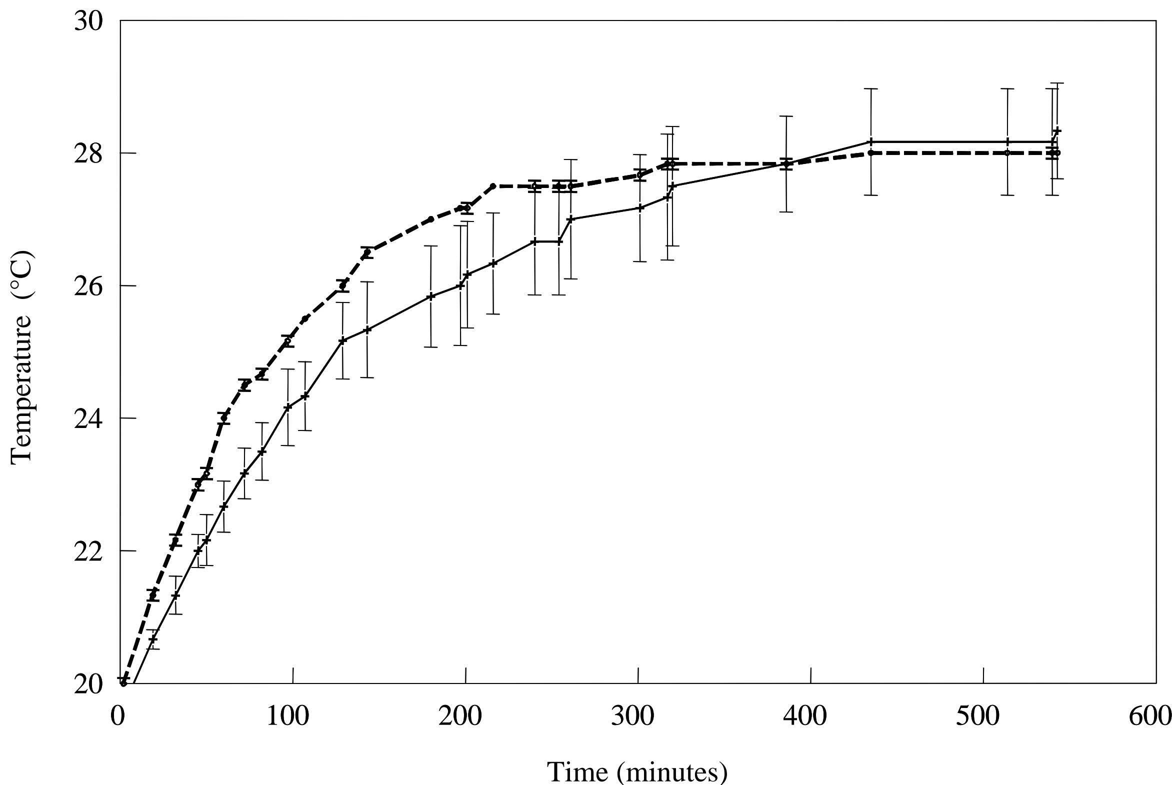


Figure 3. Temperature comparison between tank system - - • - - (using 2 x 1 kW titanium heaters in the main tank) and stand-alone tanks —+— (using individual aquarium glass heaters (300 W). Data reflects average and standard errors between 6 replicated tanks in the system or stand-alone.

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5.1.3 Automated Microdiet Dispenser operated by Programmable Logic Controller

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5.1.3.1 Automatic feeding system

24 Automated Microdiet Dispensers (AMD) are installed in a 24 tank experimental system (described by Kolkovski et al, 2004) to feed up to 8 different experimental or commercial microdiets (MD's) for larval weaning and/or nutrition experiments. Each treatment would have 3 or more replicate tanks, which are fed simultaneously via a single output from the Programmable Logic Controller (PLC). Specific PLC output programs are directed to operate particular AMD's within each feeding regime, via a network of 8 input – single output rotary switches. Each rotary switch is specific to an AMD that enables the program output from the PLC to be selected. This then connects a 12VDC power supply via a solenoid switch that is operated by the PLC. The PLC, power supplies and switches are all situated within a single control station, which is hardwired using 2 – core insulated flexible cable to the AMDs.

5.1.3.2 Automated microdiet dispenser

The AMD is designed to periodically administer a small amount of microdiet to larvae culture tanks, in order to spread the allocation of the required daily amount of feed evenly across the whole day. This prevents the need to manually feed the larvae, and provides a more constant availability of feed across the whole photoperiod, and outside working hours. This should reduce opportunities for bacterial proliferation on unconsumed feed particles, a consequence of feeding greater amounts less often. The feeder can cope with a diet particle size range of up to 1.5 mm.

The AMD mechanism operates by using a 12VDC piston solenoid to pull a slotted plate across the bottom opening of a vertical 20 mm tube (at a 45° angle) containing the diet. The slotted feeder plate rests on another smaller, slotted base plate. When not in operation, the slots on each plate overlap with the bars on the other plate, thus preventing the MD from falling through.

When the AMD operates, the feeder plate is pulled by the solenoid between the bottom of the feed tube and the base plate. The openings line up when the plate is half pulled, then closed when fully pulled. The piston is returned to its resting position when power is off to the solenoid by a stainless steel coil spring. The spring is situated around the piston, between the feeder plate and the solenoid housing. The feeder releases a dose of MD through the openings at the moment the slots line up, when passing in both directions. The plate can be pulled singularly or multiple times in any one feeding event, depending on the MD flow characteristics and the amount of MD required. With the current feeder plates and piston, each feeding event releases approximately 30 mg of MD. A larger amount of MD can be released at each feeding event by replacing existing plates with plates that have wider slots or by increasing the number of feeder action repetitions for any given feeding event.

5.1.3.3 Programmable logic controller

A Mitsubishi, Alpha 20 PLC is used to operate 24 AMD's using 8 outputs. The PLC has 8 x 24VDC outputs, which are used to operate 8 x 24VDC / 240V solenoid switches. The switches connect a 1.5 amp, 12VDC power supply to a maximum of 12 AMD's per switch, via a network of 24 – 1 pole sealed rotary switches. Each of the 24 AMD's has its own rotary switch with an input line from all 8 PLC-operated solenoid switch outputs. The rotary switch for each feeder can select an output program from the PLC that is designed to feed the daily amount of the specific MD, for the treatment to which the AMD is allocated. Generally, any one PLC program operates 3 to 4 replicated AMD's via their respective rotary switches.

The PLC operates the solenoids for 0.3 sec on and 0.1 sec off for multiple dosing, during any one feeding event. Multiple dosing is engaged when larger amounts of feed are required at any one time, or if a MD has slow flow characteristics. The PLC can be programmed to “flicker”, that is feed at regular intervals throughout the day or feed at prescribed ‘real’ times according to the internal clock and calendar. The “flicker”- feeding regime is selected if the MD is required to be fed in small amounts relatively frequently for the whole light period. The timer clock is used for a few larger doses at prescribed times, for example 3 morning feeding events at 15 min intervals and 3 afternoon feeding events at 20 min intervals.

Figure 1. Feeding system installed in the larvae rearing system.

5.1.3.4 Experimental applications

To this point the AMD system has been operated during 5 experiments relating to the nutritional requirements of barramundi (*Lates calcarifer* Bloch) larvae and pink snapper (*Pagrus auratus*) larvae.

Commercial MDs that have been fed by the AMD system during these experiments include; the Proton MD range (P1, P2, P3, P4, P2/3 and P3/4) and the NRD MD range (NRD3/5 and NRD4/6) (INVE, Belgium); the Gemma Micro MD range (Gemma Micro 150 and Gemma Micro 300), Gemma 0.3 MD and Skretting 600 crumble (Nutreco / Skretting Australia).

Experimental MDs using different protein sources that were produced by the Department of Fisheries, Western Australia (within particle size classes of 150 to 300 µm, 300 to 500 µm, 500 to 750 µm and 750 to 1000 µm), have also successfully been delivered to experimental aquaculture tanks by the AMD system.

The system has been continuously operated on a daily basis for periods of up to 26 days. The maintenance routine requires daily cleaning of the plates using high-pressure air and a quick inspection to ensure correct operation.

Figure 2. Individual feeder and the microdiet canister.

5.2 LIVE FOOD ENRICHMENTS

5.2.1 Nutritional and bacterial profiles of juvenile *Artemia* fed different enrichments and during starvation

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5.2.1.1 Abstract

Biochemical and bacterial profiles of juvenile *Artemia* (1.5 mm, 5 days old) were examined in two experiments designed to manipulate the nutritional composition of *Artemia*. In Experiment 1, *Artemia* were enriched for up to 36 h with the diatom *Chaetoceros muelleri*, the chromist *Schizochytrium* (Algamac 3050®) or a squid oil emulsion. In Experiment 2, *Artemia* were enriched with the same diets for 6 h, and then starved for 24 h at 4, 18 or 28°C. *Artemia* did not survive beyond 24 h enrichment on Algamac and survival was low on oil emulsion, contrasting with the rapid growth and high survival on *C. muelleri*. Fatty acid (FA) content of *Artemia* increased marginally after enrichment with *C. muelleri*, with a marked increase over 36 h enrichment in the percent long-chain polyunsaturated FA (LC-PUFA). FA content increased by 60% and 140% after enrichment with Algamac and squid oil emulsion, respectively, and % LC-PUFA also increased. FA content and % LC-PUFA in starved *Artemia* declined by up to 57% at 28°C whereas the proportional loss was < 30% at 4°C. Ascorbic acid (AsA) content in *Artemia* increased 4-fold over 36 h enrichment with *C. muelleri* and declined or did not change with the other enrichments. AsA was retained during starvation of 6 h-enriched *Artemia* with all treatments. α–tocopherol (α–T) concentration increased 3-fold to a peak after 6 h on oil emulsion and increased 2-fold after 24 h on *C. muelleri* while decreasing by 50% during 12 h on Algamac. α–T declined during starvation by up to 50% (depending on temperature) following enrichment with oil emulsion although there was no change with *C. muelleri* or Algamac. For *Artemia* enriched 12 h on *C. muelleri* or 6 h on oil emulsion, bacterial abundance was not elevated but further enrichment resulted in increases by 2- to 3-fold. Bacterial abundance in *Artemia* enriched for 6 h on Algamac increased 4-fold and by 24 h, most *Artemia* had perished. Total numbers of heterotrophic bacteria and *Vibrio* spp. were similar after enrichment for 6 h with *C. muelleri* or oil emulsion and after 24 h starvation at 18 or 28°C. Starvation at 18 or 28°C after Algamac enrichment increased bacterial numbers up to 4-fold. For *Artemia* starved at 4°C for 24 h, there was a marked reduction in bacterial numbers for all enrichments, indicating that cool temperatures may be bactericidal. Enrichment of juvenile *Artemia* for 6 h achieved substantial biochemical improvement while minimising bacterial contamination, and is regarded as a suitable protocol before feeding to target species such as spiny lobster phyllosoma larvae.

5.2.1.2 Introduction

Artemia are used widely for the feeding of larvae of many crustaceans and finfish. The larvae of some aquaculture species, such as spiny lobster phyllosoma (*Jasus edwardsii*), prefer larger *Artemia* juveniles (≥1.5 mm long, 5 day old) as prey to meet their food intake requirements (Ritar et al.

2003a). Although *Artemia* are not the natural prey of these animals, they are simple to prepare, are attractive in texture and behaviour and have been used effectively as the major or sole component to culture phyllosoma (Kittaka, 1994; Tong et al. 1997; Moss et al. 2000; Ritar et al. 2002, 2003a), presently making them a diet of choice.

The biochemical composition of *Artemia* is regarded as important in optimizing larval nutrition for survival and growth of aquaculture species (McEvoy and Sargent, 1998; Narciso et al. 1999). Most enrichment procedures and products have been developed to improve the nutritional quality of *Artemia* metanauplii (Rees et al. 1994; McEvoy and Sargent, 1998; Sorgeloos, et al. 2003) but these may not be suitable for juvenile *Artemia*. The behaviour of juvenile *Artemia* appears to be significantly different to metanauplii in that they ingest and metabolise enrichment diets more rapidly (Dhont and Lavens, 1996). However, the systematic investigation of the most appropriate protocol for enrichment of juveniles and nutrient losses during starvation has yet to be undertaken.

Research on enriching *Artemia* metanauplii has concentrated on increasing the lipid content and especially the amounts of the long-chain polyunsaturated fatty acids (LC-PUFA) docosahexaenoic, eicosapentaenoic and arachidonic acids (DHA, EPA and ARA, respectively) (Barclay and Zellar, 1996; Sargent et al. 1999a,b; Sorgeloos et al. 2001), with a recent focus on the ratio of DHA:EPA (Evjemo et al. 1997; McEvoy et al. 1996; Narciso et al. 1999). These LC-PUFA, which are usually low in abundance in *Artemia*, are regarded as essential for decapod crustaceans (Castell, 1982; Kanazawa, 1982; Cahu and Fauvel, 1986; Sargent, 1995) and must be supplied in the diet (Kanazawa et al. 1979; Deering et al. 1997). Understanding the accumulation and loss of essential FA (EFA) in *Artemia* metanauplii during enrichment and starvation has helped the development of feeding strategies for finfish larvae (Estévez et al, 1998; Evjemo et al. 2001) and this is also needed for juvenile *Artemia*.

The lipid composition of unenriched juvenile *Artemia* is considerably different to phyllosoma at hatch (Phleger et al. 2001; Smith et al. 2002) and during the development of later stages of wild larvae (Phleger et al. 2001). This suggests that *Artemia* should be enriched to improve their lipid profile for feeding to phyllosoma. Indeed, it is possible to alter the composition of developing larvae in culture by feeding them enriched *Artemia* (Nelson et al. 2002; Smith et al. 2002). Other nutritional components, such as vitamins C (ascorbic acid) and E (tocopherols), may also need to be boosted to further improve phyllosoma performance (Smith et al. 2004a). However, spiny lobster larval cultures are often plagued by sudden mortalities, which have been attributed colonisations by bacterial pathogens. Therefore, enrichment procedures that also minimise the transfer of bacteria via *Artemia* to larval cultures will be as vital as improvements in their biochemical composition.

Various enrichments that have been used to improve the composition of *Artemia* fall into three groups: live micro-algae, spray-dried algae and marine oil emulsions. This study used one representative from each of these groups: a live micro-algal diatom *Chaetoceros muelleri*, a commercial spray-dried alga Algamac 3050 and a custom-made squid oil emulsion, with both of the later enrichments high in DHA. Nutritional and bacterial profiles of juvenile *Artemia* were examined during enrichment for up to 36 h and following starvation for 24 h at 4, 18 or 28°C, mimicking the changes during chilled storage or when fed to phyllosoma from temperate or tropical environments, respectively.

5.2.1.3 Materials and methods

Artemia production

Decapsulated *Artemia* cysts (E.G. grade, *Artemia* Systems, INVE, Belgium) were hatched in 50 L conical tanks at 28°C in 1 µm-filtered seawater (SW) with vigorous aeration and a 150-W incandescent light suspended 0.5 m above the water surface. Hatched nauplii were rinsed for 5

min and stocked in 670 L tanks at 5 *Artemia* ml^{-1}, followed by the addition of *C. muelleri* on the first day. *Artemia* were then fed twice-daily on a commercial brine shrimp diet (Eyre Peninsula Aquafeeds Pty Ltd, South Australia) consisting of rice pollard, soya flour and wheat flour. The diet was prepared by homogenising ingredients in 500 ml seawater in a blender for 15 min (Sunbeam, Australia) and screening to a particle size of 40 µm. *Artemia* were cultured for 5 days to reach approximately 1.5 mm.

Experimental Design

Experiment 1 – Time-course enrichment

Three enrichments were used to examine the time-course uptake of FA, ascorbic acid (AsA; vitamin C) and tocopherols (T; vitamin E) in juvenile *Artemia*:

1. *C. muelleri* - pre-heated to 28°C and completely exchanged every 6 h after rinsing *Artemia*. The concentration was $2.7 \pm 0.1 \times 10^6$ cells ml^{-1}. Algae were cultured according to the method of Coutteau (1996) and harvested on day 5 during logarithmic phase.

2. Algamac 3050 (spray-dried cells of *Schizochytrium* sp. algae, Aquafauna Bio-Marine Inc, USA) - blended for 3 min in 500 ml SW pre-heated to 28°C and completely exchanged every 12 h at a rate of 0.3 g l^{-1} after rinsing *Artemia*.

3. Emulsion containing 54% squid oil (prepared at Fisheries Western Australia) – blended for 3 min in 500 ml SW pre-heated to 28°C and completely exchanged every 12 h at a rate of 0.3 g l^{-1} after rinsing *Artemia*. The oil emulsion included AsA as mixed sodium and calcium salts of ascorbyl phosphate (Rovimix, Stay-C 35, Roche) at 40 mg g^{-1} with <0.2 mg g^{-1} of free AsA, and mixed tocopherols (Cognis Australia Pty Ltd) at 40 mg g^{-1}.

Experiment 2 – Enrichment and starvation

Six enrichments were used to examine the depletion during starvation of FA, AsA and tocopherols in juvenile *Artemia*:

1. *C. muelleri* logarithmic ($2.1 \pm 0.1 \times 10^6$ cells ml^{-1}) - produced and exchanged as for Treatment 1, Experiment 1 and harvested on day 5 during the logarithmic phase.

2. *C. muelleri* stationary ($2.6 \pm 0.1 \times 10^6$ cells ml^{-1}) - produced and exchanged as for Treatment 1, Experiment 1 and harvested on day 8 during the stationary phase.

3. Algamac 0.15 g l^{-1} - prepared and exchanged as for Treatment 2, Experiment 1 at 0.15 g l^{-1}.

4. Algamac 0.3 g l^{-1} - prepared and exchanged as for Treatment 2, Experiment 1 at 0.3 g l^{-1}.

5. Oil emulsion 0.3 g l^{-1} - prepared and exchanged as for Treatment 3, Experiment 1 at 0.3 g l^{-1}.

6. Oil emulsion 0.6 g l^{-1} - prepared and exchanged as for Treatment 3, Experiment 1 at 0.6 g l^{-1}.

For both experiments, *Artemia* were enriched in triplicate 25 L buckets. Buckets received 200,000 x 1.5 mm *Artemia* and the volume was made up to 20 L with pre-heated (28°C) 1 µm filtered SW after addition of enrichment and maintained at 28 ± 1°C. In all treatments, *Artemia* were aerated vigorously (large bubbles), with *Artemia* in Algamac or oil emulsion receiving additional oxygenation through fine air stones to maintain > 90% oxygen saturation. *Artemia* were harvested by pouring through a 125 µm screen and rinsing with 1 µm-filtered SW for approximately 5 min before the initial enrichment and then at each further exchange of enrichment. In the first experiment, *Artemia* were continuously enriched up to 36 h with *C. muelleri* or oil emulsion, and sampled at 0, 3, 6, 12, 24 and 36 h for biochemical analyses. *Artemia* were enriched with Algamac

only up to 24 h, although high mortality precluded sampling beyond 12 h. For Experiment 2, *Artemia* were enriched for only 6 h. Triplicate samples of enrichments were collected during the experiments. Each *Artemia* sample comprised 3 L of the contents from each bucket (containing approximately 30,000 *Artemia* or 180 mg dry matter, DM) screened through a 63 µm mesh, rinsed and resuspended in 1 L of 1 µm-filtered SW. Sample were screened, rinsed with 0.5 M ammonium formate and stored in liquid nitrogen before freeze-drying prior to analysis.

Density and size of Artemia

In Experiment 1, *Artemia* densities, lengths and dry matter were evaluated at each sampling from each bucket. To calculate densities, three 10 ml samples were taken from each replicate, poured through a 250 µm screen and *Artemia* were counted under a dissecting microscope. Mortalities were evident at the 12 h sampling, so at 24 and 36 h, six samples containing 15-30 *Artemia* were taken from each replicate to calculate survival. These were viewed by eye and actively swimming *Artemia* were assessed to be survivors; all others were counted as mortalities. The lengths of *Artemia* (n=10) were measured using a dissecting microscope, digital camera and Scion Image Beta 4.0.2 software (Scion Corporation, Frederick, MD, USA). In Experiment 2, only initial measurements were taken before enrichment and there were negligible mortalities.

Bacteriology

In Experiment 1, a total of 40 *Artemia* (20 per replicate) were sampled from each of the 3 treatments to enumerate heterotrophic bacteria per *Artemia* at 0, 6, 12 and 36 h during enrichment. In Experiment 2, *Artemia* were sampled similarly at 0 h and after 6 h at the highest density of each enrichment, i.e. *C. muelleri* at stationary phase, Algamac at 0.3 g l^{-1} and oil emulsion at 0.6 g l^{-1}, and after starvation for 6 h and 24 h. These times coincided with samples collected for lipid analysis and were taken immediately prior to water exchanges and re-feeding. *Artemia* were homogenised, serially diluted (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) and plated onto ZoBells Marine agar medium or thiosulphate citrate bile salts (TCBS) agar medium (Amyl Media AM243 or AM187, respectively, Australia) for the measurement of total marine heterotrophic bacteria and number of *Vibrio* spp., respectively. After the culture media had been incubated for 24 h at 25ºC, plates containing between 50 and 300 colony forming units were selected and enumerated.

Biochemical analyses

The fatty acid content of diets, *Artemia* during enrichment and starved *Artemia* were analysed in duplicate. Fatty acid methyl esters (FAME) were formed directly by treating samples in a solution of methanol:chloroform:hydrochloric acid (10:1:1) and analysed with an HP5890A gas chromatograph (Agilent Technologies, USA) and Finnigan Thermoquest GCQ GC-mass spectrometer (Austin, Texas, USA) as detailed elsewhere (Lewis et al. 2000).

AsA was extracted from dried *Artemia* (10 mg) using metaphosphoric acid (3%) and acetic acid (8%), derivatised and analysed by HPLC (Brown et al. 1998).

For the analyses of tocopherols (α-,δ- and γ-T), dried *Artemia* (10 mg) were transferred to 10 ml plastic centrifuge tubes, together with 2 ml methanol containing 2 mg butylated hydroxy-toluene. Samples were sonicated (Labsonic 1510 sonic probe; 30 s at 100 W), vortexed (10 s) and left at room temperature for 1 h. Samples were centrifuged (1000 x g, 10 min), the supernatant filtered through a 0.45 µm syringe filter (Alltech) and analysed by HPLC (Huo et al. 1999).

Statistical analyses

Statistical analyses were performed using JMP version 5.0 (SAS Institute Inc.) for one-way ANOVA and Tukey-Kramer HSD tests for post-hoc multiple comparisons (Sokal and Rohlf, 1995). After

arcsin√ transformations were performed, survival data were normal and homogeneous. The level of significance for all analyses was $P < 0.05$. Data are presented as mean±standard error unless stated otherwise.

5.2.1.4 Results

Time-course enrichment (Experiment 1)

Artemia size, survival and dry matter

Initial *Artemia* length was 1.46 ± 0.03 mm and increased significantly during enrichment with *C. muelleri* to 1.77 ± 0.04 mm at 36 h and with Algamac to 1.60±0.04 mm at 24 h, while there was no change in *Artemia* fed the oil emulsion over the 36 h (Fig. 1a). The population density of *Artemia* before enrichment was 14.2 ± 1.0 *Artemia* ml^{-1} (Fig. 1b). Density declined to 10.3-12.6 *Artemia* ml^{-1} ($P < 0.05$) for all treatments during enrichment until 12 h and then remained constant in *C. muelleri*. Density continued to decline to 3.1 *Artemia* ml^{-1} in Algamac at 24 h and to 4.9 *Artemia* ml^{-1} in oil emulsion at 36 h. The proportion of live *Artemia*, which is an instantaneous assessment of survival, was high at 24 h and 36 h when enriched in *C. muelleri* (97 ± 1% and 92 ± 1%, respectively), and was lower ($P < 0.05$) when enriched in oil emulsion (65 ± 2% and 44 ± 1%, respectively). The proportion of live animals was only 14 ± 2% for *Artemia* in Algamac at 24 h after which this treatment was discontinued. The changes in *Artemia* length, density and survival were reflected in biomass per litre (DM l^{-1}) (Fig. 1c). For *Artemia* before enrichment, biomass was 0.075 g l^{-1} and there was little change during the first 12 h for any enrichment. However, after 36 h biomass was reduced by 63% for the oil emulsion and increased by 137% in *C. muelleri*.

Microbiology

The total number of heterotrophic bacteria per *Artemia* immediately before enrichment was 15.5 ± 4.7 x 10^3 (Fig. 2). Bacterial counts remained at this level in *Artemia* enriched with *C. muelleri* at 6 h and 12 h but had increased ($P < 0.05$) at 36 h by 190%. For the oil emulsion, counts also remained low after 6 h enrichment, then increased progressively at 12 and 36 h (190% and 415%, respectively). By contrast, counts in Algamac enrichment increased markedly by as early as 6 h and remained elevated at 12 h (442% and 345%, respectively).

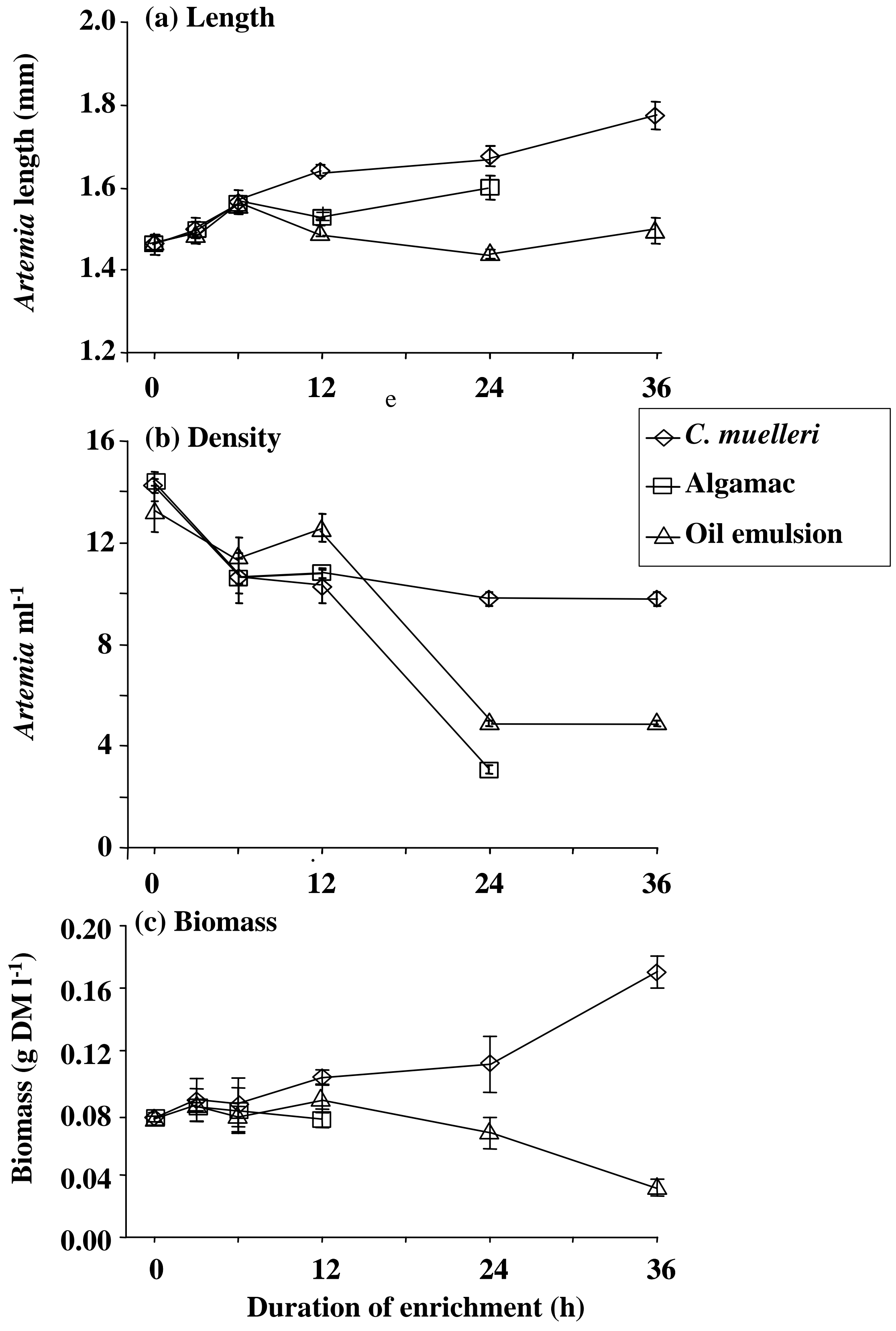

Figure 1. The effect of enrichment of juvenile *Artemia* with *Chaetoceros muelleri* (logarithmic phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.3 g l^{-1}) on (a) length (mm), (b) density (*Artemia* ml^{-1}) and (c) biomass (g DM l^{-1}). Data are mean ± se. (Experiment 1).

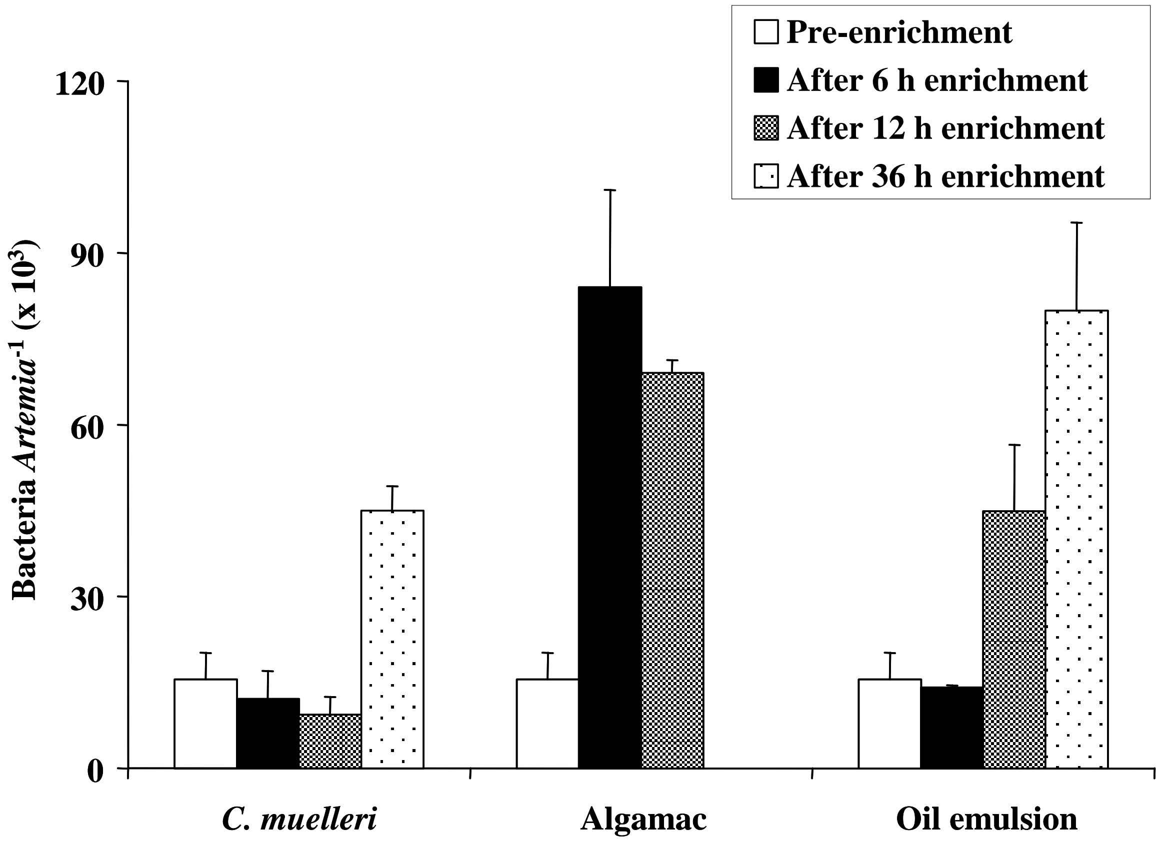

Figure 2. Total number of heterotrophic bacteria (mean ± se) on juvenile *Artemia* during enrichment with *Chaetoceros muelleri* (logarithmic phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.3 g l^{-1}). (Experiment 1).

Fatty acid composition

The FA content of logarithmic phase *C. muelleri* was 150 mg g^{-1} and % abundance of individual FA, in decreasing order, was: 16:3n-4 26%, EPA 21%, 16:1n-7 20%, 14:0 8%, palmitic acid (16:0) 7% and ARA 2% (Table 1). FA content in Algamac was 587 mg g^{-1} and proportional abundance of individual FA, in decreasing order, was: DHA 36%, 16:0 28%, DPA(6) (22:5n-6) 15%, 14:0 11%, EPA 2% and ARA 2%. FA content in the oil emulsion was 655 mg g^{-1} and the proportional abundance of individual FA, in decreasing order, was: 16% each for DHA, 16:0 and oleic acid (18:1n-9c), and EPA 11%.

Pre-enriched *Artemia* contained 123 mg g^{-1} FA and were low in ARA, EPA and DHA (Table 1, Fig. 3) and high in 18:1n-9 (39%) and 18:2n-6 (31%). FA content increased with all enrichments with the degree of increase and changes in profiles reflecting the composition of the diets. Maximum FA content of *Artemia* in *C. muelleri* was achieved after only 6 h enrichment (144 mg g^{-1}) and the composition continued to change over the following 30 h in which EPA doubled (from 8% to 17%) and C_{18} FA reduced by 50%. FA content of *Artemia* fed Algamac rapidly peaked by 6 h (198 mg g^{-1}) with increases in ARA (2%), EPA (4%), DPA(6) (7%) and DHA (17%) and C_{18} FA reduced by 50%.

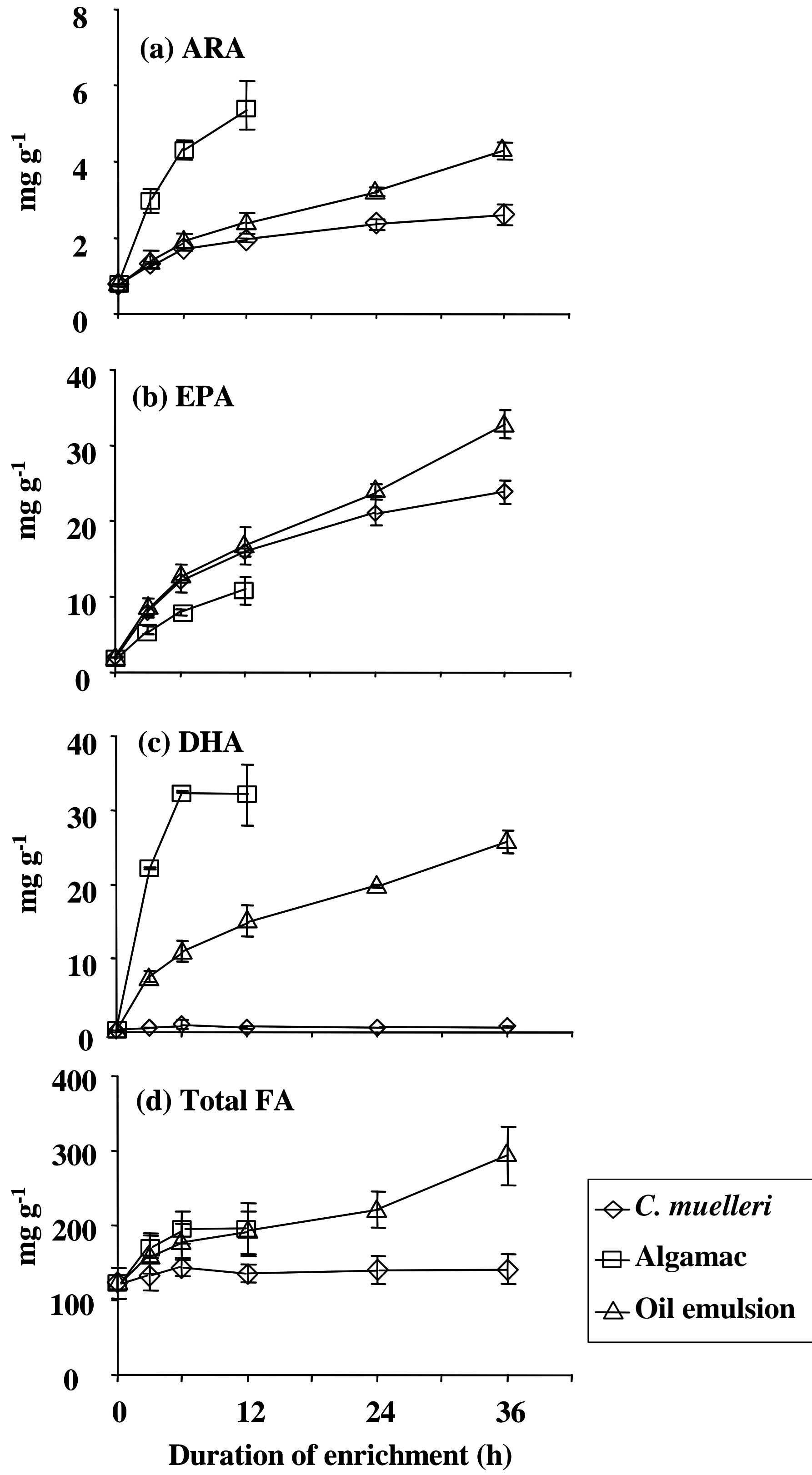

Figure 3. Content (mean±sd, mg g^{-1} DM) in juvenile *Artemia* of (a) arachidonic acid (ARA), (b) eicosapentaenoic acid (EPA), (c) docosahexaenoic acid (DHA) and (d) total fatty acids during enrichment with *Chaetoceros muelleri* (logarithmic phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.3 g l^{-1}). (Experiment 1).

The highest FA content was achieved in *Artemia* enriched with oil emulsion for 36 h (293 mg g^{-1}). EFA were incorporated into *Artemia* at high rates somewhat reflecting the enrichment, although the relative increase in DHA were considerably less than for ARA and EPA. The percentage sum PUFA was elevated after enrichment on all diets and increased over the enrichment period, reaching its highest after 12 h in Algamac (to 52%), which was at the expense of MUFA.

Ascorbic acid (AsA) and tocopherol (T) concentations

Concentrations of AsA in *C. muelleri* and Algamac were 0.60 and 0.04 mg g^{-1}, respectively. The assay did not detect free AsA in the oil emulsion as the ascorbate was present as a stable phosphate ester. *Artemia* initially contained 0.14 mg AsA g^{-1} and there was no significant increase after enrichment with Algamac or oil emulsion (Fig. 4a). In contrast, the concentration of AsA in *Artemia* fed *C. muelleri* increased more than 4-fold over the enrichment period to 0.63 mg g^{-1}.

C. muelleri and Algamac contained 0.30 and 0.001 mg α-T g^{-1}, respectively. Low concentrations of γ-tocopherol (γ-T; 0.02 mg g^{-1}) were also detected in *C. muelleri*. The oil emulsion contained high concentrations of mixed tocopherols; 9.9 mg α-T g^{-1}, 19.6 mg γ-T g^{-1}, and 5.2 mg δ-T g^{-1}. Concentrations of tocopherols in enriched *Artemia* reflected that of their diets. After 6 h enrichment with the oil emulsion, there was a 3-fold increase in α–T from an initial concentration of 0.10 mg g^{-1}, although further enrichment caused a reduction in α–T (Fig. 4b). Enrichment with *C. muelleri* produced an 80% increase in α–T after 24 h, whereas enrichment with Algamac produced a 50% reduction in α–T by 12 h.

Apart from their content of α–T, *Artemia* initially contained trace amounts of γ–T (0.02 mg g^{-1}) and δ-T (0.01 mg g^{-1}). Concentrations of these tocopherols did not change in *Artemia* enriched with *C. muelleri* and Algamac. However, both their concentrations increased significantly in *Artemia* fed the oil emulsion to over 0.50 mg g^{-1} at 6 h for γ–T and 12 h for δ-T (Fig. 4c).

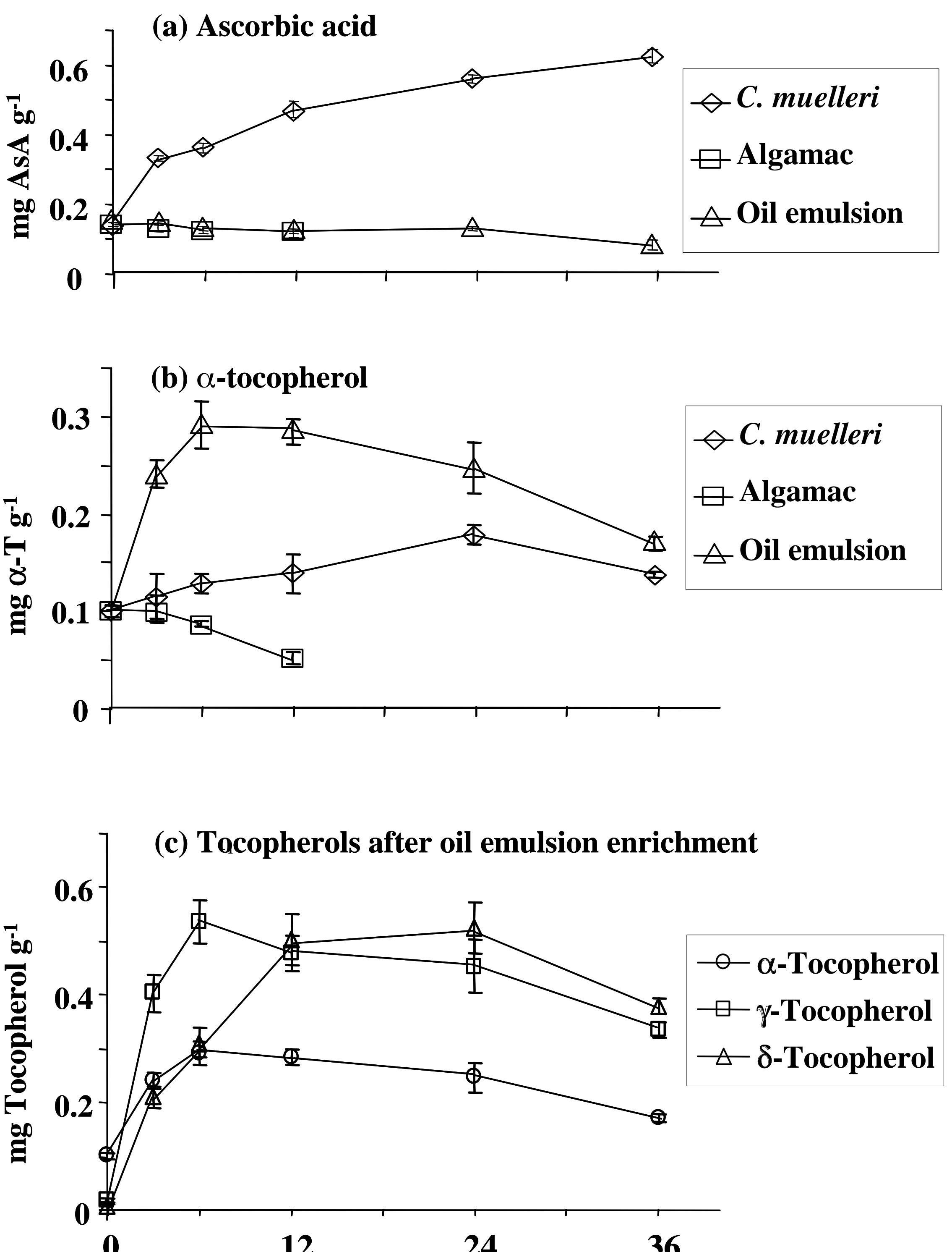

Figure 4. Content (mean±sd, mg g^{-1} DM) in juvenile *Artemia* of a) ascorbic acid, or b) α–tocopherol (α–T) during enrichment with *Chaetoceros muelleri* (logarithmic phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.3 g l^{-1}), and of c) α–, γ– and δ-T during enrichment with oil emulsion (0.3 g l^{-1}). (Experiment 1).

Enrichment and starvation (Experiment 2)

Artemia size and microbiology

Initial *Artemia* length was 1.52 ± 0.04 mm. The total number of heterotrophic bacteria per *Artemia* immediately before enrichment was 10.5 ± 0.8 x 10^3 (Fig. 5a). For *Artemia* enriched for 6 h at 28°C, bacterial counts declined by 81% in *C. muelleri*, increased by 29% after enrichment with Algamac or did not change after enrichment with oil emulsion. After 24 h starvation at 4°C, counts mostly declined by 90-99% for *Artemia* in all enrichments and were lowest in *C. muelleri*-enriched *Artemia*. After 24 h starvation at 18°C, counts were 31% and 65% less in *Artemia* enriched with *C. muelleri* and oil emulsion, respectively, but increased by 182% with Algamac. After 24 h starvation at 28°C, bacterial numbers were elevated for *Artemia* in Algamac (170%) and oil emulsion (20%) but were still 70% lower for *Artemia* enriched with *C. muelleri* than pre-enriched *Artemia*. *Vibrio*. spp. counts were generally 30-70% lower and followed a similar pattern to total counts, except for Algamac-enriched *Artemia* held at 18°C in which counts remained low (Fig. 5b).

Fatty acid composition

FA contents of logarithmic and stationary phases of *C. muelleri* were similar (92 and 108 mg g^{-1}, respectively) but compared to Experiment 1, were 39% lower. However, the proportional abundances of individual FA were similar to Experiment 1. FA contents of Algamac and oil emulsion were 529 and 789 mg g^{-1}, respectively, and the FA profiles were similar to Experiment 1.

FA content and profile of *Artemia* after 6 h enrichment reflected the three diets and were similar to Experiment 1. FA content increased by 20% after enrichment with *C. muelleri* (to 125-133 mg g^{-1}) regardless of whether logarithmic or stationary phase cultures were used (Fig. 6d), and the FA profiles were also similar. FA content increased by 60% and 100% for *Artemia* enriched with Algamac (to 169-173 mg g^{-1}) and oil emulsion (to 192-210 mg g^{-1}), respectively, and the FA profiles between enrichment rates were similar. There was no difference in the improvement in DHA, EPA or ARA content after enrichment of *Artemia* within diet type i.e. logarithmic or stationary phase of *C. muelleri*, or either dose rate of Algamac or oil emulsion. ARA content was about 40% higher after enrichment with Algamac than with oil emulsion or *C. muelleri* (5.3, 3.2 and 3.1 mg g^{-1}, respectively; Fig. 6a). In contrast, EPA content of enriched *Artemia* was highest with oil emulsion, and similar with Algamac and *C. muelleri* (21, 9 and 8 mg g^{-1}, respectively; Fig. 6b). DHA was highest using Algamac (19% of total fatty acids), followed by oil emulsion (10%) and *C. muelleri* (0.2%), which equated to 34, 21 and 0.3 mg g^{-1} *Artemia*, respectively (Fig. 6c).

The FA profile of starved *Artemia* progressed towards that of pre-enriched *Artemia* (Fig. 6) i.e. lower amounts of LC-PUFA. With all diets, FA content of starved *Artemia* was lower than immediately after enrichment and the reduction was greater during starvation at warmer temperatures. After starvation for 6 h, the % loss of FA was lowest with *C. muelleri*, and for the three enrichments was less at 4°C (0-15%) than at 18°C (2-21%) or 28°C (8-21%). The loss was greater after 24 h starvation, i.e. 20-29% at 4°C, 19-32% at 18°C and 41-57% at 28°C, and was similar for all enrichments. The loss of DHA from *Artemia* during starvation at 28°C was >75% for Algamac and oil emulsion treatments, with reduced losses at cooler temperatures, while there were comparatively minor reductions in EPA and ARA.

Ascorbic acid (AsA) and tocopherol (T) concentrations

AsA concentrations in pre-enriched *Artemia* (0.13 mg g^{-1}) and in *Artemia* after 6 h of the respective enrichments were similar to Experiment 1. AsA did not increase in *Artemia* enriched with oil emulsion or Algamac, whereas *Artemia* enriched with *C. muelleri* (either logarithmic or stationary) increased to 0.50 mg g^{-1}. There were no significant changes in AsA concentrations during starvation at 4°C, 18°C or 28°C in *Artemia* fed any of the diets.

α–T concentrations in pre-enriched *Artemia* (0.04 mg g^{-1}) were approximately one-third of those in Experiment 1, while after 6 h enrichment, were similar to Experiment 1. *Artemia* concentrations doubled after enrichment with *C. muelleri* (0.07 mg g^{-1}, logarithmic or stationary). Oil emulsion (at either rate) produced 10-fold increases in α–T (0.31-0.39 mg g^{-1}) but there was no change for Algamac. There were also no changes following 6 h starvation of *Artemia* enriched with *C. muelleri* or Algamac. However, in *Artemia* enriched with oil emulsion and starved for 6 h, the α–T decline depended on temperature, i.e. from 0.39 mg g^{-1} initially to 0.29, 0.23 and 0.20 mg g^{-1} at 4°C, 18°C and 28°C respectively, while after 24 h starvation, the concentrations decreased by 50% or more.

As in Experiment 1, δ–T and γ–T were only present in *Artemia* after enrichment with oil emulsion with concentrations reaching 1.8 mg γ–T g^{-1} and 0.5 mg δ–T g^{-1}. Depletion during starvation showed similar temperature-dependant trends to α–T, although the rates differed. Hence, concentrations of α–T, δ–T and γ–T reduced by 47%, 33% and 80%, respectively, after *Artemia* were starved for 24 h at 28°C.

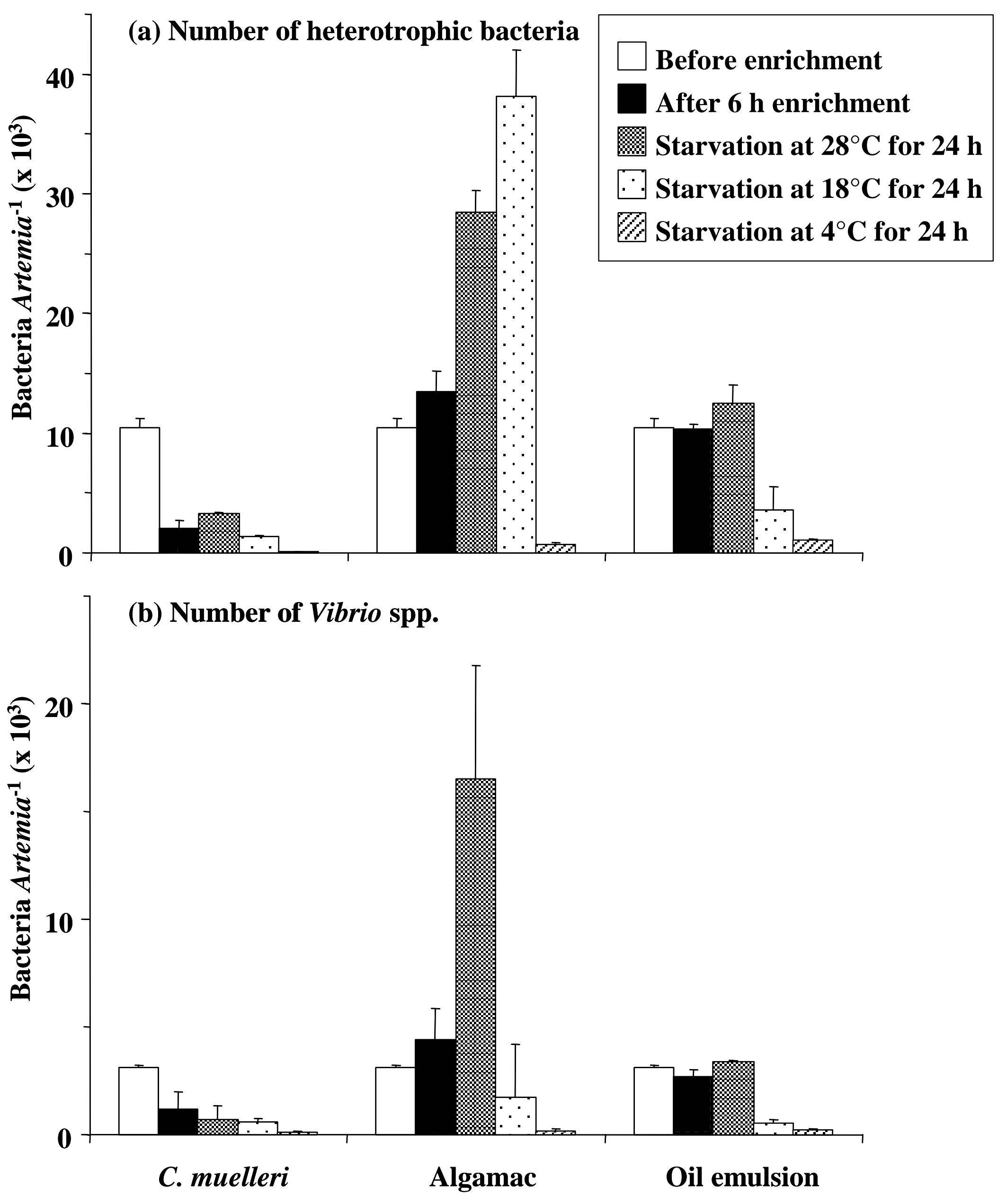

Figure 5. Total numbers of (a) heterotrophic bacteria and (b) *Vibrio* spp. (mean±sem) in juvenile *Artemia* during enrichment with *Chaetoceros muelleri* (stationary phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.6 g l^{-1}) and after 24 h starvation at 4, 18 or 28°C. (Experiment 2).

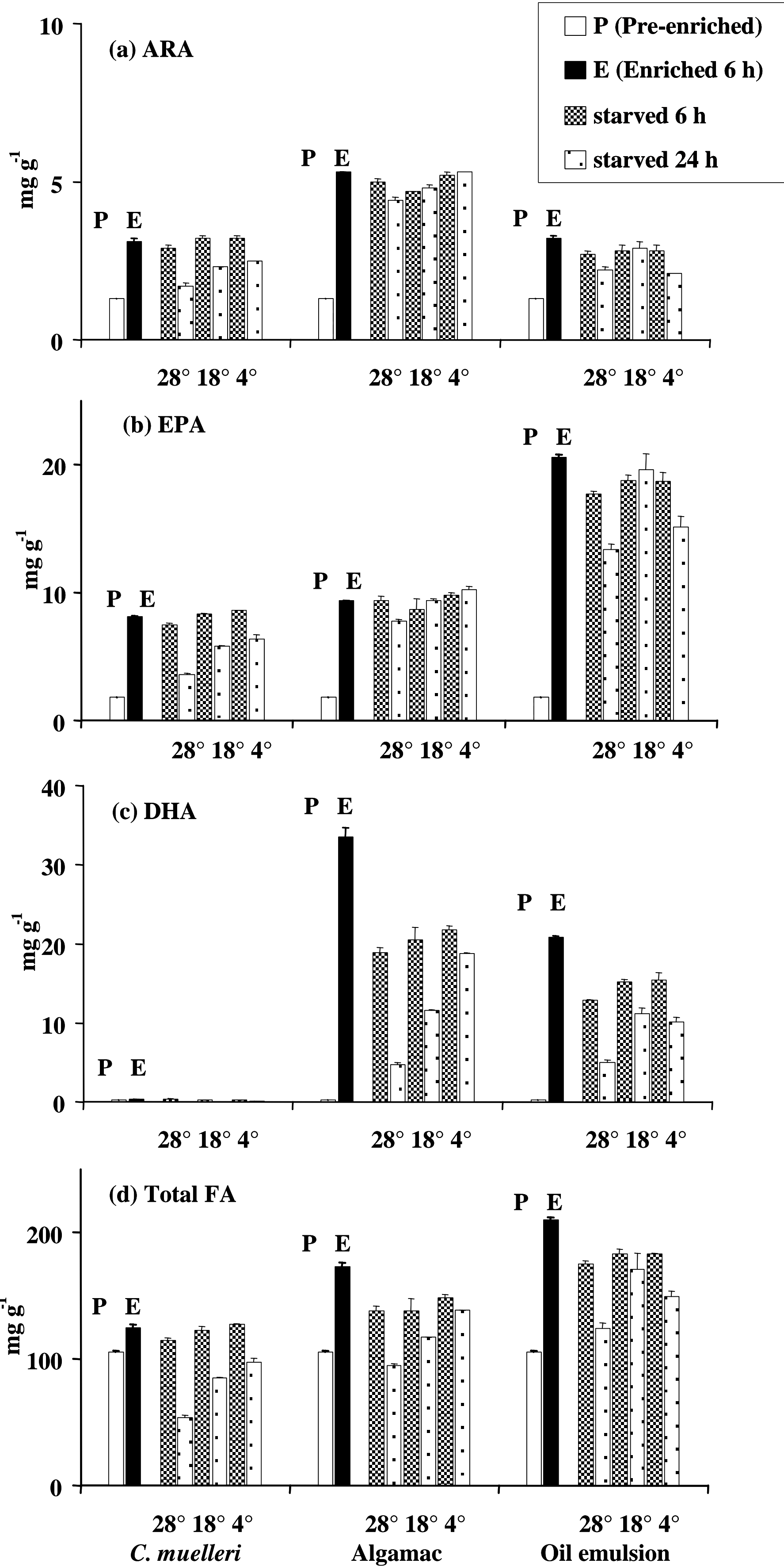

Figure 6. Content (mean ± sd, mg g^{-1} DM) in juvenile *Artemia* of (a) arachidonic acid (ARA), (b) eicosapentaenoic acid (EPA), (c) docosahexaenoic acid (DHA) and (d) total fatty acids during enrichment with *Chaetoceros muelleri* (stationary phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.6 g l^{-1}). (Experiment 2).

Table 1. Percentage composition of major (>3%) fatty acids in pre-enriched *Artemia* and in *Artemia* enriched with *C. muelleri* (logarithmic phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.3 g l^{-1}) for up to 36 h. (Experiment 1).

	Newly-hatched phyllosoma[1]	Enrichments			Pre-enriched *Artemia*	*Artemia* enriched with									
		C. muelleri	Algamac	Oil emulsion		*C. muelleri* for:				Algamac for:		Oil emulsion for:			
					0 h	6 h	12 h	24 h	36 h	6 h	12 h	6 h	12 h	24 h	36 h
14:0	1.5	8.4	10.5	4.2	0.5	2.3	2.8	3.6	4.1	2.8	2.1	1.0	1.0	1.0	1.3
16:2n-4	–	3.3	0.1	–	–	0.9	1.1	1.5	0.4	0.1	0.1	–	–	–	–
16:3n-4	–	26.0	0.1	0.1	0.5	5.9	8.0	10.3	11.3	0.3	0.4	0.4	0.3	0.3	0.4
16:1n-7	3.9	20.4	0.3	4.7	1.8	7.0	9.3	12.0	14.9	1.1	1.2	2.7	2.8	3.1	3.7
16:0	8.3	6.6	28.3	15.9	9.1	8.7	8.6	8.3	8.5	14.8	13.2	10.3	10.1	9.3	10.3
18:2n-6	1.5	0.8	0.0	4.1	31.2	22.9	19.2	14.8	11.7	17.4	17.0	21.1	18.3	16.0	13.8
18:1n-9c/ 18:3n-3	15.7	3.1	0.1	15.7	38.7	28.3	22.8	17.4	13.3	22.4	21.9	31.1	29.5	28.8	27.6
18:1n-7	4.6	0.7	0.1	3.7	4.4	3.7	3.7	4.0	4.4	2.7	2.8	4.5	4.7	4.7	5.0
18:0	4.2	0.7	0.6	3.0	4.9	4.2	4.4	4.5	5.0	3.3	3.4	4.1	3.8	3.2	3.2
20:4n-6 ARA	7.1	2.0	2.1	1.4	0.6	1.2	1.4	1.7	1.9	2.2	2.8	1.1	1.2	1.5	1.5
20:5n-3 EPA	17.8	20.9	2.3	11.3	1.7	8.4	11.6	15.1	17.0	4.0	5.6	7.0	8.8	10.9	11.3
22:5n-6 DPA(6)	–	–	15.2	0.4	–	0.2	0.1	0.1	0.1	6.5	6.7	0.2	0.3	0.3	0.5
22:6n-3 DHA	14.8	1.0	36.1	16.2	0.1	0.8	0.5	0.4	0.6	16.6	16.5	6.1	7.9	8.9	8.9
Other[2]	14.8	6.0	4.3	19.4	6.4	5.7	6.3	6.3	6.8	5.9	6.3	10.4	11.1	12.0	12.6
Sum branched	–	–	0.2	1.1	1.7	0.6	1.2	1.0	0.9	0.8	0.9	1.3	1.1	1.0	1.2
Sum SFA	16.1	17.0	40.1	24.4	16.0	16.7	17.1	17.8	19.1	21.9	19.8	16.8	16.2	14.6	15.8
Sum MUFA	31.9	26.4	0.9	33.4	47.3	41.2	38.0	35.5	34.9	27.7	27.5	42.7	41.7	41.8	42.2
Sum PUFA	46.5	56.6	58.8	41.1	35.0	41.4	43.6	45.7	45.1	49.6	51.8	39.2	41.0	42.5	40.8
Total FA mg g^{-1} DM	70	150	587	655	123	144	136	140	141	198	195	178	191	222	293

Data are mean ± sd values; n = 2; (–), below detection.

ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA(6), docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[1]Newly-hatched phyllosoma profile (target predator species) is included for comparison (Smith et al. 2003b).

[2]Other minor fatty acids included: 12:0, i14:0, C_{14} PUFA, 14:1, br15:0, i15:0, a15:0, 15:0, C_{16} PUFA, i16:0, 16:1n-7t/16:2n-7, 16:1n-9c, 16:1n-5c, 16:1n-13t, 16:0 fatty aldehyde (Falde), 16:1 Falde, br17:0, i17:0, a17:0, 17:1, 17:2, 17:0, C_{18} PUFA, 18:3n-6, i18:0, 18:4n-3, 18:1n-7t, 18:1n-5c, 18:0 Falde, 18:1 Falde, i19:0, 19:1, 20:3n-6, 20:4n-3, 20:2n-6, 20:1n-7c, 20:2, 20:1n-5, 20:1n-11c, 20:1n-9c, 20:0, C_{21} PUFA, 21:0, 22:4n-6, 22:5n-3, C_{22} PUFA, 22:1n-9, 22:1n-7, 22:1n-11, 22:0, C_{24} PUFA, 24:1, 24:0.

5.2.1.5 Discussion

Fatty acids

The nutritional improvement of *Artemia* with diets rich in FA, and in particular LC-PUFA, has been found to be beneficial when they are fed to marine larvae, especially to finfish, although this is not yet clear for crustaceans (Sorgeloos et al. 2003). The chosen representatives of the three main groups of enrichments had lipid content ranging from >10% for the live micro-alga to >50% for the spray-dried alga and oil emulsion, and all were rich in one or more of the LC-PUFA. The target predators for nutritional improvement of *Artemia* in our laboratory were newly-hatched phyllosoma larvae of the spiny lobster *J. edwardsii*, which typically contain 7-12% ARA, 15-18% EPA and 8-15% DHA as proportions of total FA, and lipid content at hatch may range between 50-140 mg g^{-1} DM (Phleger et al. 2001; Smith et al. 2003a,b; Ritar et al, 2003b). Lipid content increases at later stages of development in wild phyllosoma and may reach >300 mg g^{-1} at the final Stage XI before decreasing markedly to <40 mg g^{-1} in the recently-settled puerulus (Phleger et al. 2001). This is due to the tremendous energy expenditure of the puerulus during its long oceanic return journey to close inshore (Jeffs et al. 2001) and metabolic maintenance before it starts to feed (Lemmens, 1994). Therefore, a diet that readily allows the accumulation of lipid reserves during larval development in culture would probably assist in the peri-metamorphic transition to puerulus and post-puerulus.

Artemia fed the oil emulsion achieved, after 36 h enrichment, the highest FA and EPA contents, reflecting their diet. In contrast, *Artemia* fed Algamac increased their total FA content more rapidly and had more ARA, DPA(6) and DHA, as did their diet. Doubling the feeding rate of Algamac (from 0.15 to 0.3 g l^{-1}) or oil emulsion (from 0.3 to 0.6 g l^{-1}) did not appreciably increase the content of ARA, EPA or DHA after 6 h enrichment. Thus, if a short enrichment of 6 h improves efficiency in the hatchery and the high *Artemia* mortalities can be remedied, Algamac may prove to be the most nutritious diet for phyllosoma. Enrichment with *C. muelleri* for 36 h resulted in negligible change in FA content compared to 6 h enrichment. The FA composition of *C. muelleri* is typical of other diatoms, with high levels of 16:0, 16:1n-7, C_{16} PUFA, ARA and EPA, and low levels of C_{18} and C_{22} PUFA including DHA. In this study, there was little difference in the compositions of logarithmic and stationary phase *C. muelleri*. The composition of *C. muelleri* was reflected in gradual changes in these FA in enriched *Artemia* with elevated EPA and low DHA. Decapod crustacean larvae have limited or negligible capacity to convert C_{18} PUFA to LC-PUFA (Suprayudi et al. 2004). With enrichment, C_{18} PUFA were reduced markedly, especially with the *C. muelleri* diet.

The LC-PUFA profile of enriched juvenile *Artemia* generally reflects that of the enrichment, and this offers opportunities for dietary manipulation to meet the needs of the phyllosoma larvae, as found previously by Smith et al. (2002). However, although ARA and EPA accumulation in *Artemia* mirrored the percentage in the enrichment, DHA increased at a lower rate. Estévez et al. (1998) also found previously that *Artemia* metanauplii accumulated less DHA during enrichment and lost it fastest during starvation compared to other LC-PUFA. This is likely to be due to an inability of juveniles to preferentially assimilate DHA during enrichment.

DHA did not undergo significant retroconversion to EPA in juveniles, as is the case with *Artemia* metanauplii (Navarro et al. 1999; Evjemo et al. 2001) because EPA was accumulated at a rate similar to inclusion levels in the enrichments and increased independently of DHA. EPA content of *Artemia* increased after enrichment with the degree dependent on the relative content of the diet. For example,% EPA was highest with the *C. muelleri* diet and increased progressively during 36 h enrichment.

The ARA level in *Artemia* was highest after enrichment with Algamac and was proportionally higher than in the diet, although retroconversion from DPA(6) may be contributing to this increase.

Spiny lobster phyllosoma contain 7-8% ARA (Smith et al. 2003b; Phleger et al. 2001; Ritar et al. 2003b), so they probably have a high requirement for this n-6 PUFA during development. Higher ARA content in larvae has been proposed as beneficial for the stress resistance and pigmentation of crustaceans and finfish (Estévez et al. 1997; D'Souza and Loneregan, 1999; Koven et al. 2001). ARA also has a major role as a precursor of eicosanoid hormones required for moulting and stress response (Lytle et al. 1990; Sargent, 1995). It has been suggested that EPA modulates eicosanoid production from ARA, and failure to supply these two FA in the appropriate balance may result in adverse biochemical responses in the predator larvae (Sargent, 1995). The ARA content can be readily incorporated into *Artemia* via supplementation in oil emulsion (Smith et al. 2002).

Smith et al. (2002) found that for juvenile *Artemia* starved at 28°C for 6 h, there were no losses in lipid content although there were significant changes in the relative levels of FA. In contrast, Evjemo et al. (2001) found a greater than 30% loss in lipid content from enriched *Artemia* metanauplii held at 26°C for 24 h while losses were only 11% at 12°C. In this study, the loss in FA during starvation for 24 h was higher, reaching 57% at 28°C while at 4°C it was only 29%. However, losses were much higher for DHA than for the other EFA, especially at 28°C, reaching 86% (in the Algamac treatment). *Artemia* do not store DHA at similar proportions to other EFA (Evjemo et al. 2001). Nevertheless, the decline in DHA appears to occur at a slower rate in enriched juveniles than in metanauplii (Evjemo et al. 2001; Smith et al. 2002) and is lowest at 4°C, making their refrigerated storage a feasible procedure for delayed feeding to predator larvae.

Vitamins

Algae are usually high in vitamins, especially in AsA and α-T, while commercial fish oil emulsions, such as the customised squid oil emulsion used here, are often supplemented with vitamins during manufacture. The greatest improvement in AsA concentrations of juvenile *Artemia* occurred after enrichment with *C. muelleri*, increasing 4-fold to 0.6 mg g^{-1} DM, which is comparable to the 5-fold increase seen previously using *Isochrysis* spp. (Tahitian strain) (Ritar et al. 2003a). These concentrations are similar to unenriched *Artemia* metanauplii, and are probably sufficient to meet normal dietary requirements of most fish and crustacean larvae (Merchie et al. 1997). However, the enrichment we observed with algae was considerably less than the 60-fold increase after direct enrichment with the particulate ascorbyl-2-phosphate (Smith et al. 2004b), which improved the stress resistance of newly-hatched phyllosoma (Smith et al. 2004a).

There was no increase in AsA after enrichment with the oil emulsion despite the inclusion of 40 mg ascorbyl phosphate g^{-1} DM. Ascorbyl phosphate is converted to free AsA in other fish and crustacean larvae, presumably by phosphatase enzymes (Gouillou-Coutans et al. 1998; Ruff et al. 2001). In our study, failure to increase AsA in *Artemia* may have been due to an insufficient inclusion in the emulsion, poor absorption or low efficiency of conversion of ascorbyl phosphate to AsA by *Artemia*. Regarding the latter, we incubated *Artemia* with acid phosphatase (Merchie et al. 1997; H. Nelis, pers. comm.) before AsA assay, but this was inconclusive as we found a poor conversion to AsA by the enzyme.

The 3-fold difference in α-T concentration of pre-enriched *Artemia* in Experiment 1 (0.10 mg g^{-1}) compared to Experiment 2 (0.04 mg g^{-1}) was not explored further, but may have reflected compositional variations in batches of the commercial on-growing diet. Enrichment with algae gave moderate (up to double) increases in α-T concentration, i.e. to 0.2 mg g^{-1} after 24 h in Experiment 1 and to 0.07 mg g^{-1} after 6 h in Experiment 2. In another study, where juvenile *Artemia* were cultured wholly on algae, α-T concentrations were 0.04-0.08 mg g^{-1} (Vismara et al. 2003). Optimal concentrations of α-T in live feeds have not been defined for larvae of many species in culture, although zooplankton containing 0.07-0.17 mg g^{-1} supported growth of fish larvae (Atlantic halibut, *Hippoglossus hippoglossus*, Rønnestad et al. 1999; fresh water walleye

Stizostedion vitreum, Kolkovski et al. 2000). It is yet to be established whether higher doses of this antioxidant are beneficial, but this may be so when larvae are fed high PUFA diets as α-T is degraded to protect PUFA against oxidation (Stéphan et al. 1995). In this context, inclusion of high α-T concentrations (and the less biologically active δ–T and γ–T) in *Artemia* enriched with marine oil emulsions may be warranted because of their corresponding high PUFA. Following the same reasoning, while Algamac-enriched *Artemia* are also rich in PUFA, their low concentrations of α-T may be sub-optimal for larvae. More rigorous experimentation is needed to test these hypotheses.

There was no reduction in AsA concentration during starvation of *Artemia* juveniles for any of the enrichments regardless of temperature, which is in accord with findings for *Artemia* metanauplii enriched with ascorbyl palmitate and later starved (Merchie et al. 1995). In contrast, tocopherols concentrations in *Artemia* enriched with oil emulsion reduced significantly following 24 h starvation, although they remained higher than for pre-enriched *Artemia*.

Survival, growth and bacterial abundance

Although the primary purpose of enrichment is to improve the nutritional composition of juvenile *Artemia*, there may be other consequences. *Artemia* will use some diets, such as the microalgae *C. muelleri*, as food resulting in a considerable increase in body size and biomass over the 36 h enrichment. In contrast, *Artemia* enriched with Algamac or oil emulsion did not grow under the regime used here. Further, the 30% mortality in *Artemia* stabilised after 6 h enrichment with *C. muelleri*, whereas there were continuing losses in the other enrichments. This was more severe with Algamac, which resulted in almost complete mortality by 24 h despite, maintaining high oxygenation during enrichment, and appeared to be associated with extensive proliferation of bacteria. For all treatments, enrichments were exchanged every 6 h (*C. muelleri*) or 12 h (Algamac and oil emulsion) after rinsing *Artemia*. Thus, it appears that a considerable remnant bacterial burden was carried over into the following enrichment period where there was a further build up. This burden was attached directly to *Artemia* despite rinsing away excess enrichment for at least 5 min in an attempt to reduce the bacterial levels in the culture water (Lavens and Sorgeloos, 1996).

Optimising the *Artemia* composition with nutrients that are needed for larval development would possibly improve phyllosoma performance in culture, although this is yet to be proven. A recent study in which juvenile *Artemia* were variously enriched failed to demonstrate improvements in larval growth, and more importantly in survival, when fed to phyllosoma (Nelson et al. 2003). It is probable that the nutritional improvement after enrichment of *Artemia* was outweighed by the higher microbial contamination attributable to some of the diets. We found that bacterial numbers increased rapidly to their maximum after 6 h of Algamac enrichment, more than 4-fold the levels prior to enrichment. Bacterial proliferation was slower with the oil emulsion, taking at least 12 h to reach comparable bacterial numbers. Elevated numbers of bacteria with the *C. muelleri* enrichment were only apparent at 36 h. *Artemia* typically contain high bacterial loads especially of *Vibrio* spp., including pathogens to marine larvae in culture (Makridis et al. 2000; Olafsen, 2001). Some enrichments may accentuate this problem, leading to complete mortality of larvae in culture when fed contaminated *Artemia* (unpublished data). In contrast, pathogenic *Vibrio* spp. and externally fouling *Leucothrix*-like bacteria are diminished in phyllosoma during starvation because they are not exposed to bacteria-laden *Artemia* (Ritar et al. 2003b) or their organic wastes. Thus, if bacterial numbers are a concern, then a short enrichment, which still allows for significant nutritional improvement while minimising the microbial burden, should be considered, especially with diets such as Algamac or oil emulsions.

Nutrients that enhance the growth of opportunistic and pathogenic bacteria (Olsen et al. 2000; Olafsen, 2001) are probably higher in the leachate from Algamac particles and in oil emulsion. If *Artemia* are enriched for longer than 6 h with either of these diets, then the high microbial content,

which is transferred to culture tanks, would probably reduce larval survival. In fish larvae, these bacteria associated with *Artemia* are consumed and have a seriously adverse impact on gut physiology (Ottesen and Olafsen, 2000) and this is also likely to be the case in lobster phyllosoma.

Bacterial proliferation in *Artemia* fed *C. muelleri* was low, even exhibiting somewhat of a reduction after 6 h enrichment. Some microalgae appear to be naturally bacteriostatic or bactericidal (Kellam and Walker, 1989; Olsen et al. 2000), disinfective properties useful during enrichment of *Artemia* at warm temperature and high nutrient load typical of the intensive, static cultures, which are otherwise conducive to bacterial growth. For example, *C. muelleri* may be used to sanitize the external and internal surfaces of *Artemia* by short-term (0.5-3 h) purging, either alone (Tolomei et al. 2004) or in combination with formaldehyde (unpublished data). These algal treatments markedly reduced the bacterial content (especially *Vibrio* spp.) compared to enriched *Artemia* remaining untreated. It must be cautioned that algal cells may themselves be carriers of bacteria including *Vibrio* spp., occurring in much higher numbers in stationary than logarithmic phase cultures (Salvesen et al. 2000).

The bacterial numbers remained high or even increased during starvation for up to 24 h at 18 or 28°C, temperatures that mimicked *Artemia* residence in culture for temperate or tropical lobster larvae, respectively. Interestingly, enriched *Artemia* held at 4ºC for up to 24 h displayed a reduction in total and *Vibrio* numbers suggesting this may be a possible means to disinfect *Artemia* of pathogenic *Vibrio* spp. prior to feeding of phyllosoma. However, recent studies showed that *Vibrio* held at low temperatures enter into a state termed "viable but non-cultureable" (VBNC) (Jiang and Chai, 1996; Johnson and Brown, 2002). For example, *V. vulnificus* could no longer be detected by direct plating on TCBS agar after 24 h at 4ºC but these previously VBNC cells were able to revert to growth following a temperature upshift before plating (Johnson and Brown, 2002). Furthermore, Oliver (1995) found that VBNC cells have the potential to cause infection following resuscitation. Further work is required to confirm whether storage of *Artemia* at 4ºC results in *Vibrio* spp. becoming VBNC or killed/inactivated before the treatment could be considered a means of disinfection.

5.2.1.6 Conclusions

The type and the duration of enrichment markedly influenced the biochemical composition and bacterial content of juvenile *Artemia*. The greater depletion rate during starvation at higher temperatures indicates that freshly-enriched *Artemia* may need to be fed more than once daily in tropical hatcheries to ensure their nutritive value. The minimal loss of enrichment at 4°C makes refrigerated storage before feeding a viable proposition, with a possible further benefit of *Artemia* disinfection. The use of *C. muelleri* was fortuitous in exemplifying both its nutritive and anti-bacterial effects, although some other algae may have equivalent properties. In contrast, Algamac and oil emulsion increased microbial levels probably due to the presence of nutrients conducive to their rapid proliferation. It may be argued that the relative changes in microbial content, particularly of the pathogenic *Vibrio* spp., are of greater importance than changes in the nutritional profile.

On the basis of all parameters examined, enrichment of juvenile *Artemia* for 6 h is most appropriate. In future, other enrichments and feeding strategies, such as mixed diets of bactericidal micro-algae in combination with Algamac or vitamin-enhanced oil emulsions, could also be examined. The potential of diets to improve lobster phyllosoma survival and growth needs to be determined in a culture environment where there is no interference from microbial pathogens.

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5.2.2 Influences of dietary n-3 long-chain PUFA on body concentrations of 20:5n-3, 22:5n-3, and 22:6n-3 in the larvae of a marine teleost fish from Australian waters, the striped trumpeter (*Latris lineata*)

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We determined the effect of dietary long-chain (greater than or equal to C(20)) PUFA (LC-PUFA), 20:5n-3 and 22:6n-3, on larval striped trumpeter (*Latris lineata*) biochemistry through early development and during live feeding with rotifers (*Brachionus plicatilis*). Rotifers were enriched using seven experimental emulsions formulated with increasing concentrations of n-3 LC-PUFA, mainly 20:5n-3 and 22:6n-3. Enriched rotifer n-3 LC-PUFA concentrations ranged from 10-30 mg/g dry matter. Enriched rotifers were fed to striped trumpeter larvae from 5 to 18 days post-hatch (dph) in a short-term experiment to minimize gross deficiency symptoms such as poor survival that could confound results. No relationships were observed between larval growth or survival with dietary n-3 LC-PUFA at 18 dph. The larval FA profiles generally reflected those of the rotifer diet, and significant positive regressions were observed between most dietary and larval FA at 10, 14, and 18 dph. The major exception observed was an inverse relationship between dietary and larval 22:5n-3. The presence of 22:5n-3 in elevated amounts when dietary 22:6n-3 was depressed suggests that elongation of 20:5n-3 may be occurring in an attempt to raise body concentrations of 22:6n-3. We hypothesize that accumulation of 22:5n-3 might be an early indicator of 22:6n-3 deficiency in larval fish that precedes a reduction in growth or survival. A possible role of 22:5n-3 as a biochemical surrogate for 22:6n-3 is discussed.

5.2.3 The effect of various *Artemia* fatty acid enrichment preparations on growth and survival in yellowtail kingfish (*Seriola lalandi*) and pink snapper (*Pagrus auratus*) larvae

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5.2.3.1 Abstract

Commercially available *Artemia* fatty acid enrichment products DHA Selco® (DS), Super Selco® (SS) and Super HUFA® (SH), oil emulsions, Algamac 2000® (A2), which is a powder enrichment based on spray dried *Schizochytrium* sp., and a control treatment (C) with no enrichment, were compared for growth and survival of yellowtail kingfish (*Seriola lalandi*) and pink snapper (*Pagrus auratus*) larvae, until 31 days post hatch (dph) and 35 dph, respectively. An experimental fatty acid enrichment (Exp.) was included in the yellowtail kingfish comparison and a newly hatched *Artemia* nauplii treatment (NH) was included for pink snapper. The larvae were reared using an up-welling flow through 'green water' technique, using *Nannochloropsis* sp., rotifers and fed *Artemia* enriched using one of the enrichment products mentioned previously.

The feeding of fatty acid enriched *Artemia* metanauplii to yellowtail kingfish significantly ($P < 0.05$) improved both growth and survival, when compared to feeding unenriched metanauplii. Larvae in treatments SH, SS, Exp, A2 and DS (150.7 ± 22.1 mg, 151.8 ± 8.8 mg, 138.6 ± 15.8 mg, 141.8 ± 7.1 mg and 151.1 ± 2.8 mg, WW respectively), were not significantly different from each other, but were significantly ($P < 0.05$) larger than larvae in treatment C (78.3 ± 3.3 mg, WW). There were significantly higher levels of essential fatty acids in *Artemia* enriched by all of the oil emulsion enrichment products, when compared to treatment A2. The experimental enrichment sustained significantly ($P < 0.05$) better survival than all other treatments, except SH, which in turn was not significantly different from the other enrichment treatments.

Pink snapper grew significantly ($P < 0.05$) better when fed unenriched *Artemia* metanauplii in treatment C (71.7 ± 6.0 mg, WW), relative to all other treatments, receiving fatty acids enriched metanauplii (treatments SS, DS, SH and A2 achieving a final WW of 57.2 ± 8.1 mg, 63.2 ± 0.7 mg, 48.2 ± 4.4 mg and 51.0 ± 5.5 mg, WW respectively). Larvae in treatment NH (61.3 ± 1.1 mg, WW) were not significantly different from any of the treatments. However, survival was significantly ($P < 0.05$) lower in both NH and C treatments (9.0 ± 1.6%, and 11.1 ± 3.4% survival, respectively), than treatments SS, DS, SH and A2. (18.7 ± 3.4%, 22.8 ± 2.5%, 29.7 ± 6.5% and 24.0 ± 2.9% survival, respectively) which were not significantly different from each other.

Bacterial levels in enriched *Artemia* nauplii were significantly ($P < 0.05$) higher in treatment A2, and the resulting bacteria levels in the larvae culture tank water were elevated after *Artemia* were administered. However, bacterial levels did not have a direct effect on growth or survival of yellowtail kingfish and / or pink snapper.

Based on the results from this experiment commercial hatcheries can choose the *Artemia* enrichment product best suited to their needs, relative to growth, survival, rigor and bacterial load data.

5.2.3.2 Introduction

The successful culture of marine finfish larvae is heavily reliant on live feeds such as rotifers (*Brachionus* sp.) and brine shrimp (*Artemia*) (Baskerville-Bridges and King, 2000; Sorgeloos et al. 2001). They are thought to stimulate larval feeding and endogenous enzyme production by their movement and chemical release, and may also provide a stimulus for ontogenetic intestinal development (Kolkovski et al. 1995; Hart and Purser, 1996; Kolkovski et al. 1997; Cahu and Zambonino Infante, 2001; Kolkovski, 2001; Aragao et al. 2004). The feeding of live prey with controlled levels of nutrients such as fatty acid composition, are amongst the most commonly used methods employed for investigating the nutritional requirements of marine finfish larvae (Izquierdo, 2004).

There are two stages at which *Artemia* nauplii are used, depending on larval development and mouth gape (Moretti et al. 1999). Newly hatched *Artemia* nauplii (instar I) are relatively small (350-400 µm) and therefore are fed at the end of the rotifer stage (Lavens and Sorgeloos, 1996). However their nutritional value is often poor (depending on strain, harvest, season, etc.), they are low in protein and amino acids and often lack essential fatty acids (EFAs) (Watanabe et al. 1978, 1980, 1982; Sorgeloos et al. 2001; Helland et al. 2003). Therefore, as soon as the fish larval mouth gape is large enough, the *Artemia* nauplii are then fed for 24 h on an enriched media containing EFAs (n-3 HUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) (Izquierdo, 2004). This process leads to significantly larger (550-650 µm) *Artemia* metanauplii, however the n-3 HUFA enrichment improves their nutritional value for fish larvae and is a standard procedure in marine finfish hatcheries (Barclay and Zeller, 1996; Sargent et al. 1999a, 1999b; Sorgeloos et al. 2001). There are a number of commercial fatty acid enrichments available in different forms, as lipid emulsions or dry powders. Some of those

available in Australia include, INVE 'Selco' products, Algamac 2000 and Super HUFA. The Selco and Super HUFA products are oil emulsions, while Algamac 2000 is a spray-dried, heterotropically grown algae (*Schizochytrium* sp.).

However, complicating factors such as high levels of pathogenic bacteria can be associated with live feeds and can adversely affect fish larvae performance (Moretti et al. 1999; Makridis et al. 2000; Olsen et al. 2000; Olafsen, 2001). Specifically, Olsen et al. (2000) larvae demonstrated a correlation between microbial levels in live feeds and rates of mortality. It has been shown in previous work by the authors, that enrichment products often encourage the proliferation of bacteria, leading to significant levels of pathogenic bacteria such as *Vibrio sp*. (Curnow et al. 2004b).

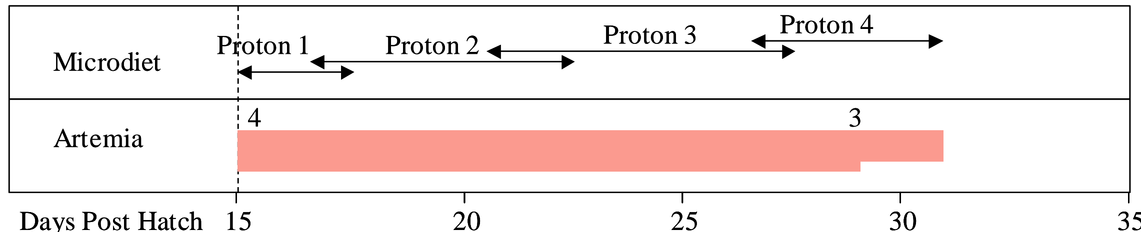
The current trial compares the effects that five *Artemia* fatty acid enrichment products and the associated levels of bacteria have on the growth and survival of yellowtail kingfish (*Seriola lalandi*) and pink snapper (*Pagrus auratus*) larvae.

5.2.3.3 Methods

Yellowtail kingfish larvae rearing protocol

Yellowtail kingfish larvae were reared from eggs, obtained from wild broodstock by Cleanseas SA, using a static 'green water' technique (Moretti, 1999) using *Nannochloropsis* sp. and fed on enriched rotifers (Challenger TAFE, Aquaculture Development Unit, Fremantle). Starting at 7 dph, newly hatched *Artemia* nauplii were fed to larvae at a rate of 1 *Artemia* ml^{-1}. At 15 dph the larvae were stocked into the experimental system (Kolkovski et al. 2004a) and fed according to the protocol in Fig. 1. Twenty-four 270 L conical tanks were each stocked with 380 larvae.

Yellowtail kingfish



Pink snapper

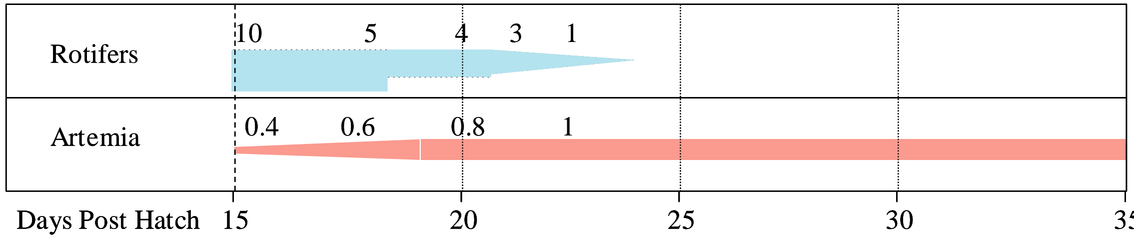


Figure 1. Weaning protocols, from 15 dph to 35 dph, are shown. MD was fed to apparent satiation, rotifer figures are expressed as 'number of rotifers ml^{-1}' continuously and *Artemia* figures are expressed as 'number of *Artemia* ml^{-1} day^{-1}'. Temporal MD size class distribution is shown for Proton MD, with an overlap during each transitional period.

First feeding of enriched *Artemia* nauplii occurred at 15 dph. *Artemia* were enriched according to (Kolkovski et al. 2004b) using commercial enrichment products DHA Selco®, Super Selco® and Super HUFA®, oil emulsion fatty acid enrichment products, and Algamac 2000® a powder

enrichment, based on spray dried *Schizochytrium* sp., (DS, SS, SH and A2) respectively. An experimental oil emulsion enrichment (treatment Exp), prepared by Nutrakol, was used to the same specification as the commercial oil emulsion enrichment products. Treatment C, fed *Artemia* that had been through the same enrichment process, but the enrichment products were substituted with fresh water.

Proton (INVE) microdiet was fed according to relative particle size in accordance with larvae size, starting from the smallest size class and increasing as the larvae developed. During the transition from one size range to the next largest, the diets were administered as a blend for a 3 day period (Figure 1).

Periodically, 5 random larvae from each tank were removed and sacrificed using iced water. These larvae were measured for standard length, weight and then dry weight after 48 h at 80°C. The final observations were based on 20 larvae for each tank.

Algae was automatically supplied (Kolkovski et al. 2004a), while enriched *Artemia* metanauplii (method according to Kolkovski et al. 2004b) were manually supplied 4 times daily, and MD was administered at intervals of 1 h. Algae concentration was maintained in the larvae tanks in order to give a secchi disc depth of average 72 ± 16 cm. The marine bore water temperature averaged 21.2 ± 0.35°C and was supplied at a flow rate of 1.5 L min^{-1}. The physiochemical parameters were maintained at pH 7.99 ± 0.03, D.O. at 7.2 ± 0.5 ppm, salinity at 33.5‰ and a photoperiod of 14 h light / 10 h dark.

Pink snapper larvae rearing protocol

Pink snapper larvae were reared according to the protocol followed for yellowtail kingfish, using eggs obtained from F2 broodstock (Aquaculture Development Unit, Challenger TAFE, Fremantle). However, starting at 12 dph, newly hatched *Artemia* nauplii were fed to larvae at a rate of 0.5 nauplii ml^{-1}. At 15 dph the larvae were stocked into experimental tanks and fed according to the protocol (Figure 1). Twenty-four 270 L conical tanks (Kolkovski et al. 2004a) were each stocked with 4,000 larvae. The larvae were reared using up-welling flow through 'green water' technique, using *Nannochloropsis* sp. and rotifers.

First feeding of enriched *Artemia* nauplii occurred at 15 dph. *Artemia* that were fed to treatments C, DS, SS, SH and A2 concur with those that were fed to yellowtail kingfish, in regards to method and enrichment. In treatment NH, *Artemia* that had been incubated at 28°C and harvested at 24 h (newly hatched nauplii) were supplied.

Periodically, 3 random larvae from each tank were removed and sacrificed using iced water. These larvae were measured for standard length, weight and then dry weight after 48 h at 80°C. The final observations were based on 20 larvae for each tank.

Algae was automatically supplied (Kolkovski et al. 2004a), while rotifers and enriched *Artemia* (method according to Kolkovski et al. 2004b) were manually supplied 4 times daily and maintained at prescribed concentrations in the larvae culture tanks. Algae concentration was maintained in the larvae tanks in order to give an average secchi disc depth of 48 ± 14 cm. The marine bore water temperature averaged 19.0 ± 0.5°C, with a flow rate of 1.0 L min^{-1}. The physiochemical parameters were maintained at pH 8.10 ± 0.01, D.O. at 6.9 ± 0.8 ppm, salinity at 33.5‰ and a photoperiod of 14 h light / 10 h dark.

Stress Test

Following the trial (at 35 dph), stress tests were conducted on pink snapper larvae by pouring a beaker of water containing 10-15 larvae from each tank through a screen in order to capture the larvae on the screen. The screen was then blotted dry from behind with paper towel. The larvae

were left on the dried screen for 2 min and then returned to a container of water. The proportion of survivors after 15 min was recorded and averaged for 4 treatment replicates.

Artemia nauplii

Artemia cysts (GSL strain, INVE) were decapsulated using chlorine and sodium hydroxide for approximately 5 minutes, then rinsed with seawater until all traces of chlorine was eliminated (Van Stappen, 1996). Excess water was then removed using a suction filter prior to storage at 4°C in an airtight bag, until used within 24-48 h.

Artemia nauplii hatched from decapsulated cysts were used in all enrichment treatments and the control treatment. *Artemia* were hatched in a static culture of marine bore water (35‰) over a 22 h period at 28.0°C ± 0.1°C. Cysts were added to hatching tanks at a rate of 1.2 g l^{-1} of hydrated decapsulated cysts, which corresponded to 0.6 g l^{-1} dry non-decapsulated cysts.

Artemia nauplii were harvested using light in order to separate them from the unhatched cysts and shell debris. The light promotes the migration of the *Artemia* nauplii away from the cyst debris and allows the wastes to be removed with minimal loss of live *Artemia* (Lavens and Sorgeloos, 1996). *Artemia* nauplii were rinsed at a rate of 10 L min^{-1} for 20 min (1 vol min^{-1}) in a bucket fitted with a 100 µm outlet screen.

Artemia nauplii were enriched over an 18 h period in a static culture of marine bore water (35‰) at a rate of 1.25 x 10^5 nauplii l^{-1} in 20 L of water. Enrichments were added at a rate of 0.45 g l^{-1} of seawater. Enrichments were mixed with 1 L of fresh water using a kitchen blender (Kenwood chef) at high speed for 3 min at 30°C. They were then added to the *Artemia* culture in two equal portions, the first at 3:30 pm and the second automatically at 2:00 am the following morning. Enrichments were kept cool with ice bricks before introducing them into the *Artemia* enrichment tanks.

Bacterial assay

Sampling for bacteria occurred at 11:30 am following hatching and during harvest of enriched *Artemia* at 9:30 am following enrichment, the subsequently day. Enriched *Artemia* were rinsed at a rate of 10 L min^{-1} for 30 min (1 vol min^{-1}) in a bucket fitted with a 100 µm outlet screen.

Sampling equipment was pre-sterilized using ethanol spray. A 100 µm screen was used to separate *Artemia* from the enrichment or hatching media then rinsed with distilled water and excess water was allowed to drain off. The resultant *Artemia* nauplii were at a concentration of 2,160 ± 20 ml^{-1}. *Artemia* nauplii were kept on ice until processed and were then homogenized using a high-speed homogeniser (IKA). *Artemia* cysts were homogenized using a sterile mortar and pestle.

Enrichment media were sampled using sterile 5 ml sampling vials. Samples were taken from the outlets of the harvesting containers (100 µm screened 15 L buckets, holding capacity 10 l).

Larvae rearing tank water was sampled by immersing a sterile 100 µm screen into the tank and filling a 5 ml sterile sample vial beneath the surface from within the screen.

Both *Artemia* nauplii and culture media samples were serial diluted (Quinn et al. 1994) and a 100 µl sample was spread onto MSA-B (Marine Salt Agar-Blood, composed of Trypticase Soya agar, 3% horse blood and 2% NaCl) . Dilutions for *Artemia* nauplii and cysts were to 10^{-5} dilution, and culture media samples were diluted to 10^{-4}, in order to get a suitable number of CFU (Colony Forming Units) $plate^{-1}$ for counting. Samples were plated at each of the dilutions produced for *Artemia* nauplii, cysts and culture media.

All agar plating was carried out in a laminar flow cabinet in sterile conditions. All equipment was sterilized using flame, ethanol spray or autoclave. Dilution media was tested for microbial activity to guarantee sterility of media and methods. Prior to use, marine bore water, algae and enrichments, were tested for microbial levels.

The plates were left at room temperature (≈25°C) for 24 h before counting colonies formed per plate. Whole plates were counted when possible or two quarter plate areas were counted, averaged and multiplied by four, when colony numbers were high (Quinn et al. 1994).

Data analysis

Replicate tank survival was calculated by dividing the total number of larvae removed from each tank at 31 dph (yellowtail kingfish) and 35 dph (pink snapper), by the number of larvae stocked into each tank at 15 dph, minus the mortalities removed directly after initiation (not resulting from treatment effects) and those larvae removed for ongoing developmental observations. Treatment survival was averaged for four replicate tanks.

All values are means ± standard error unless otherwise specified. Data were analysed for homogeneity of variances using Cochrans test. Effects of enrichment type on larvae growth and survival were examined by one-way ANOVA. Significance of differences were determined using Tukeys HSD test, with limits for critical ranges being set at $P < 0.05$.

Artemia lipid analysis

Total lipid content was determined gravimetrically following the extraction of the lipids using the method of Folch et al. (1957). Fatty acid levels were determined using gas chromatography (Department of Agriculture, Western Australia).

5.2.3.4 Results

Yellowtail kingfish

The resulting average WW of larvae at 31 dph in treatments SH, SS, Exp, A2 and DS (150.7 ± 22.1 mg, 151.8 ± 8.8 mg, 138.6 ± 15.8 mg, 141.8 ± 7.1 mg and 151.1 ± 2.8 mg, WW respectively), were not significantly different from each other, but were all significantly ($P < 0.05$) larger than larvae in the treatment C (78.3 ± 3.3 mg, WW) (Figure 2). The growth in yellowtail kingfish was linked to the total essential fatty acids (EPA and DHA) levels and total lipid levels in enrichment treatments (Figure 3).

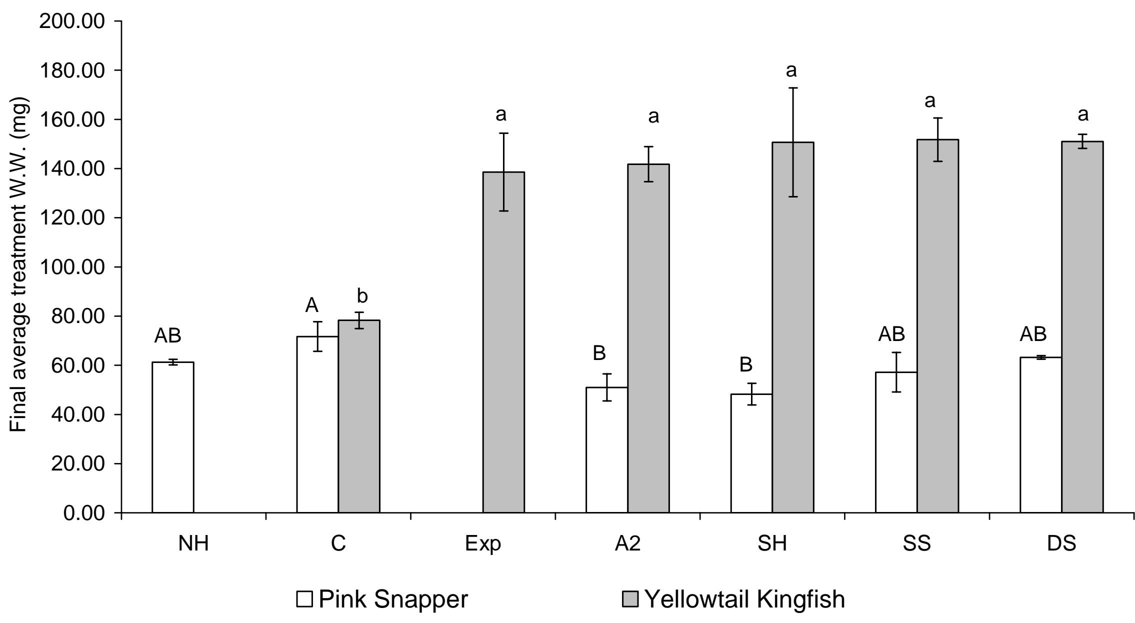


Figure 2. Average final wet weight (mean ± SE, n=4), for yellowtail kingfish at 31 dph and pink snapper at 35 dph. Lower case letters denote significant ($P < 0.05$) differences between yellowtail kingfish treatments, and capital letters denote significantly between pink snapper treatments.

The percentage survival for treatment Exp (58.8 ± 7.2% survival) was not significantly different from treatment SH (50.1 ± 5.3% survival), but was significantly ($P < 0.05$) higher than treatments SS, DS, A2 and C (42.0 ± 3.1%, 41.5 ± 5.1%, 38.6 ± 8.6%, and 20.7 ± 1.3% survival, respectively), which were not significantly different from treatment SH (Figure 4). Treatment C showed significantly ($P < 0.05$) lower survival than all other treatments. The survival in yellowtail kingfish was linked to the total essential fatty acids (EPA and DHA) levels in enrichment treatments (Figure 3).

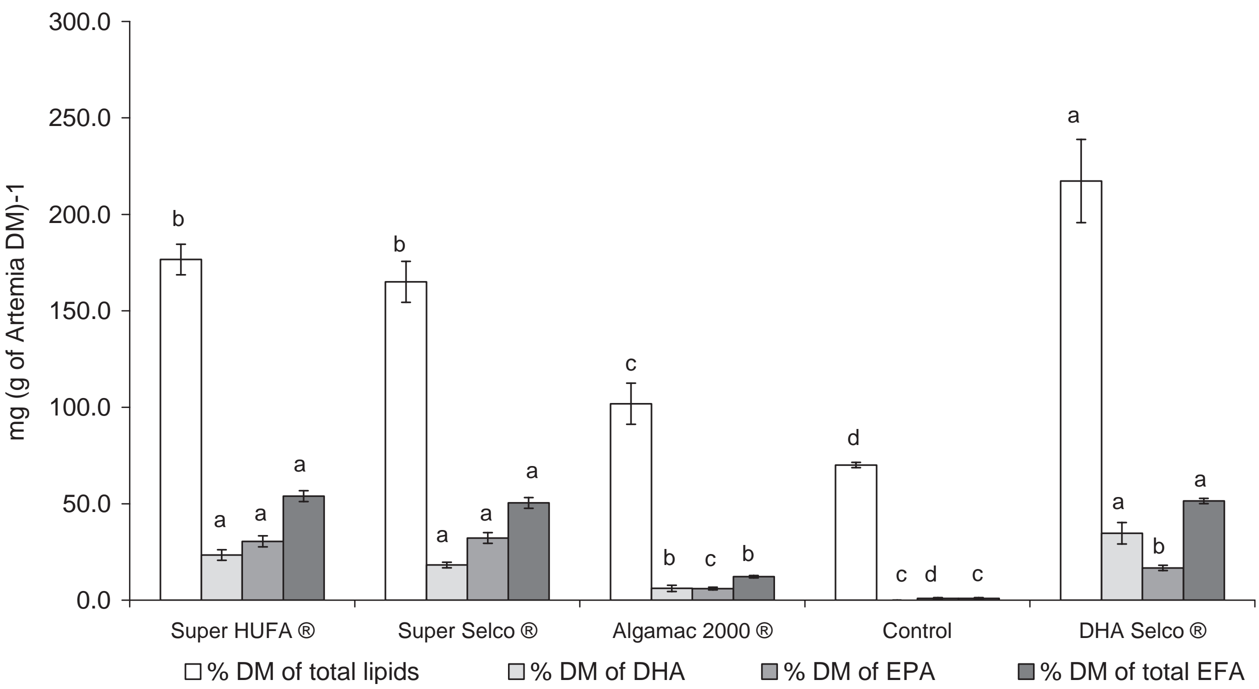


Figure 3. Total lipid, EFAs eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) levels, and total EFA levels in *Artemia* enriched with 4 commercial enrichment products, compared to no enrichment (Control). Different letters denote significant ($P < 0.05$) differences within groups.

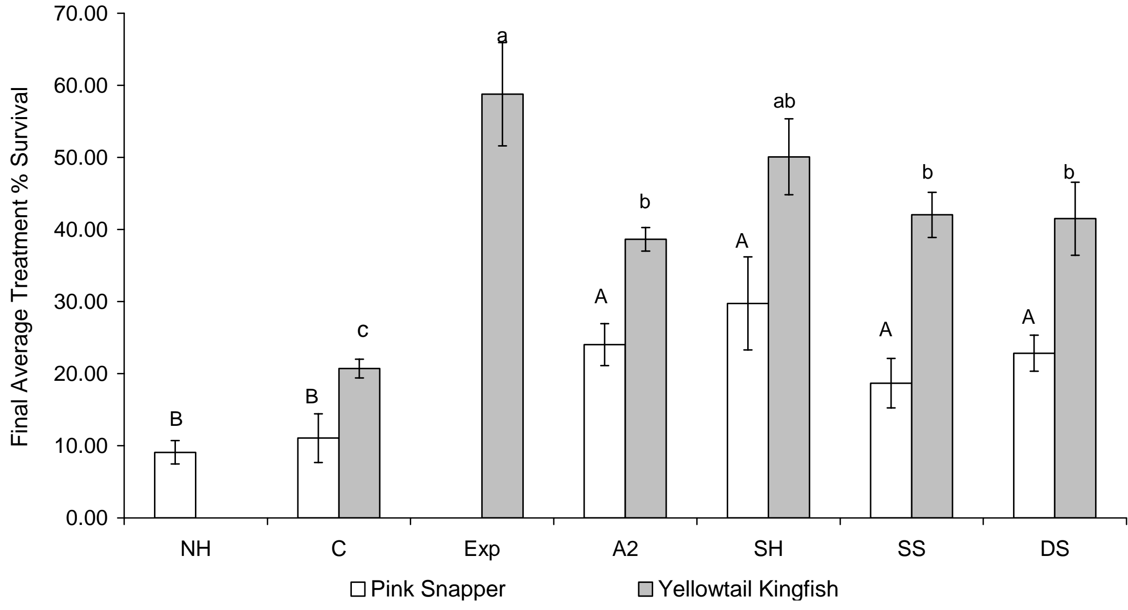


Figure 4. Average percentage survival (mean ± SE, n=4), for treatment replicates. Lower case letters denote significant ($P < 0.05$) differences between yellowtail kingfish treatments, and capital letters denote significantly between pink snapper treatments.

The resulting average WW of larvae at 35 dph in treatment C (71.7 ± 6.0 mg, WW), were significantly (P < 0.05) larger than larvae in the treatments A2 and SH (51.0 ± 5.5 mg and 48.2 ± 4.4 mg, WW respectively). Treatments NH, SS and DS (61.3 ± 1.1 mg, 57.2 ± 8.1 mg and 63.2 ± 0.7 mg, WW respectively), were not significantly different from any other treatment (Figure 2).

The percentage survival for treatments SH, SS, DS and A2 (29.7 ± 6.5%, 18.7 ± 3.4%, 22.8 ± 2.5% and 24.0 ± 2.9% survival, respectively) were not significantly different from each other, but were significantly (P < 0.05) higher than treatments NH and C (9.0 ± 1.6%, and 11.1 ± 3.4% survival, respectively), which were not different from each other (Figure 4).

Survival after stress

Pink snapper larvae rigor was measured as the percentage survival of a stress event. Larvae in treatments DS, SH, A2 and SS (80.6 ± 16.0%, 65.6 ± 3.1%, 56.2 ± 9.6%, and 39.4 ± 15.7%, stress survival respectively) were not significantly different from each other, however were significantly higher than larvae in treatments C and NH, in which no larvae survived the stress test (Figure 5).

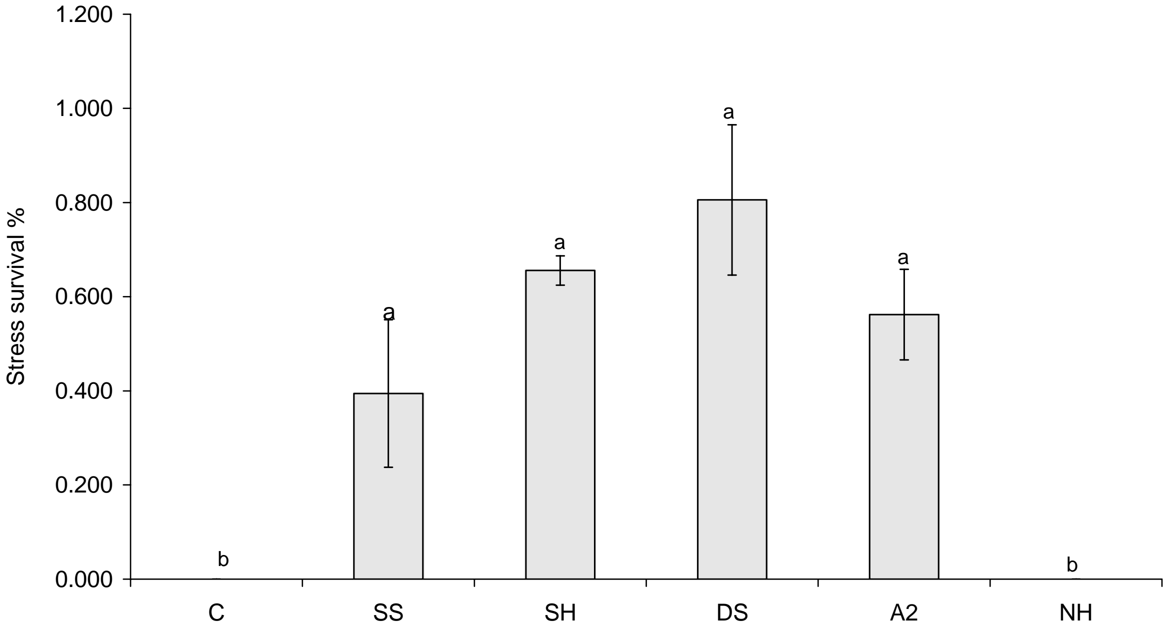


Figure 5. Larvae survival after stress, determined as the percentage of pink snapper larvae that survived (mean ± SE, n=4) a period of increased stress. Different letters denote significant (P < 0.05) differences.

Artemia lipid analysis

Lipid analysis has shown *Artemia* in treatment DS (21.7 ± 2.2% DM) to be 4% higher in total lipid levels than the *Artemia* fed to treatment SS and SH (16.5 ± 1.1% and 17.7 ± 7.9% DM, respectively), whereas the total lipid level in treatment A2 (10.2 ± 1.1% DM) was significantly lower than all three of these treatments, but significantly higher than treatment C (7.0 ± 0.1% DM). The ratio of DHA to EPA differed between treatment DS, SH and SS (2.1, 0.8 and 0.6, DHA/EPA, respectively) there was no significant difference between the sum of essential fatty acid levels found in any of the three treatments (5.1 ± 0.7% EFA, 5.4 ± 0.6% EFA and 5.0 ± 0.4 EFA% DM, respectively). In contrast, treatment A2 (1.5 ± 0.9% EFA DM) showed significantly lower levels of DHA and EPA, and treatment C (0.1± 0.5% DM) showed no DHA present and significantly lower EPA levels than treatment A2 (Figure 3).

Bacterial assay

Larvae tank bacterial levels were on average 1.6 x 10^3 ± 1.5 x 10^2 CFU (ml of tank water)$^{-1}$ before feeding. Algamac-2000® and the control treatment showed significantly different ($P < 0.05$) microbial levels to each other resulting in residual environmental microbial levels of 2.2 x 10^3 ± 2.4 x 10^2 CFU (ml of tank water)$^{-1}$ and 1.0 x 10^3 ± 1.3 x 10^2 CFU (ml of tank water)$^{-1}$ respectively, but both were not significantly different from the rest of the treatments (Figure 6).

Following feeding, the highest microbial level was 9.9 x 10^3 ± 2.5x10^2 CFU (ml of tank water)$^{-1}$ in Algamac-2000®, while the other treatments were significantly less ($P < 0.05$), on average 3.7 x 10^3 ± 6.4 x 10^2 CFU (ml of tank water)$^{-1}$ with no significant difference ($P > 0.05$) between them. The microbial counts in larvae tank water showed that all treatments increased significantly ($P < 0.05$) in microbial levels following the feeding of *Artemia* nauplii. The control (no enrichment) showed the lowest microbial counts before and after feeding *Artemia* nauplii. These microbial levels were significantly lower ($P < 0.05$) than the Algamac-2000®, however not significantly different ($P > 0.05$) than the remaining oil based enrichments (Figure 6).

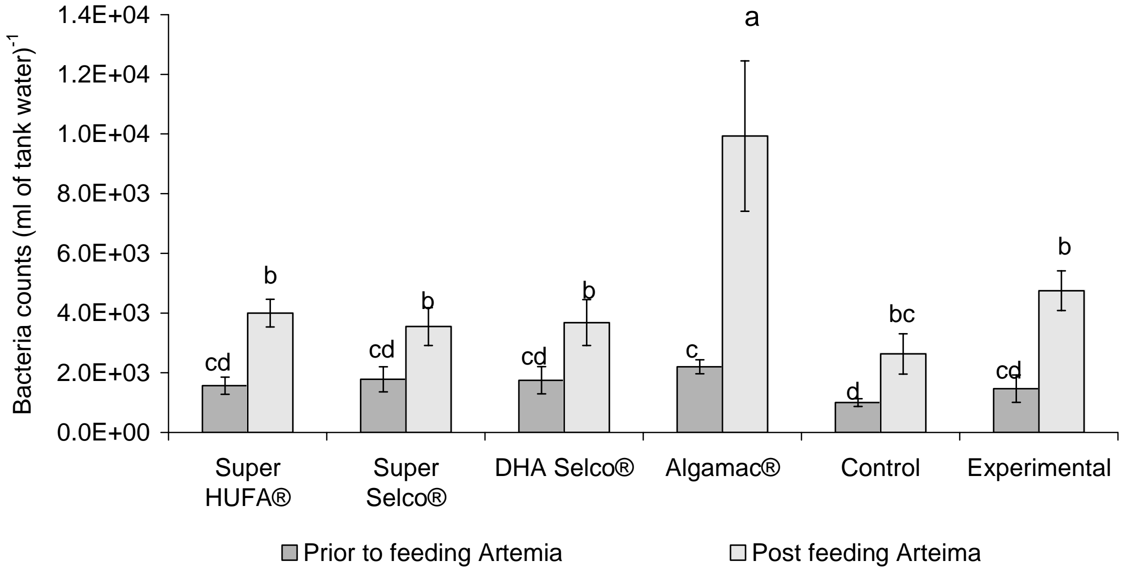


Figure 6. Bacterial levels (Means ± S.E., n=6) in yellowtail kingfish (*Seriola lalandi*) larvae rearing tanks before and after feeding enriched *Artemia* nauplii and the introduction of algae. Means not sharing a common letter are significantly different ($P < 0.05$).

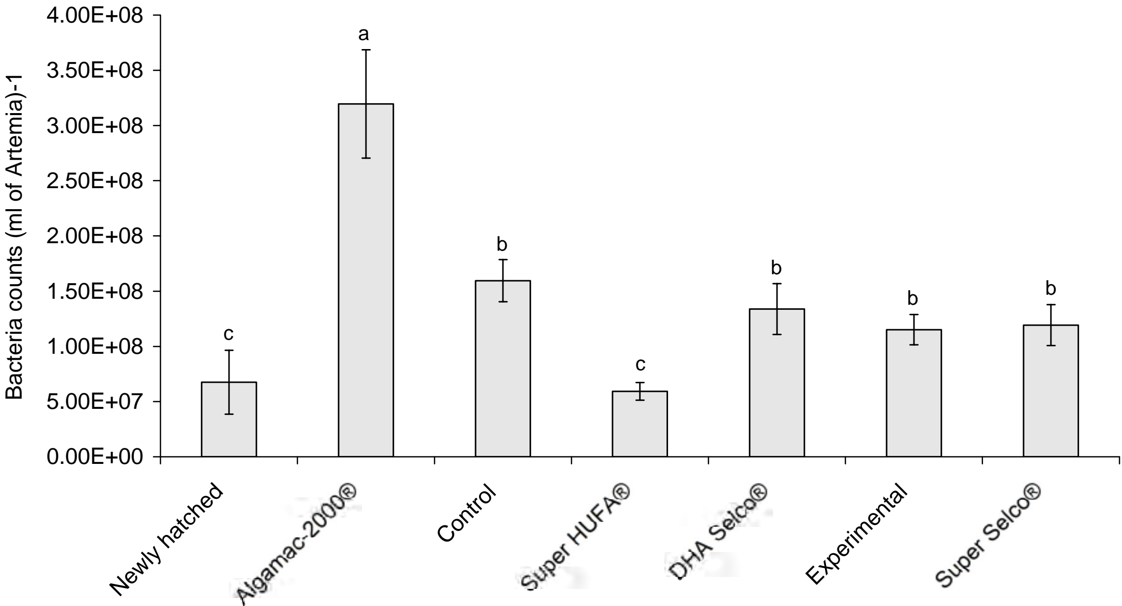


Figure 7. Bacterial levels (Means ± S.E., n=6) of *Artemia* nauplii following enrichment treatments. Averages were taken from the entire harvest procedure as no significant change (P > 0.1) in bacterial levels associated with *Artemia* nauplii occurred after rinsing with fresh seawater. Newly hatched data refers to decapsulated cysts after rinsing. Means not sharing a common letter are significantly different (P < 0.05).

The microbial levels associated with enriched *Artemia* nauplii in the majority of enrichment treatments showed no significant change (P > 0.05) from the newly hatched *Artemia* nauplii hatched from decapsulated cysts $6.8 \times 10^7 \pm 2.9 \times 10^7$ CFU (ml of *Artemia*)$^{-1}$ before they were enriched (Fig. 6). However, the control treatment showed significantly higher (P < 0.05) microbial levels at $1.6 \times 10^8 \pm 1.9 \times 10^7$ CFU (ml of *Artemia*)$^{-1}$ than the newly hatched *Artemia* nauplii. Algamac-2000® enriched *Artemia* nauplii were significantly higher (P < 0.05) in microbial levels at $3.2 \times 10^8 \pm 4.9 \times 10^7$ CFU (ml of *Artemia*)$^{-1}$ than the control. Super HUFA® at $5.9 \times 10^7 \pm 8.0 \times 10^6$ CFU (ml of *Artemia*)$^{-1}$ showed a reduction in microbial levels after treating the newly hatched *Artemia* nauplii (Figure 7).

The Algamac-2000® enrichment treatment resulted in significantly higher (P < 0.05) microbial counts per ml of *Artemia* nauplii compared to all other treatments, while Super HUFA® was significantly lower (P < 0.05) than all other treatments. There was no significant difference (P > 0.05) detected between the control and DHA Selco® and Super Selco®, (Figure 7).

5.2.3.5 Discussion

Larval growth is promoted by high energy levels in their food, supplied as neutral lipid and phospholipid mixtures (Cahu and Zambonino Infante, 2001). Optimal growth in European sea bass (*Dicentrarchus labrax*) larvae is obtained using a diet of 30% lipid (Zambonino Infante and Cahu, 1999), while Brinkmeyer and Holt (1995) found that a diet containing 18% lipids gave the best results for red drum (*Sciaenops ocellatus*) larvae. Higher lipid levels and / or the ratio of lipid groups (i.e. phospholipids) found in the enriched *Artemia* nauplii are thought to contribute positively to growth (Salhi et al. 1994; Coutteau et. al., 1997). *Artemia* enriched to levels greater than 10% total lipids (dry matter), significantly promoted growth of yellowtail kingfish larvae, but with no further improvement at higher levels. This may indicate a relatively low requirement for total lipid intake in this species, or perhaps indicate a greater importance placed on the levels and ratio of essential n-3 HUFAs. The EFA content is one of the principal factors affecting the nutritional value of live prey organisms (Watanabe et al. 1983). The total level of EFAs in the diet

of yellowtail kingfish may have caused an effect on growth, however other variations in enrichment formulations (i.e. vitamins) that were not taken into account may also have been influential on growth. Whereas, pink snapper appeared to require relatively low levels of lipids in their diet, with no improvement on growth relative to total lipid or total EFA intake. These specific requirements may be due to the different growth rates and subsequent differences in metabolic rates. Yellowtail kingfish has a much faster growth rate than snapper possibly resulting in a higher demand for total lipids and EFAs.

Pink snapper larvae sustained higher growth rates when fed *Artemia* nauplii with significantly lower levels of total lipid and total EFAs present. However, these treatments also resulted in relatively lower percentages of larvae surviving to 35 dph. The higher growth rates are thought to be the result of some larvae developing a cannibalistic behaviour. This phenomenon has been reported (Curnow et al. 2004a) in the past, relating to the cannibalistic behaviour of barramundi (*Lates calcarifer*, Bloch) larvae elevating when a low bioavailability of nutrients occurs, which can cause greater growth variation within the tank (Goldan et al. 1998). However, barramundi larvae are more obvious in their behaviour by being observed to actively hunt smaller larvae, whereas pink snapper are more likely to graze on weak or already dead larvae. Therefore the effect on growth caused by a requirement for higher lipid levels than those found in newly hatched *Artemia* nauplii and unenriched metanauplii may have been masked by this behaviour.

Zambonino Infante and Cahu (1999) reported improved survival in European sea bass larvae, obtained using diets containing high total lipid levels. The effect on both pink snapper and yellowtail kingfish showed a significant improvement in survival when fed enrichments containing elevated total lipid levels. However, EFAs such as EPA and DHA, are thought to be more important for fish larvae requirements (Lavens and Sorgeloose, 1996). Total EFA levels were shown to be the highest in *Artemia* enriched with Super HUFA®, which appeared to affect yellowtail kingfish survival more strongly than either total lipid levels or independent EFA levels or ratios. However, other variations in enrichment formulations (i.e. vitamins) that were not taken into account may also have been influential towards promoting higher survival.

It is considered that all inputs into the marine fish larvae rearing tanks can affect the bacterial levels and the development of pathogens (Gomez-Gil et al. 2000; Olafsen, 2001). The establishment of the gut flora of the larvae is therefore influenced by the bacteria associated with invertebrates fed as live feed organisms and also that of their environment (Gomez-Gil et al. 2000; Villamil, 2003). This study has shown that the application of *Artemia* nauplii, as live feed organisms to the larvae culture tanks, does in fact increase the environmental bacterial levels. These bacterial levels were shown to persist within the tanks in numbers relative to the *Artemia* enrichment treatments. However, the bacteria levels associated directly with enriched *Artemia* nauplii are of higher significance to the finfish larvae. Cultured fish larvae will inevitably consume the bacteria that are associated with the *Artemia* nauplii, which can in turn impact on the gut flora of the larvae and ultimately larvae performance (Igarashi et al, 1989; Ottesen and Olafsen, 2000). However, yellowtail kingfish growth and both yellowtail kingfish and pink snapper survival were more closely related to the total lipid and EFA levels associated with the enrichment preparations, with no relationship evident to the bacterial levels of the *Artemia*.

5.2.3.6 Acknowledgements

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Note: *The manufacturer of Algamac 2000® (Bio-Marine, Aquafauna) was approached regarding the high bacteria levels in the enrichment product. Following an internal review of manufacturing procedures, the company identified methods of production that were responsible*

for the unsatisfactorily high bacteria counts. The company stated that due to the results in the report, procedures of manufacture were amended, which lead to higher quality control of their products. They thanked Department of Fisheries, Western Australia for the work done leading to this discovery.

Note: *This study was sent to all the manufacturers of the products that were tested. One of the producers, INVE emphasised that the study involved application rates of their product that were lower than the recommended rates (in a preliminary trial, no differences in fatty acids levels were found between the recommended doses and the lower doses used in the current trial). An additional trial is proposed which includes the recommended application rates and the rates used in this trial.*

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5.2.4 The effect of PUFA enriched *Artemia* on growth, survival and lipid composition of western rock lobster, *Panulirus cygnus*, phyllosoma

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5.2.4.1 Abstract

Western rock lobster, *Panulirus cygnus*, phyllosoma were grown from hatch to stage IV. Larvae were fed with *Artemia* enriched with a (1) Base enrichment (Base) containing 52% squid oil or tailor made enrichments in which oils high in polyunsaturated fatty acid (PUFA) have been added at the expense of squid oil. These treatments were (2) Base enrichment supplemented with docosahexaenoic acid (DHA) rich oil, (3) Base enrichment supplemented with arachidonic acid (AA) rich oil, or (4) Base enrichment supplemented with DHA and AA (D + A) rich oils. Total survival of phyllosoma to stage IV was high, with no significant difference between treatments (range 12.3–17.5%). By stage IV, the larvae fed the DHA or AA enriched *Artemia* were significantly larger (3.33 mm length) than larvae fed the Base or D + A enriched *Artemia* (3.18–3.24 mm length). Phyllosoma were sampled at stages II and III for biochemical analysis. The major lipid class (LC) in all phyllosoma was polar lipid (PL) (88.9–92.4%), followed by sterol (ST) (6.2–9.7%). Triacylglycerol (TAG), free fatty acid (FFA) and hydrocarbon/wax ester (HC/WE) were minor components (≤1%) in all phyllosoma samples. In contrast, the major LC in all enrichments and enriched *Artemia* was TAG (76.3–85.1% and 53.4–60.2%, respectively), followed by PL (11.4–14.8% and 30.6–38.1%, respectively). The main fatty acids (FA) in phyllosoma were 16:0, 18:1n-9c, 18:1n-7c, 18:0, AA, eicosapentaenoic acid (EPA) and DHA. Addition of AA, and to a lesser extent DHA, to enrichments resulted in increased levels of those FA in *Artemia* and phyllosoma compared to the Base enrichment. This was particularly evident for stage III larvae. Comparatively, elevated growth and survival rates for phyllosoma to stage IV were achieved with DHA and AA enriched diets. Our findings highlight the importance of lipids and in particular essential long-chain PUFA, as nutritional components for phyllosoma diets.

5.2.4.2 Introduction

Interest in the aquaculture of rock lobsters has increased proportionally to growing fishing pressure on wild stocks of these species. An area of real promise is the closure of their life cycle for commercial production. The western rock lobster, *Panulirus cygnus*, is a valuable species with many biological traits that make it a potential candidate for aquaculture (Phillips, 1985). However, the protracted larval phase of *P. cygnus*, as is the case of other spiny lobsters, has been an impediment to their successful aquaculture. High mortalities during culture of the phyllosoma stages, linked to a number of aspects, including poor nutrition (Kittaka, 1997; Hart et al. 2001; Nichols et al. 2001; Jeffs et al. 2004), currently make commercial production unviable.

Phyllosoma have been reared on *Artemia* and mussel gonads with varying success (Kittaka, 1988; Illingworth et al. 1997), however, improved nutrition requires the identification of essential elements within the diet (Kanazawa and Koshio, 1994). Appropriate levels of those essential elements then need to be provided in the diet in an available form. However, the lack of diets designed with lipid

class (LC) and fatty acid (FA) profiles appropriate for phyllosoma has been a major limitation to the successful culture (Nelson et al. 2004).

Recent examination of lipids and FA has provided valuable insight into the lipid requirements of phyllosoma (Phleger et al. 2001; Nelson et al. 2003; Ritar et al. 2003; Smith et al. 2003). Liddy et al. (2004a) analysed cultured stage I and II *P. cygnus* larvae, which had been fed (*Artemia*) or starved, in an attempt to determine essential nutrients depleted or accumulated in the larvae. Total lipid content increased in fed larvae, with a decrease in starved larvae compared to initial stage samples. The major LC in newly hatched and fed phyllosoma samples were polar lipid (PL) (87–94%), followed by sterol (ST) (5–8%), while in starved larvae PL was the major LC to decrease. The major FA present were 16:0, 16:1n-7c, 18:1n-7c, 18:1n-9c, arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3); all FA decreased gravimetrically in starved larvae and increased in fed larvae. However, on a relative basis (% of total FA), small decreases were seen in EPA, DHA, 16:1n-7c and 18:1n-9c, while AA increased in starved larvae. In fed larvae, most of the major FA (AA, EPA, DHA, 16:1n-7c) remained at similar % levels, with larvae accumulating AA and EPA above the relative level (%) observed in *Artemia*, but not DHA.

A wide range of potential wild prey for *Jasus edwardsii* phyllosoma have been examined (Nichols et al. 2001; Jeffs et al. 2004). DHA was often the dominant FA, on average 26% of total fatty acids, markedly higher than in the *Artemia* used in previous enrichment trials (14%) with *P. cygnus* (Liddy et al. 2004a).

Artemia have been used as a food source for phyllosoma rearing with moderate success, however, their LC and FA profiles have not been formulated to the requirement of phyllosoma. Recent trials have shown enrichment can be used to not only change the biochemical composition of the *Artemia*, but that the lipid composition of dietary *Artemia* can in turn affect the lipid composition of early stage phyllosoma for *J. edwardsii* (Hart et al. 2001; Phleger et al. 2001; Nelson et al. 2002; Ritar et al. 2004). The current study examines the use PUFA-enriched diets that have been formulated for *P. cygnus* phyllosoma.

Lipid nutrition, in particular long chain polyunsaturated fatty acids (PUFA), appears to be a crucial factor in phyllosoma culture. This study examined the effects of enriching the live feed diet with the essential marine FA, AA and DHA, on the growth, survival and composition of early stage *P. cygnus* phyllosoma.

5.2.4.3 Materials and methods

Broodstock

The study was undertaken at the Western Australian Marine Research Laboratories, Perth, Australia. Broodstock animals were kept in the laboratory under 12L:12D light cycle and fed daily with live mussels (*Mytilus edulis*) and routinely with fish and abalone. Larvae used in the experiment hatched from a lobster (81.3 mm carapace length) on 6 April 2002, after incubation in a tank at 21–24°C (32–34 g L^{-1}).

Experimental system

The culturing system used has allowed southern rock lobster (*J. edwardsii*) phyllosoma to be reared to their final stage (Ritar, 2001). Seawater used in the experiments was heated to 23°C, filtered to 1 µm and UV sterilised (UViVF-9, 30 W) before entering the circular 30 L plastic rearing aquaria. Water entered the aquaria through four equally-spaced nozzles (jets) positioned close to the bottom perimeter and two towards the bottom centre of the aquarium, providing circular water

flow, thereby keeping phyllosoma moving in the water column. Each aquarium had a water flow of approximately 1 L min^{-1} and the volume was maintained at 10 l. Excess water exited through screens positioned on the side of the aquaria. Phyllosoma were fed daily with *Artemia* at 3 ml^{-1}. Every morning remaining *Artemia* were removed from the system by replacing the usual screens, "feeding filters" (200 µm), with "cleaning filters" (1500 µm) for approximately three hours. The feeding filters were replaced and freshly enriched *Artemia* were then added to the tubs. Phyllosoma were transferred to clean aquaria weekly. 1,100 larvae were counted into each aquarium for this experiment.

Artemia

Phyllosoma were fed with *Artemia* (Great Salt Lake) that had been on-grown for 3 days using Algamac 2000™ (Biomarine, Aquafauna) and an *Isochrysis* marine algal concentrate (Reed Mariculture, USA). *Artemia* were enriched for 18 hours (two feedings, 16:00 and 01:00) with one of four different enrichments at 0.6 g l^{-1} (split over the two feedings) prior to feeding to the phyllosoma.

Four enrichments were used (Nutra-kol, Western Australia), three of which were formulated from a base enrichment:

1. Base enrichment (Base) – 52% squid oil (plus water, emulsifier and vitamins);
2. Base + added Docosahexaenoic acid (DHA) – 30.6% squid oil, 20.4% DHA oil (DHASCO®, Martek Biosciences Corporation, 42.6% DHA manufacturer formulation);
3. Base + added Arachidonic acid (AA) – 30.6% squid oil, 20.4% AA oil (ARASCO®, Martek Biosciences Corporation, 42.5% AA manufacturer formulation); and
4. Base + added DHA and AA (D + A) – 25.5% squid oil, 12.7% DHA oil, 12.5% AA oil.

(See Tables 1 and 3 and Figures 4 and 6 for details of enrichment composition.)

Artemia tanks used for enrichment were supplied with air and additional oxygen to maintain the oxygen levels above 4 mg l^{-1}. *Artemia* were rinsed with filtered (1µm) and UV sterilised seawater before being fed to the phyllosoma at approximately 3 ml^{-1}.

Sampling protocol

Three replicate tubs were fed one of the four enriched *Artemia*, giving a total of 12 tubs. All phyllosoma samples for biochemical analysis were taken in triplicate, i.e. three tubs were used for each sample. For analyses, phyllosoma were sampled at hatch (Day 0), during stage II (Day 16; 305–317 larvae tub^{-1}), and during stage III (Day 25; 85–120 larvae tub^{-1}). Samples of enriched *Artemia* (n=3) and of the enrichments themselves (n=2) were also taken for analysis.

To determine size, larval stages were measured from the anterior margin of the cephalic shield between the eyestalks to the posterior of the abdomen, and staged according to Braine et al. (1979). The size (10 from each tub; three replicate tubs) of larvae fed each diet was measured after moulting to stage II, III and IV. Larvae remaining after biochemical sampling were cultured to stage IV for survival estimates. Survival rates were corrected for the numbers used for sampling.

Lipid analysis

Lipid extraction

The methods used for lipid extraction, lipid class (LC) and fatty acid (FA) analysis were as described in detail by Liddy et al. (2003). Briefly, samples which had been filtered and rinsed to remove salt were quantitatively extracted overnight using a modified Bligh and Dyer (1959) one-phase methanol:chloroform:water extraction (2:1:0.8 v/v/v) (Liddy et al. 2003).

Lipid classes

Briefly, LC were quantified using an Iatroscan MK V TH 10 thin layer chromatography-flame ionisation detector (TLC-FID) (Iatron Laboratories, Tokyo, Japan) (Liddy et al. 2003).

Fatty acids

Briefly, an aliquot of the TSE was *trans*-methylated to produce fatty acid methyl esters (FAME) using methanol:chloroform:conc. hydrochloric acid (10:1:1 v/v/v). FAME were then quantified using gas chromatographic (GC) analyses with a Hewlett Packard 5890A GC eqiped with a HP-5 cross linked methyl silicone fused silica capillary column, a FID, a split/splitless injector and a HP 7673A auto sampler, with Helium used as the carrier gas (Liddy et al. 2003). GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-mass spectrometer (Austin, TX, USA) fitted with an on-column injector. The GC was fitted with a capillary column similar to that described above.

Statistical analysis

Percentage data was arcsine √ transformed and gravimetric data √ transformed prior to analysis. Data was normally distributed with homogeneous variances. Larval stage II and III compositional results were analysed using a factorial ANOVA with Tukey's test used for multiple comparisons. Differences in survival, *Artemia* and enrichment analytical results were analysed using a One-way ANOVA with Tukey's test used for multiple comparisons. The size of larvae at stage II, III and IV was analysed using a factorial ANOVA comparing tubs and diets with Tukey's test used for multiple comparisons. Statistical analyses were performed using Statistica software (StatSoft Inc., USA, version 6). Data is presented as mean ± SD, and results were considered significantly different at $P \leq 0.05$.

5.2.4.4 Results

Survival

Total survival from hatch to stage IV was high in larvae fed the Base (16.5 ± 0.9%), DHA (17.5 ± 0.4%) and AA (16.5 ± 1.9%) enriched *Artemia*. Although not significant due to the large variation, larvae fed the D + A enriched *Artemia* had a lower, although more variable, survival (12.3 ± 5.1%) than larvae fed the other enriched *Artemia* diets.

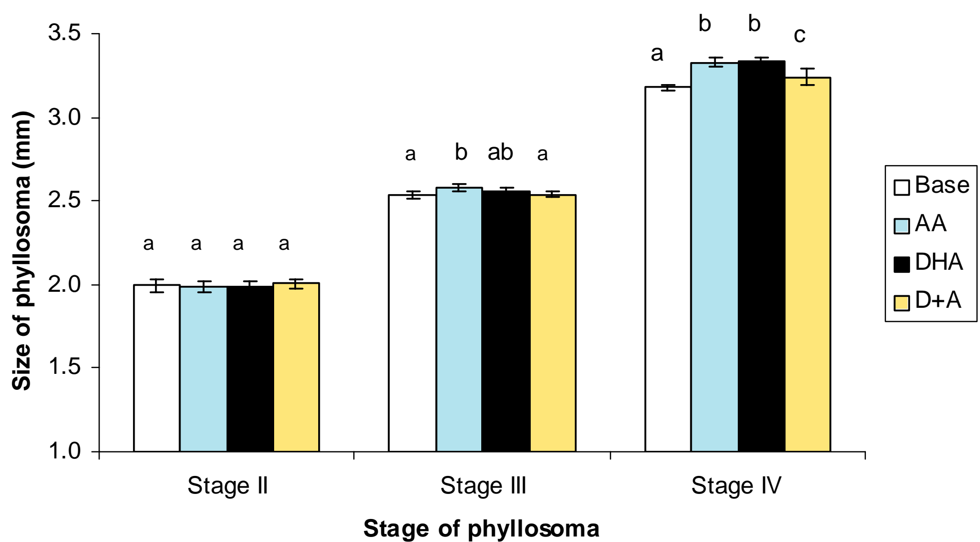

Figure 1. Size of western rock lobster (*Panulirus cygnus*) phyllosoma (mm) after moulting to stage II, III and IV fed the different enrichments (10 larvae from each tub counted, 3 tubs per enrichment). Data not sharing a common superscript within stages are significantly different (α=0.05).

Size

There was no difference in the size of larvae at stage II, 1.99–2.01 mm (Figure 1). At stage III, larvae fed the Base and D + A enriched *Artemia* (2.54 mm) were significantly smaller than larvae fed AA enriched *Artemia* (2.58 mm), and although DHA larvae were also larger (2.56 mm), this difference was not significant (Figure 1). By stage IV larvae fed the DHA or AA enriched *Artemia* (3.33 mm length) were significantly larger than larvae fed the Base or D + A enriched *Artemia*, and in turn larvae fed the D + A enriched *Artemia* (3.24 mm length) were significantly larger than those fed Base enriched *Artemia* (3.18 mm length) (Figure 1).

Total lipid

Base, DHA and AA enrichments had similar lipid content (9310.5–961.5 mg g^{-1}), however the D + A enrichment was significantly lower (886.3 mg g^{-1}) (Table 1). This was also seen in the enriched *Artemia* (208.3–214.5 mg g^{-1}), with D + A enriched *Artemia* having lower lipid content (189.9 mg g^{-1}) than the other treatments, although the differences were not significant. This difference in lipid content was evident by stage III, as larvae fed the D + A enriched *Artemia* had significantly lower lipid content (62.5 mg g^{-1}) than larvae fed the Base and DHA enriched *Artemia* (76.5 and 78.4 mg g^{-1}, respectively) (Table 1).

Lipid classes

All enriched *Artemia* were dominated by TAG (53.4–60.2%; 101.4–126.1 mg g^{-1}), followed by PL (30.5–38.1%; 63.3–72.6 mg g^{-1}), FFA (4.5–7.0%; 8.5–14.8 mg g^{-1}) and ST (2.7–3.6%; 5.6–7.3 mg g^{-1}) (Table 1, Figure 2). There was little variation between the different enriched *Artemia* in the content of the major LC (Table 1, Figure 2).

The enrichments were dominated by TAG (76.3–85.1%; 710.1–772.9 mg g^{-1}), followed by PL (11.4–14.8%; 100.8–142.1 mg g^{-1}) and FFA (1.6–4.2%; 15.4–39.0 mg g^{-1}), with little variation between the enrichments in the major LC (Table 1, Figure 2). However on an absolute basis, the D + A enrichment (100.8 mg g^{-1}) did have significantly less PL than the other enrichments (131.1–142.1 mg g^{-1}) (Figure 2).

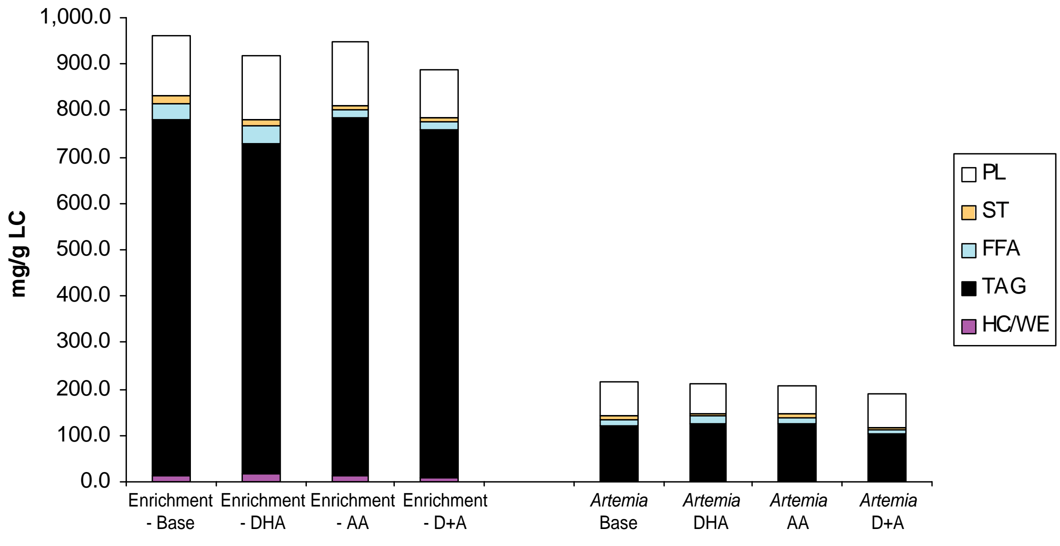

Figure 2. Lipid class content (mg/g DM) of enrichments (n=2) and enriched *Artemia* (n=3). LC, lipid class; PL, polar lipid; ST, sterol; HC/WE, hydrocarbon/wax ester; TAG, triacylglycerol; FFA, free fatty acid.

As with newly hatched larvae, stage II and III larvae fed all enriched *Artemia* were dominated by PL (88.8–92.4%) (Table 1, Figure 3). Stage III larvae fed the D + A enriched *Artemia* did, however, have a significantly lower content of PL (57.2 mg g^{-1}) than those fed the Base or DHA enriched *Artemia* (70.6–72.4 mg g^{-1}) (Figure 3). ST were the next most abundant LC in all larvae (6.2–9.7%, 3.8–5.9 mg g^{-1}) (Table 1, Figure 3). Triacylglycerol (TAG), free fatty acid (FFA) and hydrocarbon/wax ester (HC/WE) were all present at ≤1% in all phyllosoma samples.

Fatty acids

All enrichments with added PUFA showed elevated levels compared to the Base enrichment for those specific PUFA (Table 3, Figure 4). DHA enrichment had significantly higher relative levels and content of DHA (20.71% and 141.16 mg g^{-1}) than Base (12.89% and 95.60 mg g^{-1}) and D + A (15.4% and 103.48 mg g^{-1}) enrichments, which in turn had significantly higher levels than the AA enrichment (8.59% and 54.04 mg g^{-1}). The AA enrichment had significantly higher relative levels and content of AA (14.66% and 92.24 mg g^{-1}) than the D + A enrichment (8.74% and 58.62 mg g^{-1}), followed by the Base (1.14% and 8.47 mg g^{-1}) and DHA (0.64% and 4.33 mg g^{-1}) enrichments (Table 4, Figure 4). There was no significant difference in the total FA (629.08–747.12 mg g^{-1}) between the enrichments.

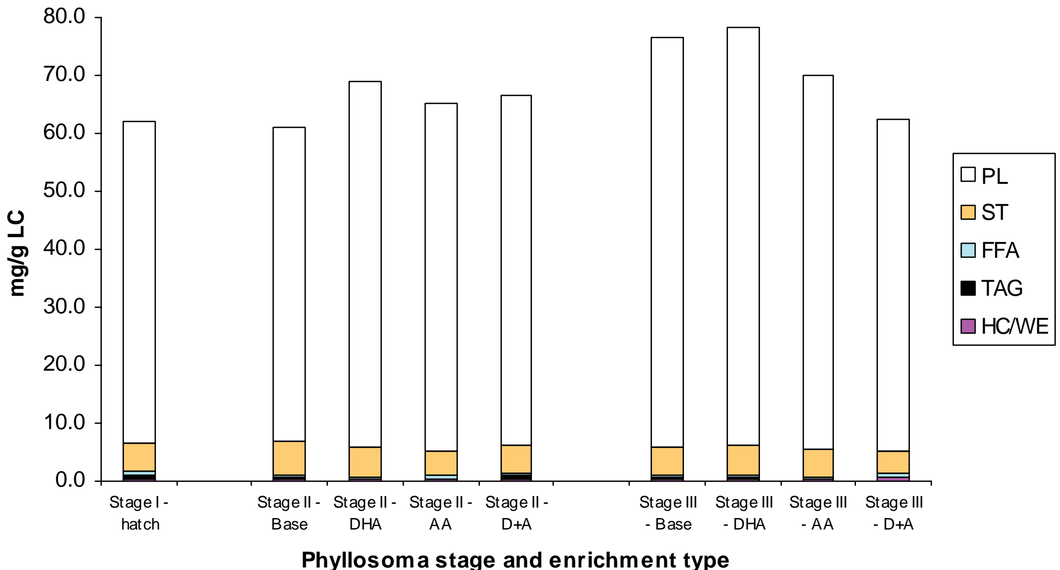

Figure 3. Lipid class content (mg/g DM) at hatch and after feeding the different enrichments at stage II and III (n=3), of western rock lobster (*Panulirus cygnus*) phyllosoma. LC, lipid class; PL, polar lipid; ST, sterol; HC/WE, hydrocarbon/wax ester; TAG, triacylglycerol; FFA, free fatty acid.

DHA enriched *Artemia* had significantly higher DHA levels and content (13.78% and 20.23 mg g^{-1}) than Base (9.84% and 12.77 mg g^{-1}) and D + A (9.47% and 12.24 mg g^{-1}) enriched *Artemia* which had significantly higher levels than AA enriched *Artemia* (6.54% and 9.68 mg g^{-1}) (Table 3, Figure 4). And as with the enrichments, AA enriched *Artemia* had significantly higher levels and content of AA (13.98% and 20.70 mg g^{-1}) than D + A enriched *Artemia* (8.43% and 10.90 mg g^{-1}), which had significantly more than the Base (2.36% and 3.09 mg g^{-1}) or DHA (1.68% and 2.46 mg g^{-1}) enriched *Artemia*.

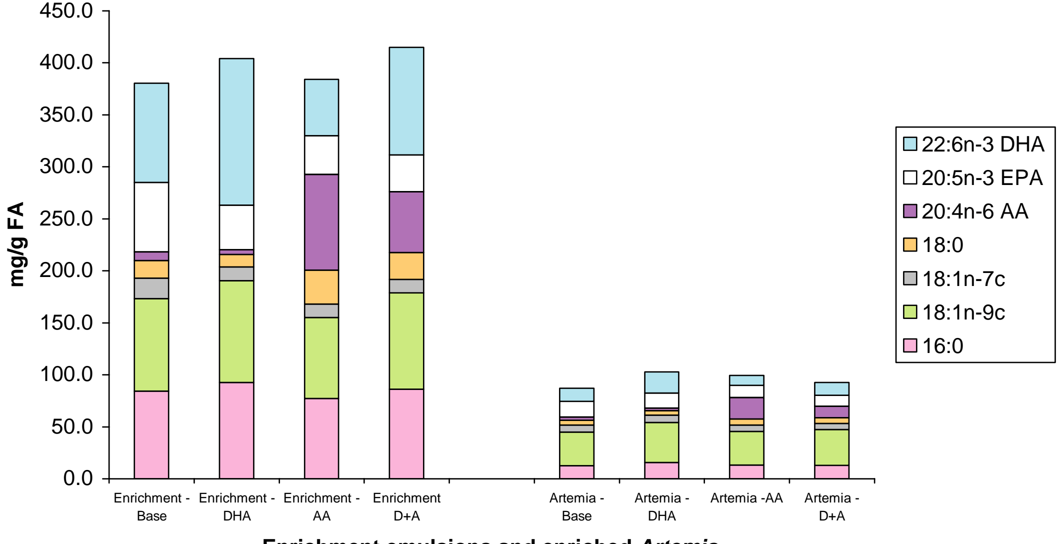

Figure 4. The content of major fatty acids (mg/g DM) in enrichments (n=2) and enriched *Artemia* (n=3). FA, fatty acids; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

The sum n-3 FA was significantly higher in the Base and DHA enrichments and enriched *Artemia*, with sum n-6 FA significantly higher in AA and D + A enrichments and enriched *Artemia* (Table 3). There was no significant difference in the total FA content (129.22–148.10 mg g^{-1}) between the *Artemia* enriched on the different diets.

Seven FA were present in phyllosoma with levels >5% (of total FA). These included 16:0 (12.23–14.54%), 18:1n-9c (20.80–25.00%), 18:1n-7c (7.70–8.77%), 18:0 (9.73–12.12%), AA (3.99–10.90%), EPA (7.45–12.21%) and DHA (3.76–6.10%) (Table 2).

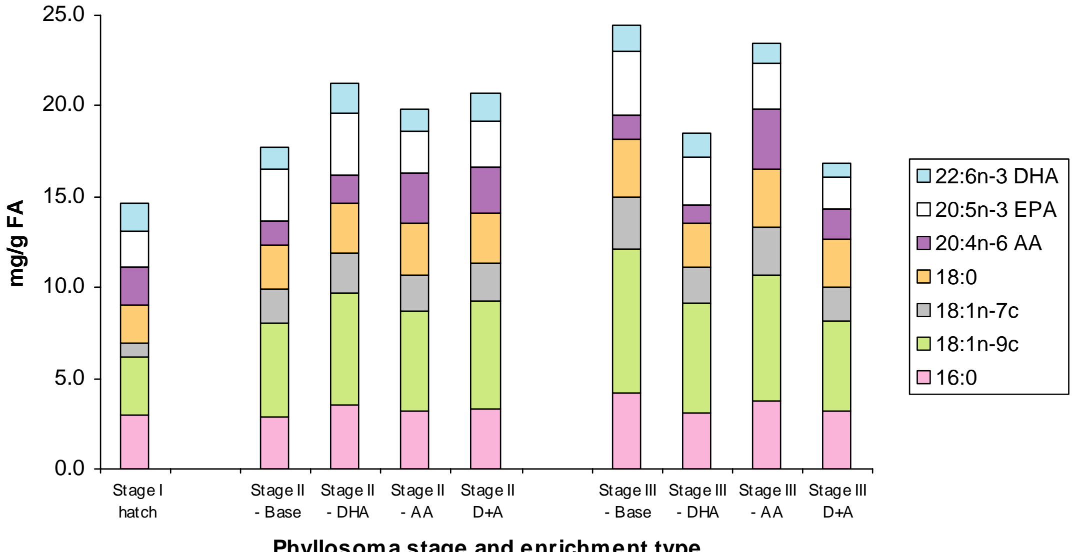

Figure 5. The content of major fatty acids (mg/g DM) at hatch, and after feeding the different enrichments at stage II and III (n=3), of western rock lobster (*Panulirus cygnus*) phyllosoma. FA, fatty acids; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

At stage II there was little difference in the DHA level and content (4.44–6.10%) between phyllosoma fed the different enriched *Artemia* (Table 2, Figure 5). However by stage III, phyllosoma fed DHA enriched *Artemia* had significantly higher DHA levels (5.45%) than phyllosoma fed other enriched *Artemia* (3.76–4.43%) (Table 2). Notably the DHA levels (%) in all phyllosoma decreased from stage II to III (Table 2).

At stage II, phyllosoma fed the AA and D + A enriched *Artemia* had significantly higher levels of AA (10.76 and 9.18%, respectively) than phyllosoma fed the Base or DHA enriched *Artemia* (5.75 and 5.67%, respectively) (Table 2). By stage III, phyllosoma fed AA enriched *Artemia* had significantly higher levels (10.90%) of AA, followed by phyllosoma fed D + A, DHA and then Base enriched *Artemia* (7.41, 4.05 and 3.99%, respectively) (Table 2, Figure 5).

Phyllosoma fed DHA enriched *Artemia* exhibited significantly higher sum n-3 FA levels, and phyllosoma fed AA enriched *Artemia* exhibited significantly higher sum n-6 FA levels (Table 2). There was no significant difference in the total FA content (mg/g) between the phyllosoma fed *Artemia* enriched on the different diets.

5.2.4.5 Discussion

As with marine fish larvae (Sorgeloos et al. 2001), increasing the amounts of key nutrients in *Artemia* through enrichments may be a way to improve their nutritional value for spiny lobsters phyllosoma. We have demonstrated that the addition of elevated levels of long-chain PUFA oils (AA and DHA) to the enrichment diets of *Artemia* was concomitant with increased PUFA in enrichments, *Artemia*, and in phyllosoma (Tables 2 and 3, Figures 5 and 6). The larval stages of rock lobster larvae, particularly the early stages, are characteristically high in PL and the PUFA, AA and DHA (Phleger et al. 2001; Nelson et al. 2003, 2004; Ritar et al. 2003; Smith et al. 2003; Liddy et al. 2004a). The enrichments used in this study resulted in *Artemia* with no significant difference in lipid content, making any differences in survival, growth and composition in the phyllosoma attributable to the quality of the lipid fraction of the *Artemia*.

Survival, size and total lipid

The highest survival of phyllosoma to stage IV occurred when fed *Artemia* enriched on the Base (16.5%), DHA (17.5%) and AA (16.5%) enriched *Artemia*. The lower average survival of the phyllosoma fed D + A enriched *Artemia* (12.3%) was due to one D + A phyllosoma aquarium having lower survival (6.8%) compared with the other two D + A aquaria (16.8 and 13.2%). The average survival in these two aquaria, 15.0%, is much closer to survival in the other phyllosoma treatments.

The survival to stage IV in the current study was similar to that found by Hart et al. (2001) for *J. edwardsii* phyllosoma (16.8%) cultured in a similar system to stage III and fed the best performing diet. Other diets trialed by Hart et al. (2001) resulted in lower survival, ranging from 1.7 to 9.9% to stage III. Nelson et al. (2004) had survival to stage V of 3–12% for *J. edwardsii* phyllosoma fed *Artemia* enriched on a number of different diets using a similar system.

Stage II larvae fed the different enriched *Artemia* showed no difference in size, however by stage III there were size differences. These differences were further enhanced at stage IV, with larvae fed the DHA or AA enriched *Artemia* significantly larger than larvae fed the Base or D + A enriched *Artemia*, and in turn larvae fed the D + A enriched *Artemia* were significantly larger than those fed Base enriched *Artemia* (Figure 1). Stage III larvae fed the D + A enriched *Artemia* also had comparatively less lipid (mg g^{-1}), significantly lower than larvae fed Base and DHA enriched *Artemia* (Table 1). Although there was no significant difference in lipid content of *Artemia*, the D + A enriched *Artemia* contained lower oil content, possibly due to the lower lipid level in the D + A enrichment (Table 1).

The larvae in our study fed all of the diets exhibited enhanced size at stage IV (3.18–3.33 mm length) compared to individually reared *P. cygnus* larvae subjected to temperature and food density (*Artemia*) treatments at stage IV (2.95–3.01 mm length) (Liddy et al. 2004b). Interestingly, the larvae in the present study were smaller at hatch (1.64 mm length) compared to newly hatched larvae used by Liddy et al. (2004b) (1.69 mm length), were a similar size at stage II and slightly larger at stage III. The results from the two experiments indicate that the PUFA-enhanced diets have had a positive effect on larval growth.

Lipid classes

Although newly hatched and wild phyllosoma are low in TAG and high in PL (Phleger et al. 2001; Liddy et al. 2004a), the use of high TAG/low PL enrichments and the resultant high TAG/low PL levels in *Artemia* did not alter the relative levels (%) of these LC, nor result in a decrease of PL content (mg g^{-1}, Figure 3) in the phyllosoma fed the enriched *Artemia* compared to newly hatched larvae (Table 1). TAG is generally the most common storage lipid in most marine animals, and is used as a short-term energy reserve (Koven et al. 1989; Olsen, 1998; Phleger et al. 2001). PL, which is used during starvation of early stage *P. cygnus* larvae (Liddy et al. 2004a), also is used as an energy store as in the puerulus stage of rock lobsters (Jeffs et al. 2001). Our results suggest that if lipid is used as an energy storage component of early stage phyllosoma, as it appears to be in late stage phyllosoma (Jeffs et al. 1999; 2001), TAG is not the storage form.

In contrast, Nelson et al. (2004) found that PL content in cultured *J. edwardsii* phyllosoma decreased from stage I–V. They suggested the phyllosoma may not efficiently metabolise and incorporate the TAG from enriched *Artemia* and that designing a feed using a high PL oil (containing high DHA), rather than TAG oil, should be considered. The present study does not show any effect of a high TAG diet on the PL levels in cultured *P. cygnus* phyllosoma, although lipid composition data for later stage phyllosoma would have been useful.

Fatty acids

The relative levels of FA in phyllosoma were similar to the values for the same parameter within *Artemia*, a result also found by Hart et al. (2001) with *J. edwardsii* phyllosoma.

D + A enriched stage III larvae, which had the lowest survival and smaller size at stage IV, had lower content of the essential PUFA, AA, DHA and EPA than newly hatched larvae. All other phyllosoma had similar or higher content of at least one of these PUFA (Figure 5). The enrichments provided significantly different relative levels of the PUFA, AA and DHA. The addition of AA to enrichments resulted in increased levels, 14.7% in the AA enrichment and 8.7% in the D + A enrichment. These levels were successfully transferred to *Artemia*, 14% for AA enriched and 8.4% for D + A enriched. These higher levels were able to maintain the AA level in the phyllosoma (7.4–10.9%) to a similar level found in newly hatched larvae (10.0%) (Table 2). Phyllosoma fed AA or D + A enriched *Artemia* showed significantly higher relative levels (%) of AA, and were at a level similar to that of newly hatched larvae. However, phyllosoma fed Base or DHA enriched *Artemia* were much lower in AA.

Levels of AA reached a maximum of 10% in phyllosoma, that found in newly hatched larvae, even when the level was higher in *Artemia*. When the level was lower in *Artemia* (Base and DHA enriched), the phyllosoma had levels of AA above the level in the enriched *Artemia* (Table 2 and 3). This suggests that phyllosoma are able to preferentially assimilate and accumulate AA from their diet. Providing levels (%) above a certain point in their diet does not further increase this level. Hart et al. (2001) and Nelson et al. (2004) suggested that cultured *J. edwardsii* phyllosoma were also able to preferentially assimilate and accumulate AA from their diet.

The addition of DHA to enrichments also resulted in increased levels, particularly in DHA enrichment (to 20.7%). This resulted in a higher level in DHA enriched *Artemia* (13.8%), with *Artemia* from the other enrichments ranging from 6.5–9.8%. Nelson et al. (2004) also found reduced levels of DHA in *Artemia* compared to the levels provided in the enrichments. In contrast to AA, not even the phyllosoma fed DHA enriched *Artemia* could maintain a DHA level found in newly hatched larvae (7.5%), with the DHA levels also decreasing further as the larvae developed (Table 2). Phyllosoma fed DHA enriched *Artemia* had the highest relative levels of DHA, however this enrichment, along with the others, resulted in phyllosoma with lower levels (3.8–6.1%) than newly hatched phyllosoma (Table 2). This suggests that much of the DHA is not digested/assimilated, or may be used as an energy source, or is retro-converted to docosapentaenoic acid (DPA). Hart et al. (2001) also found that phyllosoma exhibited reduced incorporation of DHA from enriched *Artemia*, with enriched *Artemia* having levels of DHA up to 19%, while DHA in *J. edwardsii* phyllosoma only reached 7.8% of total FA. Nelson et al. (2004) only found this decrease to be true when there where higher levels of DHA in *Artemia* (9.7%). When the DHA levels were lower in *Artemia*, ≈3%, this led to similar levels in the phyllosoma. Possible methods suggested to overcome the reduced DHA levels transferred to the phyllosoma include the use of live prey items naturally high in long chain PUFA (Hart et al. 2001) and a diet containing PL oil with a high percentage of DHA (Nelson et al. 2003; 2004). The present study confirms the requirement of *P. cygnus* for the essential long chain PUFA, especially for DHA. We suggest that DHA may be a requirement for other rock lobster species as well.

Artemia showed higher levels of monounsaturated fatty acids (MUFA) compared to the enrichments, which had higher saturated fatty acids (SFA) levels. Phyllosoma fed enriched *Artemia* showed increased levels of 18:1n-9c and 18:1n-7c compared to newly hatched larvae. Total MUFA increased and total PUFA decreased in phyllosoma fed the enriched *Artemia* compared to newly hatched larvae. If we assume that the FA composition of newly hatched larvae is physiologically optimal, then the difference in composition observed between newly hatched and cultured phyllosoma, also suggests an inadequacy of the dietary *Artemia*. Our study indicates that the current diets also were unable to maintain the high levels of PUFA, particularly DHA, found in newly hatched phyllosoma.

5.2.4.6 Conclusion

The lipid composition of dietary *Artemia* has been shown to affect the lipid composition of early stage western rock lobster larvae. Lipid enriched *Artemia* were capable of sustaining high survival and growth in early stage phyllosoma compared to previous studies with *P. cygnus* larvae (Liddy et al. 2004b).

The enrichment diets were capable of sustaining AA levels at elevated levels in phyllosoma. However, the transfer of DHA decreased from enrichment, to *Artemia*, to phyllosoma. Further research should focus on incorporation of DHA. Methods will be needed to help overcome the issue of the low and decreasing DHA levels observed in phyllosoma using current feeding practices, examining alternative forms of delivery (different live food source), or form of supply (PL versus TAG).

For the successful culture of phyllosoma, a diet needs to be provided, whether live food or formulated diet, which can provide the nutritional requirements in a form readily available to the phyllosoma.

5.2.4.7 Acknowledgements

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5.2.4.9 Tables

Table 1. Lipid class composition, as percentage of total lipid, in western rock lobster (*Panulirus cygnus*) phyllosoma (n = 3), *Artemia* (n=3) and enrichments (n=2).

	HC/WE	TAG	FFA	Sterols	Polar lipids	Lipid as mg g^{-1} dry mass	Oil mass/ind (mg)
Stage I – hatch	0.81 ± 0.20	0.95 ± 0.14	1.14 ± 0.27	7.45 ± 0.77	89.65 ± 1.08	62.03 ± 7.14	4.11 ± 0.48
Stage II – Base	0.62 ± 0.26[a]	0.38 ± 0.10[a]	0.49 ± 0.07[ab]	9.68 ± 0.44[a]	88.82 ± 0.47[a]	60.92 ± 2.25[a]	6.79 ± 0.23[a]
Stage II – DHA	0.29 ± 0.03[a]	0.44 ± 0.02[a]	0.52 ± 0.08[ab]	7.41 ± 0.02[ab]	91.34 ± 0.09[b]	68.92 ± 4.30[abc]	7.34 ± 0.39[a]
Stage II – AA	0.45 ± 0.15[a]	0.23 ± 0.02[a]	0.84 ± 0.22[ac]	6.23 ± 0.25[b]	92.25 ± 0.13[b]	65.07 ± 6.68[ab]	8.62 ± 1.10[a]
Stage II - D + A	0.45 ± 0.14[a]	1.15 ± 0.59[b]	0.34 ± 0.11[b]	7.33 ± 0.35[ab]	90.72 ± 0.72[ab]	66.51 ± 5.75[abc]	8.30 ± 1.35[a]
Stage III – Base	0.66 ± 0.02[a]	0.38 ± 0.26[a]	0.43 ± 0.12[ab]	6.16 ± 0.49[b]	92.36 ± 0.15[b]	76.47 ± 2.87[bc]	16.61 ± 0.65[b]
Stage III – DHA	0.45 ± 0.17[a]	0.22 ± 0.08[a]	0.45 ± 0.14[ab]	6.60 ± 0.49[b]	92.28 ± 0.48[b]	78.43 ± 3.76[c]	18.68 ± 0.58[a]
Stage III – AA	0.40 ± 0.17[a]	0.22 ± 0.11[a]	0.47 ± 0.13[ab]	6.90 ± 1.58[b]	91.95 ± 1.55[b]	70.04 ± 3.86[abc]	18.62 ± 0.37[b]
Stage III - D + A	0.94 ± 0.71[a]	0.29 ± 0.01[a]	1.13 ± 0.41[c]	6.16 ± 1.37[b]	91.49 ± 1.06[b]	62.53 ± 2.10[a]	16.08 ± 0.47[b]
Artemia – Base	0.34 ± 0.07[a]	56.29 ± 2.49[ab]	5.98 ± 0.22[a]	3.38 ± 0.32[ac]	34.00 ± 2.67[ab]	214.51 ± 19.98[a]	0.62 ± 0.06[a]
Artemia – DHA	0.34 ± 0.01[ab]	59.30 ± 1.50[a]	6.95 ± 0.26[b]	2.84 ± 0.23[ab]	30.56 ± 1.93[a]	212.37 ± 18.77[a]	0.63 ± 0.06[a]
Artemia – AA	0.38 ± 0.04[ab]	60.16 ± 1.41[a]	6.29 ± 0.33[ab]	2.70 ± 0.19[b]	30.47 ± 0.97[a]	208.27 ± 19.74[a]	0.52 ± 0.09[ab]
Artemia - D + A	0.47 ± 0.04[b]	53.39 ± 0.98[b]	4.49 ± 0.36[c]	3.55 ± 0.06[c]	38.10 ± 0.68[b]	189.88 ± 10.45[a]	0.41 ± 0.03[b]
Enrichment – Base	1.49 ± 0.02[ab]	79.97 ± 0.79[a]	3.70 ± 0.30[a]	1.63 ± 0.10[a]	13.71 ± 1.09[ab]	956.86 ± 25.44[ab]	
Enrichment – DHA	1.80 ± 0.04[a]	76.32 ± 2.04[a]	4.19 ± 0.31[a]	1.63 ± 0.15[a]	14.72 ± 0.27[a]	930.52 ± 9.31[ab]	
Enrichment – AA	1.27 ± 0.16[b]	80.44 ± 4.10[a]	1.60 ± 0.09[b]	0.81 ± 0.11[b]	14.77 ± 0.75[a]	961.51 ± 29.49[a]	
Enrichment – D + A	0.76 ± 0.08[c]	85.06 ± 1.19[a]	1.94 ± 0.20[a]	0.87 ± 0.24[b]	11.37 ± 0.81[b]	886.31 ± 14.10[b]	

Data, within a column and within a sample category (phyllosoma, *Artemia* or enrichments samples) not sharing a common superscript are significantly different (α=0.05).

HC/WE, hydrocarbons/wax esters; TAG, triacylglycerols; FFA, free fatty acids

Note: DAGE, diacylglyceryl ether, not present.

Table 2. Fatty acid (FA) composition at hatch and after feeding the different enrichments at stage II and III (n = 3) of western rock lobster (*Panulirus cygnus*) phyllosoma (% of total FA).

	Stage I hatch	Stage II - Base	Stage II – DHA	Stage II - AA	Stage II D + A	Stage III – Base	Stage III – DHA	Stage III - AA	Stage III D + A
16:1n-7c	2.80 ± 0.30	1.88 ± 0.04^{a}	1.76 ± 0.04^{ab}	1.40 ± 0.08^{c}	1.48 ± 0.07^{bc}	1.81 ± 0.12^{a}	1.64 ± 0.07^{abc}	1.47 ± 0.06^{bc}	1.37 ± 0.22^{c}
16:0	14.69 ± 0.09	12.45 ± 0.60^{a}	12.61 ± 0.49^{a}	12.33 ± 0.52^{a}	12.47 ± 1.28^{a}	12.79 ± 0.48^{a}	12.83 ± 0.36^{a}	12.23 ± 0.46^{a}	14.54 ± 1.71^{a}
17:0	1.05 ± 0.03	1.03 ± 0.04^{a}	0.99 ± 0.06^{a}	0.87 ± 0.08^{a}	0.94 ± 0.10^{a}	0.95 ± 0.05^{a}	0.89 ± 0.02^{a}	0.93 ± 0.04^{a}	1.03 ± 0.11^{a}
18:2n-6	1.42 ± 0.04	3.09 ± 0.05^{ab}	2.99 ± 0.10^{a}	3.80 ± 0.24^{c}	3.42 ± 0.23^{bc}	3.53 ± 0.07^{c}	3.39 ± 0.05^{bc}	4.26 ± 0.07^{d}	3.49 ± 0.17^{c}
18:1n-9c	16.37 ± 0.87	21.72 ± 0.45^{abc}	22.35 ± 0.93^{abc}	20.80 ± 0.93^{c}	21.60 ± 1.36^{bc}	24.60 ± 1.19^{ab}	25.00 ± 0.57^{a}	22.36 ± 0.23^{abc}	21.97 ± 2.21^{abc}
18:1n-7c	3.61 ± 0.10	8.29 ± 0.11^{ab}	7.70 ± 0.34^{a}	7.84 ± 0.19^{a}	7.80 ± 0.45^{a}	8.77 ± 0.25^{b}	8.44 ± 0.08^{ab}	8.21 ± 0.06^{ab}	8.33 ± 0.37^{ab}
18:0	10.54 ± 0.55	10.25 ± 0.34^{a}	9.93 ± 0.10^{a}	10.89 ± 0.72^{ab}	10.41 ± 0.81^{ab}	9.73 ± 0.34^{a}	9.93 ± 0.27^{a}	10.29 ± 0.35^{a}	12.12 ± 1.18^{b}
20:4n-6 AA	10.02 ± 0.25	5.75 ± 0.46^{a}	5.67 ± 0.34^{a}	10.76 ± 0.26^{b}	9.18 ± 0.81^{b}	3.99 ± 0.13^{c}	4.05 ± 0.21^{c}	10.90 ± 0.37^{b}	7.41 ± 1.04^{d}
20:5n-3 EPA	9.95 ± 0.37	12.15 ± 0.87^{a}	12.21 ± 0.25^{a}	8.89 ± 0.70^{cd}	9.44 ± 1.18^{bcd}	10.91 ± 0.68^{abc}	11.46 ± 0.32^{ab}	8.01 ± 0.32^{d}	7.45 ± 1.35^{d}
20:2n-6	1.04 ± 0.04	0.91 ± 0.04^{ab}	0.85 ± 0.06^{ab}	1.12 ± 0.03^{a}	0.96 ± 0.00^{ab}	0.73 ± 0.08^{b}	0.72 ± 0.02^{b}	1.09 ± 0.05^{a}	0.82 ± 0.21^{b}
20:1(n-9/11)c	2.20 ± 0.08	1.73 ± 0.02^{ab}	1.76 ± 0.03^{ab}	1.62 ± 0.04^{ab}	1.59 ± 0.13^{b}	1.86 ± 0.04^{ab}	1.96 ± 0.12^{a}	1.75 ± 0.06^{ab}	1.56 ± 0.27^{b}
22:6n-3 DHA	7.53 ± 0.35	5.18 ± 0.17^{ab}	6.10 ± 0.26^{a}	4.44 ± 0.47^{bc}	5.36 ± 0.05^{a}	4.34 ± 0.39^{bc}	5.45 ± 0.16^{a}	3.78 ± 0.08^{c}	3.76 ± 0.52^{c}
22:1(n-9)	0.43 ± 0.05	0.66 ± 0.13^{a}	0.58 ± 0.09^{a}	0.88 ± 0.22^{a}	0.83 ± 0.80^{a}	1.29 ± 0.98^{a}	0.56 ± 0.24^{a}	0.62 ± 0.36^{a}	1.69 ± 0.85^{a}
22:0	0.74 ± 0.02	1.22 ± 0.04^{a}	1.10 ± 0.01^{abc}	1.13 ± 0.03^{ab}	1.20 ± 0.13^{a}	1.00 ± 0.03^{bc}	0.98 ± 0.06^{bc}	0.97 ± 0.04^{c}	1.10 ± 0.03^{abc}
Other	8.81 ± 0.12	6.85 ± 0.28	6.70 ± 0.07	6.61 ± 0.34	6.67 ± 0.54	6.85 ± 0.31	6.35 ± 0.12	6.56 ± 0.23	6.67 ± 0.74
Sum SFAs	29.77 ± 0.76	27.58 ± 1.10^{a}	27.32 ± 0.64^{a}	27.75 ± 1.21^{a}	27.58 ± 2.63^{a}	26.98 ± 1.01^{a}	27.09 ± 0.49^{a}	26.71 ± 0.92^{a}	31.57 ± 3.16^{a}
Sum MUFAs	28.04 ± 0.88	36.62 ± 0.52^{a}	36.31 ± 1.15^{a}	34.60 ± 1.14^{a}	35.42 ± 1.15^{a}	41.02 ± 0.66^{c}	39.94 ± 0.54^{bc}	36.76 ± 0.13^{ab}	37.11 ± 2.25^{ab}
Sum PUFA	33.38 ± 1.06	28.96 ± 1.43^{abc}	29.67 ± 0.56^{ab}	31.04 ± 0.67^{a}	30.33 ± 2.04^{ab}	25.15 ± 1.08^{cd}	26.62 ± 0.62^{bcd}	29.97 ± 0.79^{ab}	24.65 ± 2.77^{d}
Sum n-3	19.02 ± 0.84	18.34 ± 1.02^{ab}	19.30 ± 0.35^{a}	14.21 ± 1.16^{cd}	15.69 ± 1.16^{bc}	16.28 ± 1.08^{abc}	17.90 ± 0.46^{ab}	12.61 ± 0.39^{d}	11.78 ± 1.95^{d}
Sum n-6	14.22 ± 0.25	10.39 ± 0.43^{a}	10.16 ± 0.34^{a}	16.83 ± 0.49^{b}	14.64 ± 0.88^{c}	8.81 ± 0.14^{d}	8.70 ± 0.21^{d}	17.36 ± 0.41^{b}	12.87 ± 0.84^{e}
Ratio (n-3)/(n-6)	1.34 ± 0.04	1.76 ± 0.04	1.90 ± 0.06	0.85 ± 0.10	1.07 ± 0.02	1.85 ± 0.11	2.06 ± 0.01	0.73 ± 0.01	0.91 ± 0.09
Ratio EPA/AA	0.99 ± 0.02	2.11 ± 0.05^{a}	2.16 ± 0.16^{a}	0.83 ± 0.09^{bd}	1.03 ± 0.04^{c}	2.73 ± 0.13^{e}	2.84 ± 0.08^{e}	0.73 ± 0.01^{d}	1.00 ± 0.04^{bc}
Ratio DHA/EPA	0.76 ± 0.01	0.43 ± 0.02^{ac}	0.50 ± 0.02^{ab}	0.50 ± 0.02^{ab}	0.57 ± 0.08^{b}	0.40 ± 0.01^{c}	0.48 ± 0.00^{ac}	0.47 ± 0.01^{ac}	0.51 ± 0.04^{ab}

Data, within a row, not sharing a common superscript are significantly different (α=0.05).

AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 3. Fatty acid (FA) composition of *Artemia* (n=3) and enrichments (n=2) (% of total FA).

	Artemia - Base	*Artemia* - DHA	*Artemia* -AA	*Artemia* - D + A	Enrichment - Base	Enrichment - DHA	Enrichment - AA	Enrichment D + A
16:1n-7c	3.63 ± 0.03^{a}	3.16 ± 0.02^{b}	2.68 ± 0.02^{c}	2.80 ± 0.03^{d}	3.27 ± 0.45^{a}	2.50 ± 0.06^{ab}	2.02 ± 0.03^{b}	2.17 ± 0.08^{b}
16:0	9.71 ± 0.21^{a}	10.65 ± 0.07^{b}	9.02 ± 0.13^{c}	9.98 ± 0.08^{a}	11.34 ± 0.75^{a}	13.61 ± 0.82^{a}	12.30 ± 0.11^{a}	12.84 ± 0.21^{a}
17:0	0.51 ± 0.06^{a}	0.35 ± 0.00^{c}	0.41 ± 0.00^{bc}	0.46 ± 0.02^{ab}	0.48 ± 0.02^{a}	0.29 ± 0.01^{b}	0.40 ± 0.02^{c}	0.37 ± 0.02^{c}
18:2n-6	2.48 ± 0.77^{a}	2.25 ± 0.41^{a}	5.64 ± 0.20^{b}	2.40 ± 0.73^{a}	0.75 ± 0.05^{a}	1.83 ± 0.24^{b}	4.65 ± 0.04^{c}	3.56 ± 0.03^{d}
18:1n-9c	25.00 ± 0.63^{a}	26.08 ± 1.35^{a}	21.81 ± 0.12^{b}	26.77 ± 0.82^{a}	11.92 ± 0.71^{a}	14.30 ± 0.41^{c}	12.38 ± 0.03^{ab}	13.86 ± 0.26^{bc}
18:1n-7c	5.20 ± 0.11^{a}	4.90 ± 1.09^{a}	4.16 ± 0.03^{a}	4.61 ± 0.02^{a}	2.67 ± 0.23^{a}	1.97 ± 0.05^{b}	2.04 ± 0.00^{b}	1.91 ± 0.08^{b}
18:0	3.44 ± 0.06^{a}	3.02 ± 0.02^{b}	3.89 ± 0.05^{c}	4.23 ± 0.07^{d}	2.25 ± 0.11^{a}	1.77 ± 0.11^{b}	5.16 ± 0.03^{c}	3.85 ± 0.06^{d}
20:4n-6 AA	2.36 ± 0.16^{a}	1.68 ± 0.01^{b}	13.98 ± 0.08^{c}	8.43 ± 0.06^{d}	1.14 ± 0.08^{a}	0.64 ± 0.03^{b}	14.66 ± 0.16^{c}	8.74 ± 0.27^{d}
20:5n-3 EPA	11.63 ± 0.08^{a}	9.80 ± 0.04^{b}	7.75 ± 0.17^{c}	8.20 ± 0.04^{d}	8.96 ± 0.46^{a}	6.27 ± 0.16^{b}	5.91 ± 0.02^{bc}	5.25 ± 0.19^{c}
20:2n-6	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.08 ± 0.14^{ab}	0.20 ± 0.02^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.28 ± 0.06^{b}	0.21 ± 0.03^{b}
20:1(n-9/11)c	1.62 ± 0.05^{a}	1.30 ± 0.01^{b}	1.23 ± 0.02^{c}	1.10 ± 0.01^{d}	2.37 ± 0.20^{a}	1.74 ± 0.15^{b}	1.70 ± 0.03^{b}	1.54 ± 0.05^{b}
22:6n-3 DHA	9.84 ± 0.12^{a}	13.78 ± 0.15^{b}	6.54 ± 0.16^{c}	9.47 ± 0.11^{a}	12.89 ± 1.66^{a}	20.71 ± 0.60^{b}	8.59 ± 0.03^{c}	15.40 ± 1.01^{a}
22:1(n-9)	0.22 ± 0.04^{a}	0.19 ± 0.02^{a}	0.17 ± 0.02^{a}	0.17 ± 0.02^{a}	0.40 ± 0.02^{a}	0.35 ± 0.06^{a}	0.35 ± 0.01^{a}	0.30 ± 0.01^{a}
22:0	0.35 ± 0.01^{a}	0.32 ± 0.01^{b}	0.40 ± 0.01^{c}	0.51 ± 0.01^{d}	0.29 ± 0.04^{a}	0.13 ± 0.02^{b}	0.59 ± 0.00^{c}	0.40 ± 0.01^{a}
Other	12.00 ± 0.18	11.25 ± 0.13	11.12 ± 0.14	10.33 ± 0.22	20.64 ± 2.34	16.95 ± 0.93	14.48 ± 0.02	14.80 ± 0.34
Sum SFAs	16.81 ± 0.23^{a}	18.23 ± 0.09^{b}	16.25 ± 0.22^{c}	18.30 ± 0.20^{b}	18.42 ± 1.11^{a}	22.90 ± 1.16^{b}	22.36 ± 0.33^{b}	23.22 ± 0.40^{b}
Sum MUFAs	40.59 ± 0.58^{a}	39.43 ± 0.40^{ab}	33.59 ± 0.14^{c}	38.61 ± 0.77^{b}	28.74 ± 1.79^{a}	26.46 ± 0.28^{ab}	23.89 ± 0.17^{b}	25.08 ± 0.78^{ab}
Sum PUFA	30.59 ± 0.49^{a}	31.08 ± 0.54^{a}	39.05 ± 0.42^{b}	32.75 ± 0.87^{c}	32.20 ± 0.56^{a}	33.70 ± 0.51^{ab}	39.27 ± 0.14^{c}	36.91 ± 1.51^{bc}
Sum n-3	24.33 ± 0.22^{a}	26.12 ± 0.14^{b}	16.43 ± 0.33^{c}	19.70 ± 0.16^{d}	25.91 ± 2.34^{ab}	30.03 ± 0.04^{a}	16.61 ± 0.04^{c}	22.46 ± 1.25^{b}
Sum n-6	5.95 ± 0.68^{a}	4.69 ± 0.40^{b}	22.38 ± 0.29^{c}	12.85 ± 0.71^{d}	5.87 ± 2.92^{a}	3.32 ± 0.46^{a}	22.32 ± 0.15^{b}	14.21 ± 0.26^{b}
Ratio (n-3)/(n-6)	4.13 ± 0.51	5.59 ± 0.46	0.73 ± 0.02	1.54 ± 0.07	5.15 ± 2.96	9.13 ± 1.25	0.74 ± 0.01	1.58 ± 0.06
Ratio EPA/AA	4.94 ± 0.35^{a}	5.85 ± 0.03^{b}	0.55 ± 0.01^{c}	0.97 ± 0.01^{d}	7.87 ± 0.16^{a}	9.74 ± 0.16^{b}	0.40 ± 0.00^{c}	0.60 ± 0.00^{d}
Ratio DHA/EPA	0.85 ± 0.01^{a}	1.40 ± 0.02^{b}	0.84 ± 0.01^{a}	1.15 ± 0.01^{c}	1.44 ± 0.11^{a}	3.30 ± 0.01^{b}	1.45 ± 0.01^{a}	2.93 ± 0.09^{b}

Data, within a row, not sharing a common superscript are significantly different (α=0.05).

Abbreviations as per Table 1.

5.2.5 Effects of commercially available bacterial retardant and nutrient enrichment treatments on microbial levels associated with *Artemia* nauplii and fish larvae cultures

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5.2.5.1 Abstract

Artemia sp. is considered to be a standard live food in marine finfish and crustacean hatcheries. Techniques for hatching, enriching and harvesting have become relatively uniform, although a range of commercial treatments may be used. However, it is evident that *Artemia* nauplii can have high indigenous microflora levels and may harbour pathogenic bacteria, thus serving as vectors for the transfer of these pathogens to cultured fish. This prompted questions regarding the management of the microflora associated with *Artemia* nauplii.

The microbial levels in four hatching and seven enrichment treatments were compared and the development of the resultant bacteria associated with *Artemia* nauplii was tracked during the hatching phase. The total number of viable bacteria was calculated by counting the bacterial colonies formed per agar plate colony forming units, (CFU), after plating serial diluted samples and allowing 24 h of incubation at room temperature.

A large proportion of microbial flora associated with the *Artemia franciscana* nauplii was shown to originate from within the cysts and to proliferate upon hatching. Unhatched cysts were found to have an indigenous microflora at levels of $9.8 \times 10^4 \pm 1.4 \times 10^4$ CFU (ml of cysts)$^{-1}$ (±SE). Microbial levels associated with newly hatched *Artemia* nauplii were on average $6.4 \times 10^7 \pm 5.7 \times 10^6$ CFU (ml of *Artemia*)$^{-1}$ and $4.7 \times 10^7 \pm 1.0 \times 10^7$ CFU (ml culture media)$^{-1}$. The process of cyst decapsulation was shown to have no effect on the indigenous microflora associated with unhatched *Artemia* and the subsequent microbial levels associated with newly hatched *Artemia* nauplii. The use of Hatch Controller® significantly reduced the total microbial levels in newly hatched *Artemia* to an average of $3.0 \times 10^5 \pm 1.2 \times 10^5$ CFU (ml of *Artemia*)$^{-1}$ and $6.5 \times 10^2 \pm 3.5 \times 10^2$ CFU (ml culture media)$^{-1}$.

Powder enrichment (Algamac-2000®), based on spray dried *Schizochytrium* sp. was shown to promote significantly higher microbial growth levels directly associated with the *Artemia* nauplii $3.2 \times 10^8 \pm 4.9 \times 10^7$ CFU (ml of *Artemia*)$^{-1}$ when compared to oil based emulsion enrichments that showed on average $2.9 \times 10^7 \pm 3.3 \times 10^6$ CFU (ml of *Artemia*)$^{-1}$. DC DHA Selco® significantly reduced the microbial levels associated with *Artemia* nauplii to $2.4 \times 10^6 \pm 8.1 \times 10^5$ CFU (ml of *Artemia*)$^{-1}$. Microbial levels in finfish larval rearing tanks showed a positive correlation between those associated with *Artemia* nauplii treatments.

After establishing the origin of the bacteria and analysing the effects of common practices and treatments used in commercial marine hatcheries, it is possible to develop more appropriate microbial management for the control of pathogenic bacterial populations in *Artemia* nauplii culture. This can contribute to maintaining fish health, while maximizing productivity in the early stages of fish rearing in the hatchery.

Key words: Bacteria, microflora, microbial, *Artemia*, enrichment, culture media.

5.2.5.2 Introduction

Produced as a live food organism for aquaculture, *Artemia* nauplii are considered essential in the culture of many marine species (Sorgeloos et al. 2001). Generally, newly hatched *Artemia* nauplii and later enriched *Artemia* nauplii are fed to marine fish larvae following their initial rearing stage

on rotifer *Brachionus plicatilis* and before weaning onto dry micro diets (Watanabe et al. 1982; Moretti et al. 1999). There are two stages in which *Artemia* nauplii are used, depending on the larvae development and mouth gape (Moretti et al. 1999). Newly hatched *Artemia* nauplii (instar I) are relatively small (400-500 µm) and therefore are fed immediately after the rotifer stage (Lavens and Sorgeloos, 1996). However their nutritional value is variable and they often lack essential fatty acids needed to fulfill the requirements of developing fish larvae (Watanabe et al. 1978, 1980, 1982; Sorgeloos et al. 2001). Therefore, as soon as the larvae are able to consume enriched *Artemia* metanauplii, which are significantly larger (± 660 µm), *Artemia* nauplii are on grown for 6-8 h after hatching until they have become second-instar metanauplii and begin to actively up take food particles (Sorgeloos et al. 2001). The *Artemia* nauplii are then fed for 24 h on an enriched media containing essential fatty acids (n-3 HUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6). This process of n-3 HUFA enrichment is aimed at boosting the *Artemia* nauplii's nutritional value for fish larvae and is a standard procedure in marine finfish hatcheries (Barclay and Zeller, 1996; Sargent et al. 1999a, 1999b; Sorgeloos et al. 2001). Currently there are several commercial products that are designed to boost the n-3 HUFA's in fish larvae. These products are based on marine oils (Ozkizilcik and Chu, 1994) for example the Selco® (INVE) product range or on dried *Schizochytrium* sp. such as Algamac-2000® (Bio-Marine Inc., Aquafauna).

However, it has been observed and well documented that live food organisms such as *Artemia* nauplii can have high indigenous microflora levels and may harbour pathogenic bacteria, thus serving as vectors for the transfer of these pathogens into the larvae culture environment (Moretti et al. 1999; Makridis et al. 2000; Olafsen, 2001). Specifically, Olsen et al. (2000) demonstrated a correlation between microbial levels in live feeds and larvae mortality rates. One approach to this problem is to control the bacteria by reducing total microbial levels during the culture of the live food organisms. A number of commercial products that claim to control microbial levels at the hatching and/or enriching stages are now available. In the present study, microbial levels were determined by comparing cultures supplied with different commercially available and experimental products at the hatching and enrichment stages, including some reputed to control microbial levels. The microbial levels were estimated at all the stages in both, the culture media and the *Artemia* itself and in the larvae rearing tank water.

5.2.5.3 Materials and methods

Experimental Design

Artemia cysts (Great Salt Lake (GSL) origin, INVE premium grade) were hatched and then treated with commercial or experimental enrichment and/or disinfectant products (Table 1). Total microbial levels associated with *Artemia* nauplii and their hatching and enrichment culture media were determined.

Both *Artemia* and associated hatching and enrichment culture media were sampled at various stages of production. All hatching treatments were added at specified application rates according to the manufacturers' directions, however, all enrichments were added at a rate of 0.45 g l^{-1} in 20 L of seawater. This rate is below the rates recommended by some of the manufacturers (0.6g l^{-1}, INVE) for enriching *Artemia*, however, in order to gain a direct comparison between all the products, a standardised rate of application was needed. Also, we found that the *Artemia* fatty acid profile was equally good and DO levels in the tanks remained higher (Kolkovski et al. unpublished data).

System Design

The system is comprised of eight 50 L conical fibre reinforced plastic (FRP) tanks, held within a 1,000 L temperature controlled water-bath, providing highly reproducible conditions. Inlet flow-

rates are preset to 10 L min^{-1} marine bore water (35‰), which is also used to rinse during harvest as a flow-through into harvesting containers. The bore water was sampled and found to be free of bacteria.

Aeration is provided using a top-capped 25 mm PVC standpipe with an air inlet fitted to the cap and outlet holes at the base, which provides total mixing with no 'dead spots'. During the enrichment period, oxygen is supplied via fine air diffusers connected to a manifold; dissolved oxygen levels were constantly maintained above 5.0 mg l^{-1}.

External lights have been fitted below and above individual tanks in order to allow separation of the live *Artemia* from the empty shells and unhatched cysts. Each tank has a clear section at the apex of the bottom cone and its own light proof cover in order to block out natural light during harvesting, when bottom lighting is required (Kolkovski et al. 2004 b).

Artemia nauplii

Artemia cysts were decapsulated using chlorine and sodium hydroxide for approximately 5 minutes, then rinsed with seawater until all traces of chlorine was eliminated (Van Stappen, 1996). Excess water was then removed using a suction filter prior to storage at 4°C in an airtight bag, until used within 24-48 h.

Artemia nauplii hatched from decapsulated cysts were used in all enrichment treatments and the control treatment, except for Hatch Controller®, where the manufacture specified decapsulation was not required. A control treatment of non-decapsulated cysts was also hatched. A1 DHA Selco® was applied in two ways; firstly according to the manufacturers' directions where it was used as a hatching treatment and then extra product was added directly to this media as an enrichment. Secondly, a rinsing process was performed between hatching and enrichment, as in other enrichment treatments.

Artemia were hatched in a static culture of marine bore water (35‰) over a 22 h period at 28.0°C ± 0.1°C. Cysts were added to hatching tanks at a rate of 1.2 g l^{-1} of hydrated decapsulated cysts, which corresponded to 0.6 g l^{-1} dry non-decapsulated cysts.

Artemia nauplii were harvested using light in order to separate them from the unhatched cysts and shell debris. The light promotes the migration of the *Artemia* nauplii away from the cyst debris and allows the wastes to be removed with minimal loss of live *Artemia* (Lavens and Sorgeloos, 1996). *Artemia* nauplii were rinsed at a rate of 10 L min^{-1} for 20 min (1 vol min^{-1}) in a bucket fitted with a 100 µm outlet screen.

Artemia nauplii were enriched over a 18 h period in a static culture of marine bore water (35‰) at a rate of 1.25×10^5 nauplii l^{-1} in 20 L of water. Enrichments were added at a rate of 0.45 g l^{-1} of seawater. Enrichments were mixed with 1 L of fresh water using a kitchen blender (Kenwood chef) at high speed for 3 min at 30ºC. They were then added to the *Artemia* culture in two equal portions, the first at 3:30 pm and the second automatically at 2:00 am the following morning. Enrichments were kept cool with ice bricks before introducing them into the *Artemia* enrichment tanks.

Sampling occurred at 11:30 am following hatching and during harvest of enriched *Artemia* at 9:30 am following enrichment, the subsequentl day. Enriched *Artemia* were rinsed at a rate of 10 L min^{-1} for 30 min (1 vol min^{-1}) in a bucket fitted with a 100 µm outlet screen.

Enriched *Artemia* nauplii were introduced to 270 L marine fish larvae rearing tanks as a live food at a rate of 1.5 million *Artemia* nauplii in 2.5 L of water, in order to achieve a final concentration of 5.6 *Artemia* ml^{-1}. All tanks, pipes, air stones, harvest buckets etc. were sterilized after use with oxalic acid and/or ethanol.

Sampling Procedure

Sampling equipment was pre-sterilized using ethanol spray. A 100 µm screen was used to separate *Artemia* from the enrichment or hatching media then rinsed with distilled water and excess water was allowed to drain off. The resultant *Artemia* nauplii were at a concentration of 2160 ± 20 ml^{-1}. *Artemia* nauplii were kept on ice until processed and were then homogenized using a high-speed homogeniser (IKA). *Artemia* cysts were homogenized using a sterile mortar and pestle.

Enrichment media were sampled using sterile 5 ml sampling vials. Samples were taken from the outlets of the harvesting containers (100 µm screened 15 L buckets, holding capacity 10 l).

Larvae rearing tank water was sampled by immersing a sterile 100 µm screen into the tank and filling a 5 ml sterile sample vial beneath the surface from within the screen.

Microbial Assay

Both *Artemia* nauplii and culture media samples were serial diluted (Quinn et al. 1994) and a 100 µl sample was spread onto MSA-B (Marine Salt Agar-Blood, composed of Trypticase Soya agar, 3% horse blood and 2% NaCl) . Dilutions for *Artemia* nauplii and cysts were to 10^{-5} dilution, and culture media samples were diluted to 10^{-4}, in order to get a suitable number of CFU (Colony Forming Units) $plate^{-1}$ for counting. Samples were plated at each of the dilutions produced for *Artemia* nauplii, cysts and culture media.

All agar plating was carried out in a laminar flow cabinet in sterile conditions. All equipment was sterilized using flame, ethanol spray or autoclave. Dilution media was tested for microbial activity to guarantee sterility of media and methods. Marine bore water, algae and enrichments, prior to use, were tested for microbial levels.

The plates were left at room temperature (≈25°C) for 24 h before counting colonies formed per plate. Whole plates were counted when possible or two quarter plate areas were counted, averaged and multiplied by four, when colony numbers were high (Quinn et al. 1994).

The Animal Health Laboratories, Department of Agriculture Western Australia using standard methods outlined in Quinn et al. (1994), completed bacterial identification. Identification focussed on *Vibrio* sp., as this genus of bacteria are known marine finfish pathogens and are commonly found associated with *Artemia* (Olafsen, 2001; Verschuere et al. 2000).

Larvae rearing tanks

Artemia supplied with one of six enrichments were fed to yellowtail kingfish (*Seriola lalandi*) larvae. 380 14-day old larvae were reared in each of 270 L conical flow through tanks. Each treatment (enrichment) had four replicates (tank). The larvae were reared in marine bore water (35‰, 20°C) flow at 2 L min^{-1} through an up welling system (Kolkovski et al. 2004 a). Algae, *Nannochloropsis* sp. was added automatically at set intervals to maintain 30-40 cm secchi-disk visibility ('green water' method). Samples were taken from tanks where the fish had been previously fed on their respective treatments.

During a larval rearing cycle bacteria counts were recorded in the morning prior to feeding and algae addition and 2 h after algae and *Artemia* were added to the rearing tank.

Statistical Design and Analysis

Treatments were randomly assigned, using a block design, to tanks and over time with duplicate samples taken from three replicates for each treatment. Treatments were repeated if no result could be obtained because the CFU $plate^{-1}$ was too numerous to count. The CFU ml^{-1} of sample was calculated according to the following formula:

$$CFU\ ml^{-1} = (\text{plate count (CFU)} \times \text{dilution}) / \text{Sample volume (ml)}$$

Where,

Plate Count is CFU (MSA-B plate)$^{-1}$ inoculated with 0.1 ml of sample of dilution 10^x.

duplicates from 3 replicate tanks was determined in order to calculate the microbial levels per ml of sample and per *Artemia* cyst or nauplii.

The variance of microbial levels within treatments over time and between treatments at the same time point was assessed by a one-way ANOVA and Tukey's Post Hoc Comparison. All data of variance were tested for normality (Shapiro – Wilk W test) and homogeneity (Bartlett Window). The critical level for significance was set at $P < 0.05$. All variation estimates given in the paper are ± SE.

Table 1. Microbial levels in treatments when prepared for administering to *Artemia* nauplii culture tanks.
** Manufacturers claim that these products have an antibacterial effect.*The average CFU from 2

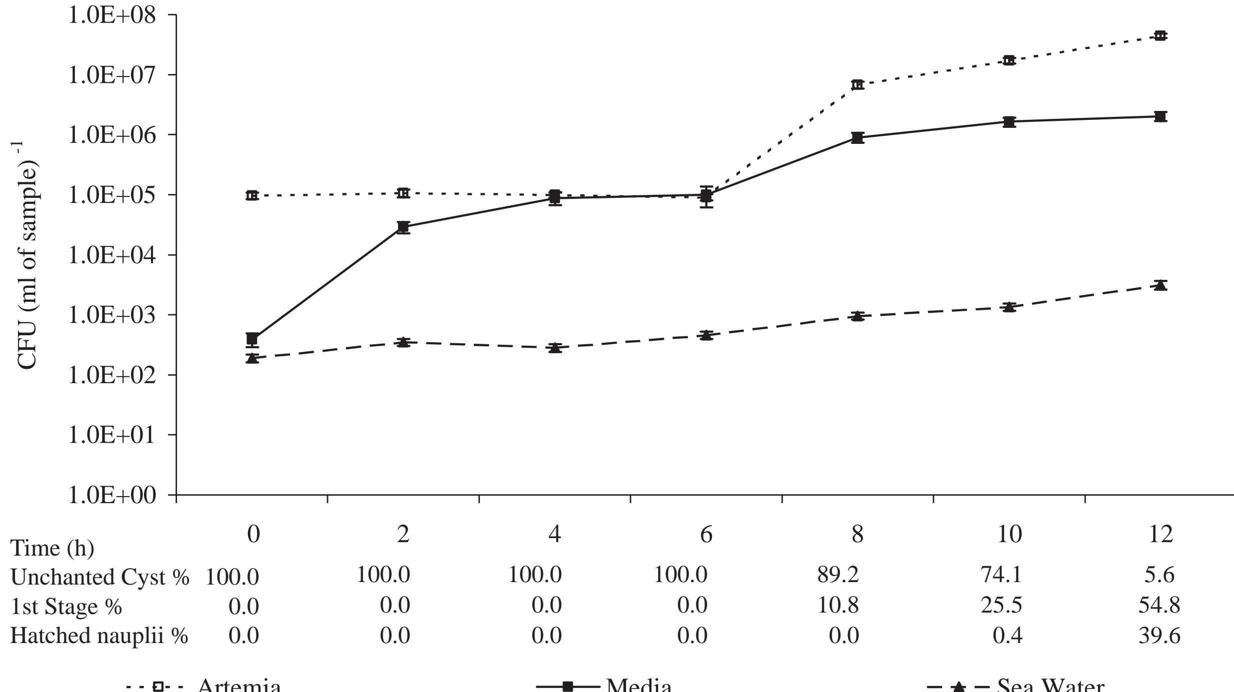
Treatment	Source	Type	CFU·ml^{-1}± Std. Err.
Algamac-2000®	Bio-Marine, Inc., Aquafauna	Spray Dried *Schizochytrium* sp.	1903 ± 283
Super Selco®	INVE Aquaculture	Oil based emulsion	35 ± 10
DHA Selco®	INVE Aquaculture	Oil based emulsion	72 ± 41
A1 DHA Selco®*	INVE Aquaculture	Oil based emulsion	90 ± 52
DC DHA Selco®*	INVE Aquaculture	Oil based emulsion	75 ± 53
Hatch Controller Selco®*	INVE Aquaculture	Oil based emulsion	10 ± 4
Super HUFA®	Salt Creek, Inc.	Oil based emulsion	5450 ± 521
Experimental enrichment	Dr S. Kolkovski	Oil based emulsion	297 ± 32
Cyst decapsulation		NaOH Buffered Chlorine	N/A

5.2.5.4 Results

Artemia Nauplii Hatching Procedure

High microbial levels, associated with *Artemia* nauplii and their respective culture media, were recorded throughout the production process at indicated times following the initiation of the incubation period. The initial microbial levels associated with the hatching culture media of decapsulated cysts were not significantly different ($P < 0.05$) from marine water (without cysts) held in the same system, showing $3.9 \times 10^2 \pm 2.5 \times 10^2$ CFU ml^{-1} and $1.9 \times 10^2 \pm 6.6 \times 10^1$ CFU ml^{-1} respectively. The microbial levels in the hatching culture media increased steadily until the cysts began to hatch after 6 h of incubation. Following this, it increased at an accelerated rate until at 12 h of incubation, the microbial levels in the hatching culture media were significantly higher ($P < 0.05$) than the marine water levels at $2.0 \times 10^6 \pm 1.0 \times 10^7$ CFU (ml of solution)$^{-1}$ and $3.1 \times 10^3 \pm 1.2 \times 10^3$ CFU (ml of solution)$^{-1}$ respectively (Fig. 1). Both the hatching culture media and

marine water microbial levels showed a significant increase (P < 0.05) after 12 h of incubation relative to the initial microbial levels, however in the first 2 h of incubation, the microbial levels in the hatching culture media significantly diverged (P < 0.05) from the marine water microbial levels (Fig. 1). Decapsulated *Artemia* cysts showed an initial microbial level of $9.7 \times 10^4 \pm 2.9 \times 10^4$ CFU (ml of cysts)$^{-1}$, which remained constant until hatching, at which time it increased significantly until harvest at 22 h of incubation, when microbial levels were on average $2.1 \times 10^8 \pm 7.2 \times 10^7$ CFU (ml of *Artemia*)$^{-1}$ before rinsing, reducing significantly (P < 0.05) to $6.8 \times 10^7 \pm 2.9 \times 10^7$ CFU (ml of *Artemia*)$^{-1}$ after rinsing (Fig. 2).



Time (h)	0	2	4	6	8	10	12
Unchanted Cyst %	100.0	100.0	100.0	100.0	89.2	74.1	5.6
1st Stage %	0.0	0.0	0.0	0.0	10.8	25.5	54.8
Hatched nauplii %	0.0	0.0	0.0	0.0	0.0	0.4	39.6

Figure 1. Microbial levels (Means ± S.E., where n=6) associated with *Artemia* cysts and their culture media relative to the percentage of hatched and unhatched cysts, over time during the hatching process, and compared to untreated marine bore water held simultaneously in the same system. '1st stage%' refers to cysts that have elongated and shed their outer chorion layer but not fully hatched, and 'hatched%' refers to free-swimming *Artemia* nauplii.

Culture media and *Artemia* microbial levels resulting from non–decapsulated cysts and A1 DHA Selco® treated cysts after 22 h of incubation were not significantly different from those resulting from the culture of decapsulated cysts. However, A1 DHA Selco® culture media microbial levels were significantly higher (P < 0.05) than the other treatments after rinsing, at $4.4 \times 10^4 \pm 6.9 \times 10^3$ CFU (ml of solution)$^{-1}$ (Fig. 2).

Hatch Controller® significantly reduced (P < 0.05) microbial levels in the hatching media compared to the other three hatching treatments resulting in $6.5 \times 10^2 \pm 2.5 \times 10^2$ CFU (ml of solution)$^{-1}$ (Fig. 2). The microbial levels in *Artemia* treated with Hatch Controller® were also significantly lower when compared to the other treatments which averaged $3.0 \times 10^5 \pm 8.0 \times 10^4$ CFU (ml of *Artemia*)$^{-1}$ (Fig. 2).

Rinsing with fresh seawater had a significant effect (P < 0.05) on the microbial levels of hatching media in all treatments except Hatch Controller®, which remained unchanged. Rinsing had no significant effect on the microbial levels associated with *Artemia* nauplii in the A1 DHA Selco® and Hatch Controller® treatments. However, rinsing significantly reduced (P < 0.05) microbial levels in *Artemia* nauplii from decapsulated and non-decapsulated treatments (Fig. 2).

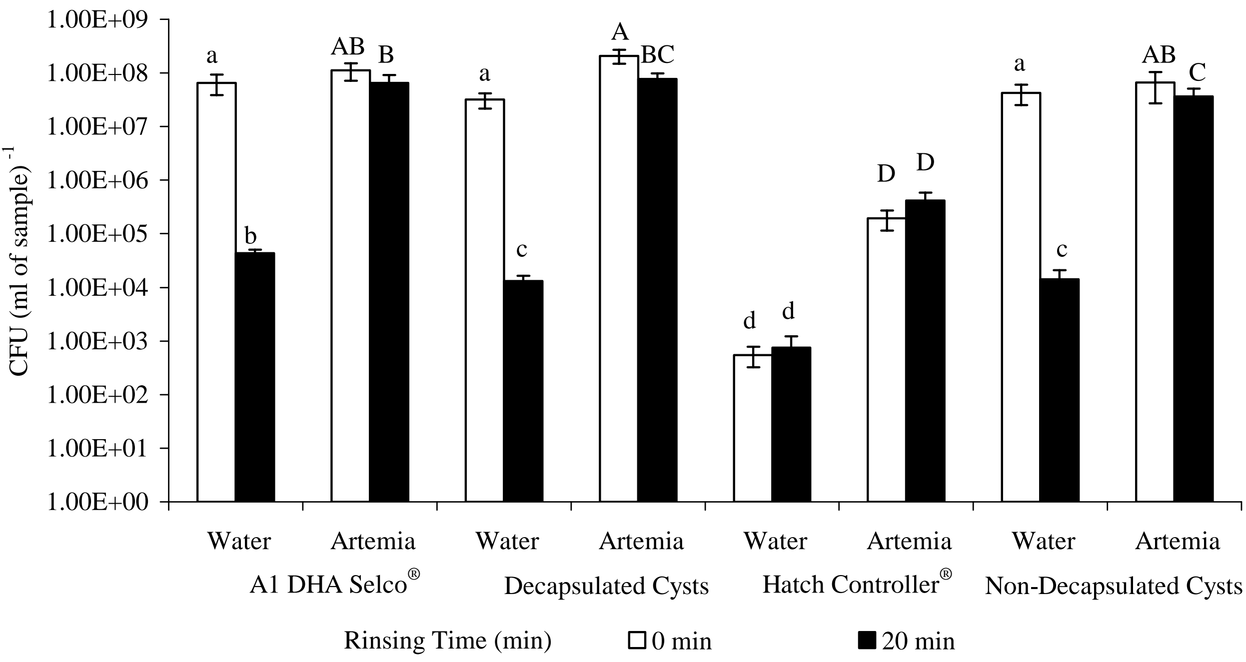


Figure 2. Microbial levels (Means ± S.E., where n=6) of newly hatched *Artemia* nauplii and their respective culture media not rinsed and rinsed for 20 minutes at one vol·min^{-1} marine bore water turnover. Lower case lettering denotes significant differences (P < 0.05) between hatching media samples. Capital lettering denotes significant differences (P < 0.05) between *Artemia* nauplii samples.

Artemia Nauplii Enrichment Procedure

A comparison of microbial levels in *Artemia* enrichment culture media after a 22 h culture period and following rinsing showed that the microflora in DC DHA Selco® at 1.3 x 10^3 ± 2.6 x 10^2 CFU (ml of solution)$^{-1}$ was the only treatment resulting in significantly lower microbial levels (P < 0.05) than the control treatment at 1.08 x 10^4 ± 3.7 x 10^3 CFU (ml of solution)$^{-1}$ (Fig. 3). All other treatments showed significantly higher (P < 0.05) bacterial levels than the control, recording on average 2.0 x 10^4 ± 4.6 x 10^3 CFU (ml of solution)$^{-1}$ with no significant difference between them. In addition, A1 DHA Selco® showed a resultant microbial level after rinsing of 3.2 x 10^4 ± 1.1 x 10^4 CFU (ml of solution)$^{-1}$, which was significantly higher (P < 0.05) than all the other commercial enrichments (Fig. 3).

The proportions of bacterial levels in enrichment culture media remained the same relative to each other, following rinsing for 30 minutes. A significant (P < 0.05) reduction in microbial levels resulted from the first ten minutes of rinsing, however there was no further significant effect due to rinsing across all treatments, except DC DHA Selco®, which remained relatively constant throughout the rinsing process (Fig. 3).

A comparison between the microbial levels of *Artemia* nauplii hatched using A1 DHA Selco® and then either rinsed before administering the enrichment or unrinsed (as in the manufacturers' instructions) was made with no significant difference between the two treatments. Therefore the final microbial levels associated with *Artemia* enriched using A1 DHA Selco® relate to the manufacturers' instructions, but the levels of enrichment were applied as described in the methods.

The microbial levels associated with enriched *Artemia* nauplii in the majority of enrichment treatments showed no significant change (P > 0.05) from the newly hatched *Artemia* nauplii hatched from decapsulated cysts 6.8 x 10^7 ± 2.9 x 10^7 CFU (ml of *Artemia*)$^{-1}$ before they were enriched (Fig. 4). However, the control treatment showed significantly higher (P < 0.05) microbial levels at

$1.6 \times 10^8 \pm 1.9 \times 10^7$ CFU (ml of *Artemia*)$^{-1}$ than the newly hatched *Artemia* nauplii. Algamac-2000® enriched *Artemia* nauplii were significantly higher ($P < 0.05$) in microbial levels at $3.2 \times 10^8 \pm 4.9 \times 10^7$ CFU (ml of *Artemia*)$^{-1}$ than the control. Both Super HUFA® at $5.9 \times 10^7 \pm 8.0 \times 10^6$ CFU (ml of *Artemia*)$^{-1}$ and DC DHA Selco® at $8.3 \times 10^6 \pm 1.4 \times 10^6$ CFU (ml of *Artemia*)$^{-1}$ showed a reduction in microbial levels after treating the newly hatched *Artemia* nauplii, however DC DHA Selco® was the only enrichment treatment resulting in significantly lower ($P < 0.05$) microbial levels than the newly hatched *Artemia* nauplii (Fig. 4).

The Algamac-2000® enrichment treatment resulted in significantly higher ($P < 0.05$) microbial counts per ml of *Artemia* nauplii compared to all other treatments, while Super HUFA® was significantly lower ($P < 0.05$) than all other treatments except DC DHA Selco® which was significantly lower ($P < 0.05$) than Super HUFA®. There was no significant difference ($P > 0.05$) detected between the control and the remaining treatments excluding Algamac-2000®, Super HUFA® and DC DHA Selco® (Fig. 4). Rinsing the *Artemia* enrichment in the harvest buckets with fresh seawater had no significant effect ($P > 0.05$) on the average bacteria levels associated with *Artemia* nauplii.

Larvae Rearing Tanks

During a larval production cycle, larvae tank bacterial levels were on average $1.6 \times 10^3 \pm 1.5 \times 10^2$ CFU (ml of tank water)$^{-1}$ before feeding. Algamac-2000® and the control treatment showed significantly different ($P < 0.05$) microbial levels to each other resulting in residual environmental microbial levels of $2.2 \times 10^3 \pm 2.4 \times 10^2$ CFU (ml of tank water)$^{-1}$ and $1.0 \times 10^3 \pm 1.3 \times 10^2$ CFU (ml of tank water)$^{-1}$ respectively, but both were not significantly different from the rest of the treatments (Fig. 5).

Following feeding, the highest microbial level was $9.9 \times 10^3 \pm 2.5 \times 10^2$ CFU (ml of tank water)$^{-1}$ in Algamac-2000®, while the other treatments were significantly less ($P < 0.05$), on average $3.7 \times 10^3 \pm 6.4 \times 10^2$ CFU (ml of tank water)$^{-1}$ with no significant difference ($P > 0.05$) between them. The microbial counts in larvae tank water showed that all treatments increased significantly ($P < 0.05$) in microbial levels following the feeding of *Artemia* nauplii. The control (no enrichment) showed the lowest microbial counts before and after feeding *Artemia* nauplii. These microbial levels were significantly lower ($P < 0.05$) than the Algamac-2000®, however not significantly different ($P > 0.05$) than the remaining oil based enrichments (Fig. 5).

Algae

The microbial levels found in the algae tanks, used for introducing algae to the larvae tanks for the implementation of the 'green water' method, were $1.8 \times 10^3 \pm 1.3 \times 10^3$ CFU (ml of tank water)$^{-1}$. No significant difference ($P < 0.05$) was detected between the microbial levels in the algae and those in the yellow kingfish larvae tanks prior to feeding *Artemia* nauplii and the introduction of algae.

Bacterial Identification

Taxonomic investigation of the bacterial flora associated with *Artemia franciscana* nauplii used in this study showed that *Vibrio alginolyticus, V. fluvialis* and *V. proteolyticus* were all present, both in the culture media and associated directly with the nauplii, at all stages of the *Artemia* culture procedure.

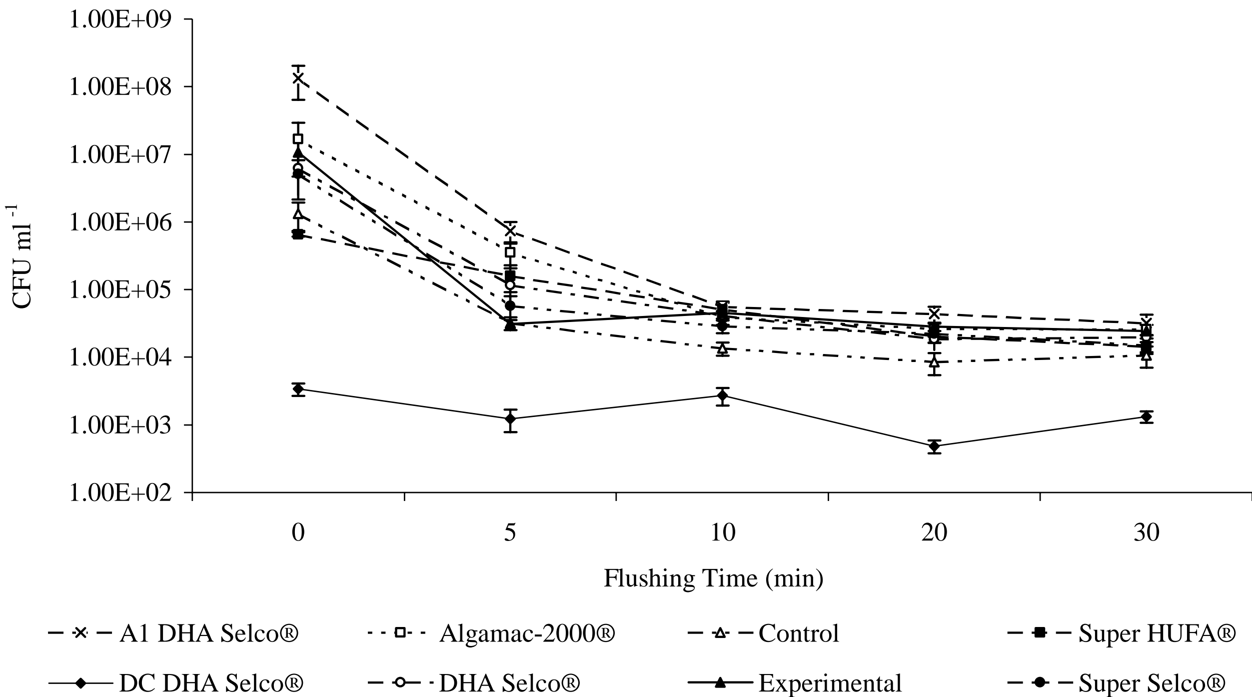


Figure 3. Microbial levels (Means ± S.E., where n=6) in enrichment solutions relative to time during rinsing, at harvest. After 10 minutes no significant reduction (P > 0.05) in enrichment microbial levels was achieved across all enrichments.

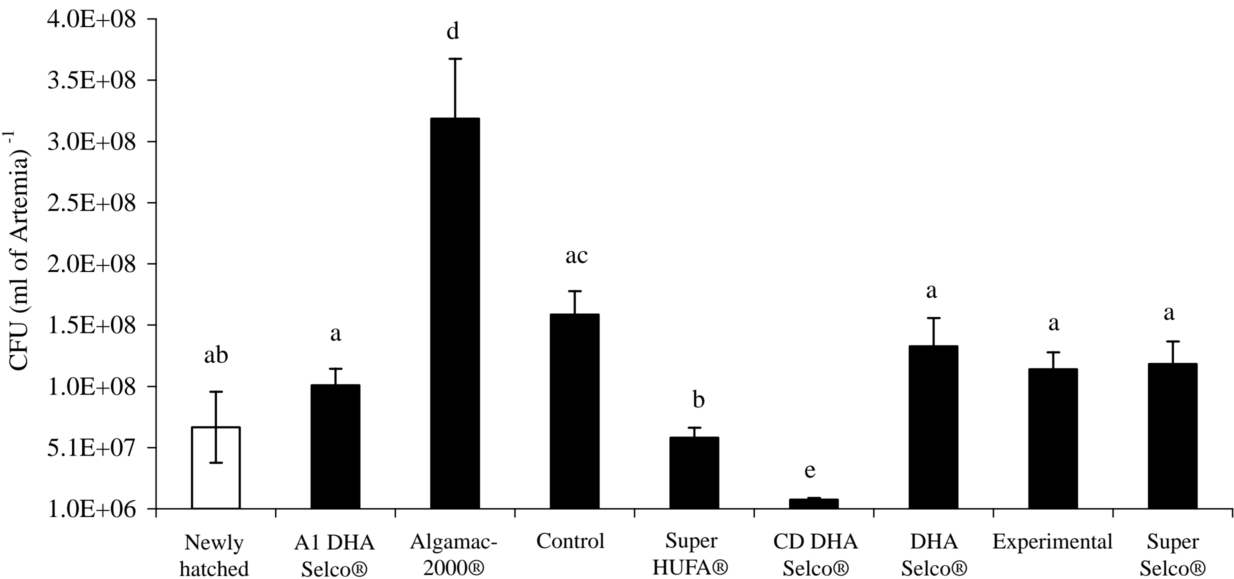


Figure 4. Microbial levels (Means ± S.E., where n=6) of *Artemia* nauplii following enrichment treatments. Averages were taken from the entire harvest procedure as no significant change (P > 0.1) in bacterial levels associated with *Artemia* nauplii occurred after rinsing with fresh seawater. Newly hatched data refers to decapsulated cysts after rinsing. Lettering denotes significant differences (P < 0.05) between *Artemia* nauplii samples.

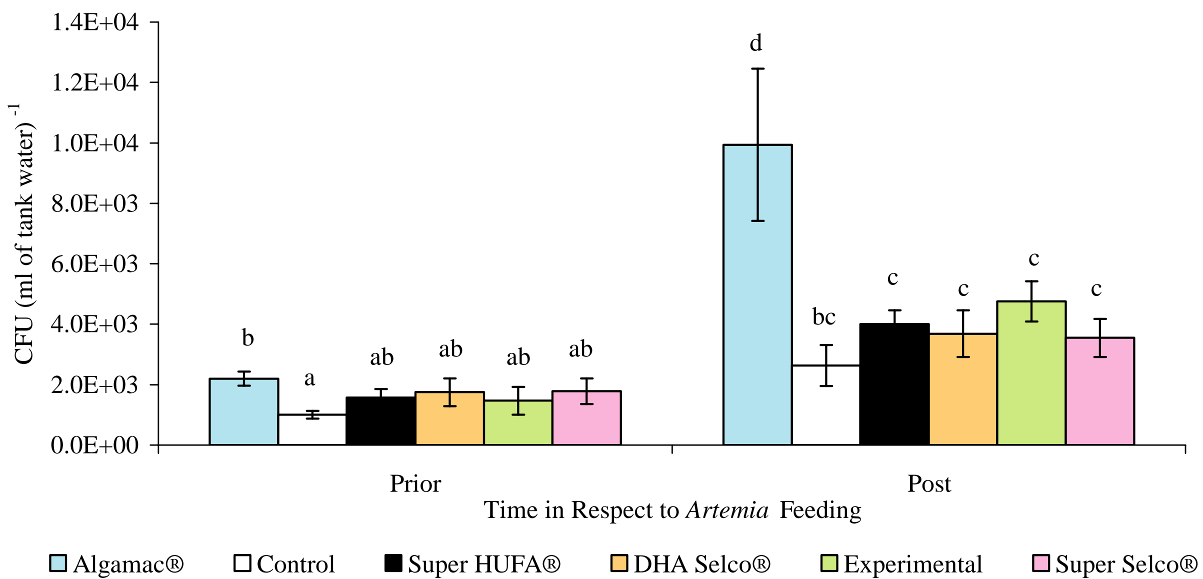


Figure 5. Microbial levels (Means ± S.E., where n=6) in Yellowtail kingfish (*Seriola lalandi*) larvae rearing tanks before and after feeding enriched *Artemia* nauplii and the introduction of algae. Lettering denotes significant differences (P < 0.05) between *Artemia* nauplii samples.

5.2.5.5 Discussion

This study investigated total microbial levels in *Artemia* nauplii when used as live food organisms under marine fish hatchery conditions. The study has shown that elevated microbial levels are associated with both individual *Artemia* nauplii and their culture medium. Further to this, it is evident that the bacteria associated with *Artemia* nauplii are transferred into the larvae culture tanks in quantities proportional to those associated with specific treatments.

Hatching Treatments

A comparison of the microbial levels in four hatching treatments was made. Decapsulated and non-decapsulated cysts were hatched untreated as well as accompanied by A1 DHA Selco® and Hatch Controller® respectively. The manufacturer suggests that both Hatch Controller® and A1 DHA Selco® have an anti-bacterial effect in their application as hatching treatments. The results show that Hatch Controller® produced microbial levels up to 100 times lower than the other hatching treatments, thus proving to be an effective control of total bacterial levels (Fig. 2). However, we recorded no significant difference between microbial levels in either the other hatching media or individual *Artemia* of the remaining treatments. Therefore, any antibacterial characteristics of either the decapsulation process (Olsen et al. 2000; Sorgeloos et al. 2001) or A1 DHA Selco® were not demonstrated by this study (Fig. 2). In addition, A1 DHA Selco® showed significantly higher microbial levels after rinsing through a 100 µm screen, which suggests that this treatment reduces the effectiveness of rinsing. Since *Artemia* will not begin to ingest food particles until after they develop into the second-instar larvae, at about 6-8 h post hatching (Sorgeloos et al. 2001), it is also of little benefit as an enrichment treatment during this phase in the production process, unless, as suggested by the manufacturer the hatching incubation period is extended to 30 h, in order to allow sufficient development to begin ingestion of the oil emulsion globules. Although the decapsulation process showed no antibacterial properties, Lavens and Sorgeloos (1996) reported other advantages such as a higher resultant energy content and weight (30-55% depending on strain) of newly hatched *Artemia* nauplii and a reduction in the amount of shell debris that can be hazardous to fish larvae, through blockage of the larval gut.

Bacterial levels in all of the hatch treatment media showed a significant drop following rinsing with seawater for 20 min, with the exception of Hatch Controller®. Densities of bacteria adhering to the surface of shell debris and cyst hatch wastes, were probably reduced. Hansen and Olafsen (1999) reported that the level of marine vibrios coincide with the densities of marine planktonic particles, therefore the reduction of shell debris in the culture media due to rinsing would coincide with a reduction in microbial numbers. However, rinsing did not lead to a significant reduction in microbial levels directly associated with newly hatched *Artemia* nauplii. This can have a far greater consequence on the transfer of bacteria to fish larvae, as they are more likely to be pathogenic strains and would be ingested in far greater quantities than water born bacteria (Olafsen, 2001).

The numbers of bacteria directly associated with *Artemia* can be extremely high, from 10^6 to 10^8 CFU·(ml of *Artemia*)$^{-1}$ in cultured commercial brand *Artemia* nauplii (Lopez-Torres and Lizarraga-Partida, 2001), and this agrees with our findings. Lopez-Torres et al. (2000) observed similarly high microbial levels, 10^7 to 10^8 CFU·(ml of *Artemia*)$^{-1}$ in newly hatched *Artemia* nauplii sourced from natural populations in Mexico. Additionally, *Vibrio* sp., which are known fish pathogens, constitute a substantial component of the bacterial flora that is naturally associated with *Artemia* (Olafsen, 2001; Verschuere et al. 2000). Qualitative investigation of the bacterial flora associated with *Artemia* nauplii used in this study showed that *Vibrio proteolyticus, V. fluvialis* and *V. alginolyticus* were all present. Olsen et al. (1999) and Villamil et al. (2003) both found that *Vibrio alginolyticus*-like bacteria dominated the microflora associated with *Artemia franciscana*. This raises great concern for the risk of pathogenic infection of marine fish larvae when fed on *Artemia*. Olsen et al. (2000) have shown that the microflora of *Artemia* nauplii introduced into halibut (*Hippoglossus hippoglossus*) larvae cultures were directly transferred to the larvae. High mortality rates have been reported during first feeding of many marine fish species and some have been attributed to the introduction of opportunistic pathogenic bacteria through live food application (Skjermo and Vadstein, 1999). Specifically, Olsen et al. (2000) demonstrated a correlation between microbial levels in live feeds and larvae mortality rates. The proliferation of opportunistic pathogens can cause high mortality in larvae due to the latent maturation of their specific immune system, until at least several weeks after hatching (Olafsen and Roberts, 1993). This implies that at this larval developmental stage, a non-specific defence against harmful bacteria would be beneficial (Ottesen and Olafsen, 2000).

In order to establish an effective control method of the microflora associated with the *Artemia* nauplii, the source of bacteria must be established. Lavens and Sorgeloos (1996) suggested that the bacteria associated with the cyst are on the chorion and develop in the water upon release of glycerol when the nauplii hatch. The decapsulation process is therefore thought to disinfect the cysts before hatching and thus reduce microbial levels associated with hatched *Artemia* nauplii (Sorgeloos et al. 2001; Moretti et al. 1999; Olsen *et* al. 2000). The results of our study do not fully support this hypothesis. This is demonstrated by no significant difference between microbial levels associated with *Artemia* hatched from either decapsulated cysts or non-decapsulated cysts (Fig. 2). The initial microbial level in the hatching culture medium was not significantly different ($P > 0.05$) from that found in the control tanks with no cysts present (Fig. 1), indicating that decapsulation had a disinfecting effect on the external cyst surface. However, there were significant levels of bacteria associated with the decapsulated cysts before incubation, remaining constant until hatching, suggesting no disinfection effect within the cysts. There was however, a clear second peak in bacterial levels directly associated with the *Artemia* nauplii and in their hatching media, possibly due to the glycerol released at hatching.

The hatching culture medium was shown to increase in bacterial levels during the incubation process with microbial levels 3 orders of magnitude higher than that found in the control water before hatching. This implies that the *Artemia* cysts did not introduce bacteria into the culture water, but introduced a source of nutrients at the initial contact with the hatching media, and not just at hatching

as Lavens and Sorgeloos (1996) have suggested (Fig. 1). The resultant environmental bacterial loading at the time of hatching is significant and would have influenced the resultant *Artemia* nauplii microflora. This is supported by Igarashi et al. (1989) who suggest that the majority of the microflora associated with *Artemia* sp. sourced from China are derived from the eggs, however the surrounding water significantly influences the resultant microflora after 24 h of incubation. If this is the case, an effective reduction in bacterial levels associated with *Artemia* nauplii may only come with the use of controlling or disinfecting agents during the hatching and or enrichment stages of the production process.

Enrichment Treatments

To assess the effects of enrichment treatments on microbial ecology, enrichments were prepared for administering to the *Artemia* culture and their microbial levels tested (Table 1). They were thought to have a negligible direct effect on the resultant *Artemia* nauplii and enrichment media microbial levels, as the bacteria were found in relatively low numbers that did not correspond with the final microflora in the relative *Artemia* enrichment solutions.

Most of the treatments showed a slight increase in media microbial levels relative to the control medium, except for Super HUFA® and DC DHA Selco® that were lower. A1 DHA Selco® and Algamac-2000® were both significantly higher in environmental bacterial levels than all the other treatments. The substantial increase in microbial levels in these enrichment solutions over those observed in the other treatments was probably due to the specific nutritive state of the treatments (Olsen et al. 1999). Olafsen and Roberts (1993) suggest that specific ingredients effect selection and growth of the micro-organisms; that adding food to culture solution provides an excellent medium for growth of heterotrophic and opportunistic bacteria. This would suggest that most of the oil-based enrichments provided a more limited availability of nutrients than Algamac-2000®, a particulate enrichment. However, bacterial growth in the oil emulsion enrichment treatments resulted in higher levels than that observed in the control treatment medium. This is due to an increased nutritive state in the media and although an extremely strong surface tension exists on the emulsified oil globules, a greater available surface area for attachment could exist for certain hydrophobic bacteria, thus facilitating a greater number of bacteria in the enrichment media (Olafsen and Roberts, 1993).

DC DHA Selco® was the only treatment that showed a significant decrease in microbial levels associated with the enrichment media from that found in newly hatched *Artemia* media. The microbial levels were found to be 10^3 times lower than that of the control treatment before rinsing. These results concur with De Wolf et al. (1998) while conducting research for INVE Technologies, who found that disinfecting continuously (DC), a precursor concept-treatment to DC DHA Selco®, resulted in bacteria levels in the hatching medium below 10^3 CFU (ml of medium)$^{-1}$ compared to levels of 10^7 CFU (ml of medium)$^{-1}$ in a control standard enrichment treatment.

All of the oil-based treatments resulted in lower bacterial levels associated with *Artemia* than that found in the control treatment and, further to this, Super HUFA® and DC DHA Selco® showed lower levels than the newly hatched *Artemia* nauplii. DC DHA Selco® achieved a reduction in resident *Artemia* bacteria by 10^2 times that of the average oil-based enrichments and by 4×10^2 times that of Algamac-2000®. While De Wolf et al. (1998) achieved a 10^4 times reduction in bacteria levels associated directly with the *Artemia*, our results show the same trend but to a far lesser degree. This may have been the effect of using a reduced concentration of DC DHA Selco® enrichment in this study. Nevertheless, this has a far greater consequence on reducing the transfer of bacteria to fish larvae than simply the reduction in media microflora that rinsing achieves, and it conclusively supports the manufacturers claims that DC DHA Selco® has anti-bacterial properties when used as *Artemia* nauplii enrichment.

In contrast, microbial levels in *Artemia* nauplii enriched with Algamac-2000® were 4 times those found in newly hatched *Artemia* nauplii. This may be the result of the nutrients in Algamac-2000®

being more suited and readily available for the growth of opportunistic bacteria than the other oil based treatments (Olafsen and Roberts, 1993). Consequently, this microflora on the food particles is transferred into the gut of the *Artemia* (Igarashi et al. 1989).

Algamac-2000® may promote higher bacterial levels due to the use of a whole cell by providing more variety of niches for bacteria to inhabit and consequently reducing competition. *Schizochytrium* sp. in Algamac-2000® is made up of about 32% total lipid, 39% protein, with the balance being ash and carbohydrates (Bio Marine Inc., Aquafauna, personal communication). However, the presence of particulate carbohydrates is more suited to the development of *V. anguillarum* than the oil based emulsions. Larsen (1985) reported a 10^3 times increase in *V. anguillarum* in coastal waters of Denmark, due to discharge of water containing carbohydrates. Also, the adhesive properties of some bacteria are more suited to particulate material rather than oil globules, for example, *V. anguillarum* has adhesive factors in its bonding mechanism and does not bond to hydrophobic surfaces (i.e. fish mucus), but binds well to all other tissues (Hansen and Olafsen, 1999). The high carbohydrate content and particulate nature of Algamac-2000®, could therefore lead to an increased predisposition to the development of high numbers of oppurtunistic *Vibrio* sp. such as *V. anguillarum*. In contrast, the growth of bacteria that are able to utilize the specific particulate nutrients in Algamac-2000® may have been restricted in the oil based enrichments (Igarashi et al. 1989; Hansen and Olafsen, 1999). Regardless of the reasons, the introduction of live food containing microbial levels of this magnitude could have quite severe implications on larval health, if they are predominantly opportunistic pathogenic strains.

Elevated microbial levels found in the control treatment *Artemia,* relative to the oil based treatments, probably resulted from a low nutritional state and a corresponding low specific immunity to bacteria (Lavens and Sorgeloos, 1996). This is supported by the fact that the majority of the bacteria in this treatment were associated directly with the *Artemia*, as their culture media showed relatively low microbial levels.

Following the *Artemia* enrichment process it is generally believed that rinsing excess enrichment away from the *Artemia* is advantageous, as it contributes to a reduction in microbial levels that are introduced into the larvae culture tank (Lavens and Sorgeloos, 1996). This is true in relation to the microbial levels associated with the enrichment culture media, as rinsing resulted in a significant reduction in relative microflora levels, consequently leading to a reduction of environmental microflora in the larvae tanks. The rinsing process was most effective in the first ten minutes, but any further rinsing had no significant reduction in bacterial levels in the media (Fig. 3).

However, the enriched *Artemia* nauplii themselves showed no reduction in associated microbial levels even after rinsing for 30 minutes at a turnover rate of 1 $vol \cdot min^{-1}$. This suggests that cultured fish larvae will inevitably consume the bacteria that are associated with the *Artemia* nauplii, which can in turn impact on the gut flora of the larvae (Ottesen and Olafsen, 2000). Rinsing with seawater is therefore not an effective method for reducing microbial levels in *Artemia*; it only has a diluting effect on the water surrounding the nauplii (Lavens and Sorgeloos, 1996). This will have a minimal contribution in preventing pathogenic bacteria from reaching the larvae, as microbial levels in the enrichment media are insignificant compared to bacteria associated with the individual *Artemia*. Therefore treatments that reduce the microbial levels that are associated directly with the *Artemia* nauplii such as DC DHA Selco®, Hatch Controller® and to a lesser extent Super HUFA® are recognised as being more effective treatments for the reduction of pathogen transfer into the fish larvae culture via live feeds.

General discussion

It is considered that all inputs into the marine fish larvae rearing tanks can affect the microflora and hence the development of pathogens (Gomez-Gil et al. 2000; Olafsen, 2001). The establishment of the gut flora of the larvae is therefore influenced by the microflora associated with invertebrates fed as live feed organisms and also that of their environment (Gomez-Gil et al. 2000; Villamil et al. 2003). This study has shown that the application of *Artemia* nauplii, as live feed organisms to the larvae culture tanks, does in fact increase the environmental microbial levels. These microbial levels were shown to persist within the tanks in numbers relative to the treatments from previous feeding. This effect was illustrated by the significant difference in microbial levels detected between the Algamac 2000® and control treatments prior to feeding. Following feeding, the proportions of microbial levels found in the tanks, relative to each other, remained the same.

In an attempt to control pathogenic bacteria, antibiotics have become frequently used in intensive rearing of marine fish (Gomez-Gil et al. 2000), but these may adversely affect the indigenous microflora of the larvae (Hansen and Olafsen, 1999) and develop antibiotic resistant bacterial strains (Gilberg and Mikkelsen, 1998; Villamil et al. 2003).

More extreme disinfection techniques eg use of ozone, formalin bath etc. can reduce the overall level of bacteria associated with *Artemia* nauplii (Olafsen, 2001; Theisen et al. 1998). However, uncontrolled recolonisation of the disinfected organisms may also enable opportunistic bacteria with high growth rates to proliferate in conditions that offer low competition for nutrients (Olafsen, 2001).

When considering the control of bacteria, a holistic approach is needed towards fish larvae health, nutrition and microflora management, instead of simply reducing or attempting to eliminate total microflora or specific pathogenic strains of bacteria that could potentially create an unstable situation (Olsen et al. 2000).

Olsen et al. (2000) have succeeded in reducing bacterial numbers and increasing microflora diversity by feeding *Artemia* nauplii on a micro alga, *Tetraselmis* sp. However, this would reduce the effectiveness of the enrichment treatment by diluting the gut contents with algae and altering the HUFA ratios.

Although continuous disinfection of *Artemia* during hatching and enrichment is becoming standard procedure in many hatcheries, probiotics is an alternative solution to controlling pathogenic strains of bacteria and achieving enhanced growth and survival of marine fish (Skjermo and Vadstein, 1999). Bio-encapsulation during the enrichment phase of *Artemia* production (Eddy and Jones, 2000; Skjermo and Vadstein, 1999) would provide a convenient method of introduction of probiotic varieties such as lactic acid bacteria, already commonly used for terrestrial animals (Sorgeloos et al. 2001; Gomez-Gil et al. 2000), which are also known to be a part of healthy fish gut microflora (Ringo and Gatesoupe, 1998).

5.2.5.6 Conclusion

In our study relatively sterile marine bore water has been used in combination with a range of commercial products to reduce bacteria and/or improve nutrient content of *Artemia*, and a "green water" larval culture system with *Nannochloropsis* that contributes only a low bacterial input. The results suggest that good practices, such as rinsing cyst and hatch debris and/or enrichments away from the culture, are effective in reducing the environmental microbial levels associated with the culture media, but not those associated with individual *Artemia* nauplii. We have found that Hatch Controller® was effective in reducing microbial levels during the hatching process and

that DC DHA Selco® and Super HUFA® did minimize bacterial growth during enrichment. The use of the particulate enrichment, Algamac 2000® could not be justified, unless it has nutritional advantages over these supplements that were not reported in this paper. Maximising the advantages conferred by the more successful products depend on establishing a diverse microflora in the larvae culture environment, which is low in pathogenic bacteria. More qualitative research is required to establish the potential for controlled recolonisation by use of probiotic bacteria in combination with appropriate bacterial retardant and nutrient enrichment products. Our study complements related research on the effects of the commercial treatments used on larval growth and survival.

5.2.5.7 Acknowledgments

This research was supported by the Australian Fisheries Research and Development Corporation (FRDC), project 2001/220. The system was built at the Aquaculture Development Unit, Challenger TAFE, Fremantle, Western Australia as part of collaboration with the Department of Fisheries, Western Australia (DoFWA). The authors acknowledge Dr. Patrick Lavens (Business Unit Manager Health, INVE), Eamon O'brien (Solution Manager Business Unit Aquaculture Nutrition, Larval Fish Nutrition, INVE), Morten Deichmann (Primo Aquaculture), Leland Lai (Bio-Marine, Inc., Aquafauna), Aquasonic (Salt Creek, Inc), Nicky Buller (Department of Agriculture Western Australia) and Dr. G. Maguire (DOFWA) for their useful comments on the manuscript.

This study was sent to all the manufacturers of products that have been tested. One of the producers, INVE emphasised that the study involved application rates of their product that were lower than the recommended rates (in a preliminary trial, no differences in fatty acids levels were found between the recommended doses and the lower doses used in the current trial). An additional trial is proposed which includes the recommended application rates and the rates used in this trial.

Bio-Marine, Inc., Aquafauna, the producer of Algamac 2000, acknowledged that the high bacterial levels found in the *Artemia* enriched with its product were a result of a problem during the product manufacturing. As a result of this study, the production process has been modified to overcome this problem (see Appendix 1).

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5.2.5.9 Appendix 1 – Email correspondence between Aquafauna and Dr Kolkovski

Subject: Re: bacteria ms

From: "Leland - Aquafauna" <lelandlai@aquafauna.com>

To: Sagiv Kolkovski@Watermans@Fisheries

CC: "Lara, Reny" (RenyLara@aquafauna.com), "Cox, Fred" (fredcox@aquafauna.com), "Insalata, Bud" (budinsalata@aquafauna.com), "Boeing, Phil" (pboeing@dc.rr.com)

Reply To: <lelandlai@aquafauna.com>

Date: 03/19/04 04:45 F00

Message Text: Message.htm

Dear Sagiv,

Its been a while since I last contacted you and I happened to run into one of your colleages in Hawaii at the WAS 2004.

This is to provide you a follow up on the bacterial loads study you did last year, which sparked an investigation into the AlgaMac-2000 in-house since the lab test here did not show similar high loads in the AlgaMac-3050 which is produced in the same facilities, and, nor did it appear in the raw inactivated cells (AlgaMac-2050) from which we spray dry into AlgaMac-2000. A couple of months after we had our conversation, our agent in Japan reported high bacterial counts in a couple of batches of the AlgaMac-2000. So, we then went back to hunt down the cause of this. Again, no loads of significance in the AlgaMac-2050 raw inactivated base product on any batches. After spray-drying, the loads were high and we then looked into third party spray-drying contamination. They clean the equipment before and after each client and we did not find material sources of contamination from possible cross contamination due to other products they spray-dry. Next step was the holding time in spray-drying since the process takes up to 6 hours before the slurry is used up from batch to batch. We found that the batches were being held at 45-50 degrees C. Running in-batch samples, we found the bacterial loads to be high before the end of the run for each batch suggesting this was the cause of the high bacterial loads which you found. Processing changes were made and now the vats containing the batch lots during processing are maintained at 60 degrees C. for the entire duration until all of it is spray dried. Post-process lab tests show normal or low bacterial counts within similar ranges as the raw product specs. As the spray-drying process is contracted out to a third party vendor, their through-put processing procedures were not shared with us and was overlooked as the cause of the highbacterial loads. This situation has now been corrected.

I wish to thank you for a first alert to this situation. All products we spray dry is now subjected to more stringent procedures due to your alert.

Thank you.

Best Regards,

Leland Lai

5.3 CO-FEEDINGS AND PROTOCOLS

5.3.1 The effect of *Artemia* and rotifer exclusion during weaning on growth and survival of barramundi (*Lates calcarifer*) larvae

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5.3.1.1 Abstract

Weaning protocols affected growth and survival with co-feeding of rotifers and microdiet allowing complete replacement of *Artemia*, albeit with a small reduction in survival of Barramundi (*Lates calcarifer*) larvae. Four replicates of 6 treatments were initiated by stocking 2 days post hatch (dph) larvae into each 270 L tank. Concentrated green micro-algae *Chlorella* sp. was added daily to all tanks. One treatment received only Gemma Micro (Skretting) microdiet (MD) (treatment-G) from initial exogenous feeding, and three treatments received rotifers for 3, 7 and 12 days including a co-feeding period with this MD and then exclusive feeding with Gemma Micro MD (treatments G3, G7 and G12 respectively). Two treatments received rotifers until 13 dph and an extended co-feeding period starting at 12 dph for 9 days with *Artemia*, and either Gemma Micro MD or Proton (INVE) MD (treatments G12A and P12A respectively), in order to approximate commercial protocols.

There was significantly better survival in treatments G12A and P12A than all other treatments, except treatments P12A and G12 did not show a significant difference in survival. Survival in all treatments was directly related to the live prey and MD lipid levels. Survival in treatments G, G3, G7 and G12 related directly ($r^2 = 0.94$) to the exposure time to rotifers. Treatment G had negligible survival. Barramundi larvae were shown to survive and grow well when rotifers were used exclusively as the live feed component of the protocol. A growth compensation effect was observed in larvae at 28 dph in treatment G12 which had a wet weight 253.4 ± 10.3 mg and standard length 21.5 ± 0.34 mm, and were significantly ($P < 0.05$) larger than those grown in either of the treatments that received additional *Artemia* and Gemma Micro MD (G12A) or Proton MD (P12A), (218.16 ± 13.65 mg and 209.04 ± 12.02 mg WW respectively, and 20.15 ± 0.39 mm and 19.79 ± 0.43 mm SL respectively). Larval growth was directly proportional with the number of days that rotifers were fed in those treatments that did not receive *Artemia*, while treatment G that received no rotifers did not develop and had very few larvae survive until 28 dph.

Treatments G12 and G12A were grown for a further 8 days in order to assess the larvae's performance as juveniles post weaning. It was found that at 36 dph there was no significant difference in either WW or SL between G12 and G12A, suggesting that the additional *Artemia* had no significant effect on growth.

5.3.1.2 Introduction

Live feeds such as rotifers (*Brachionus* sp.) and brine shrimp (*Artemia*) are considered to be essential for the production of marine fish juveniles (Moretti et al. 1999; Baskerville-Bridges and King, 2000; Sorgeloos et al. 2001). Live feed organisms are thought to stimulate larval feeding and digestive system development by their movement and chemical cues. Moreover, as live feed organisms consist of 90% water while micro-diets (MD's) are 90% dry matter, they are more readily digested (Cahu and Zambonino Infante, 2001; Hart and Purser, 1996; Kolkovski et al. 1997, 2004; Kolkovski, 2001). However, high costs and labour requirements, highly variable nutritional value of live feeds (Reitan et al. 1997) and the unreliability of mass cultures associated with the

production of live feeds, illustrate the need to find viable alternatives (Baskerville-Bridges and King, 2000).

Minimizing or eliminating the use of live feeds is economically advantageous for marine finfish hatcheries (Baskerville-Bridges and King, 2000). Person- Le Ruyet et al. (1993) found that by starting the weaning process of European sea bass (*Dicentrachus labrax*) onto MD at 20 dph instead of 35 dph, a reduction of up to 80% of live feed costs occurred during weaning, leading to a 50% reduction of the total operating cost of 3-month-old juveniles. Therefore, in an effort to reduce the reliance on live feed and its associated costs, larvae should be weaned onto formulated diets as early as practical.

In the past, early weaning has led to reduced growth and inferior quality larvae with an increased risk of skeletal deformities (Cahu and Zambonino Infante, 2001; Person-le Ruyet et al. 1993). With recent advances in microdiet (MD) formulation, the pre-weaning period has been considerably reduced, allowing the introduction of specific larval diets to marine finfish culture as early as mouth opening (Cahu and Zambonino Infante, 2001). 'Co-feeding' weaning protocols, simultaneously using inert and live diets, have also been developed to allow a fast and efficient change over period onto dry MD from live feed (Daniels and Hodson, 1999; Hart and Purser, 1996; Koven et al. 2001). This method gives higher growth and survival than feeding either live feeds or MDs alone (Kolkovski et al. 1995). Early co-feeding of an acceptable MD can improve larval nutrition and condition the larvae to more readily accept MD when live feed is withdrawn. Prolonging the live-food feeding period may even have an adverse effect on subsequent growth by conditioning the larvae to accept primarily live prey (Canavate and Fernandez-Diaz, 1999; Cahu and Zambonino Infante, 2001; Kolkovski, 2001).

The current trial investigates the effect of the rotifer-feeding period and the exclusion of *Artemia* during weaning, on barramundi *(Lates calcarifer)* larvae growth and survival. The trial also compares two commercially available MD's when using standard weaning protocols.

5.3.1.3 Methods

Experimental Design

Two commercially formulated dry MDs were used in the experiment, Gemma Micro MD and Gemma MD (Skretting Australia) and Proton MD (INVE, Belgium). Gemma Micro MD was supplied in 2 nominal particle size ranges: Gemma Micro 150 (120 - 200 µm) and Gemma Micro 300 (200 - 450 µm), along with Gemma 0.3 (300 - 600 µm). Proton MD was supplied in 3 nominal particle size ranges: Proton 1 (100-200 µm), Proton 2/4 (200 - 400 µm) and Proton 3/5 (300 - 500 µm). During the transition from one size range to the next the size categorized MDs were mixed (Figure 1).

One treatment received only Gemma Micro (Skretting) microdiet (MD) (treatment-G) from initial exogenous feeding, and three treatments received rotifers for 3, 7 and 12 days including a co-feeding period with this MD and then exclusive feeding with Gemma Micro MD (treatments G3, G7 and G12 respectively). Two treatments received rotifers until 13 dph and an extended co-feeding period starting at 12 dph for 9 days with *Artemia*, and either Gemma Micro MD or Proton (INVE) MD (treatments G12A and P12A respectively), before being exclusively fed on their respective MD, in order to approximate commercial protocols (Tables 1 and 2).

Larvae

Barramundi larvae were transported at 2 dph, within insulated fish transport containers from Darwin Aquaculture Centre NT to the Aquaculture Development Unit, Challenger TAFE, Fremantle WA.

The larvae were acclimated to system conditions over a period of 1 h, and then released into an aerated 1,000 L holding tank. Subsequently, the larvae were transferred to the experimental system in equal numbers.

Twenty-four 270 L up-welling conical tanks (Kolkovski et al. 2004) were each stocked with 2 dph barramundi larvae and fed according to the weaning protocols (Tables 1 and 2). Every 4 days, 5 larvae from each tank were randomly removed and sacrificed using iced water. These larvae were measured for standard length (SL), wet weight (WW) and then dry weight (DW) after 48 h at 80°C. The final observations at 28 dph were averaged for 20 larvae from each tank.

Rearing Protocol

Algae (concentrated *Chlorella* sp. paste, Japan, at 300 ppm) and rotifers (80-150 μm) (enriched in *Chlorella* sp. paste at 500 ppm for 22 h) were automatically supplied by the feeding system (Kolkovski et al. 2004) to those treatments receiving rotifers (Figure 1 and 2). Treatments not receiving rotifers were automatically administered concentrated *Chlorella* sp. paste diluted to 300 ppm until 13 dph, when all treatments stopped receiving algae. Enriched (DC DHA Easy Selco®) *Artemia* (GSL, INVE) were manually supplied 4 times daily to treatments P12A and G12A, and MD was administered automatically every 6 to 12 minutes. At 9 am and 4 pm the automatic feeders were checked and a manual feed was given to the tanks from the daily ration (Figure 1 and 2). The system (Kolkovski et al. 2004) supplied temperature controlled marine bore water at a flow rate of 1.0 L min^{-1} and pumped algae (and rotifers when required) every half-hour at 0.7 L min^{-1}. Green water (*Chlorella* sp.) method (Moretti et al. 1999) was used and an average secchi depth of 80 ± 17 cm was maintained and reduced proportionately with rotifer numbers according to the weaning protocols (Figure 1 and 2). The physiochemical parameters were maintained at water temperature 28.7 ± 0.8°C, pH 8.00 ± 0.02, DO 6.5 ± 0.5 ppm, salinity 35.0‰ and a photoperiod of 12 h light / 12 h dark.

Stress Test

Following the trial (at 28 dph), stress tests were conducted by pouring a beaker of water containing 10-15 larvae from each tank through a screen in order to capture the larvae on the screen. The screen was then blotted dry from behind with paper towel. The larvae were left on the dried screen for 9 min and then returned to a container of water. The proportion of survivors after 15 min was recorded and averaged for 4 replicates per treatment.

Grow-on Period

Larvae from treatments G12 and G12A were graded through a 2.5 mm grader in order to remove very fast growing fish, in order to avoid cannibalism. 362 of the remaining larvae were restocked into each of 6 tanks. The flow rate was increased to 3 L min^{-1} and the tank flow direction was reversed to bottom draining. A 1 mm screened standpipe was installed along with a protein skimmer at the water surface (Kolkovski et al. 2004). The fish were fed Skretting 600 (Skretting Australia) diet at 10% WW per day, indexed to increase at a daily increment of 10% of the previous days ration. At 36 dph, 10 fish from each tank were measured for SL, WW and DW.

Proximate Analysis

A proximate analysis of diets was conducted (Department of Agriculture WA) following the methods specified by the AOAC (1990). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyzer, based on N x 6.25. Phosphorus was determined spectrophotometrically using the vanadomolybdate method. Total ash contents were calculated gravimetrically following ignition of samples in a muffle furnace at 550°C. Crude fat content was determined gravimetrically following the extraction of the lipids using the method of Folch et al. (1957).

Diet Leaching

Leaching rates were determined by adding approximately 1 g (weighed to 4 decimal places) of each diet into a beaker containing 500 ml of marine bore water at 28°C. The samples were stirred using an automatic overhead stirrer (IKA Labortechnik, RW20) with a 25 mm propeller type agitator set to 800 rpm, for the allotted time period less filtering time. The stirrer was set to keep the majority of MD particles suspended in the water column for the duration of the leaching process (Yufera et al, 2002). A preweighed filter paper (FP) or amount of MD was dried at 80°C for 48 h and then the DW% was determined as a percentage of the initial weight. This percentage was then used to calculate initial DW of each diet sample. The filtering process was timed and used to estimate total leaching time, so that the water was removed from the filter funnel at leaching times of 1, 2, 4, 8 and 16 min ± 5 sec. Deionised water was used to wash the sample at the end of the filtering process to remove salt deposits from the MD. The MD and pre weighed FP was then dried and the losses were determined using a calculated initial dry weight (DW_i) of the MD and FP and final dry weight (DW_f) of MD and FP, as follows:

$$\text{Leaching losses} = \frac{\text{Initial (MD. } DW_i + \text{FP. } DW_i) - \text{Final (MD. } DW_f + \text{FP. } DW_f) \times 100\%}{\text{Initial (MD. } DW_i)}$$

Growth Rate Indicator

Daily Growth Coefficient (DGC) (Figure 4) was calculated using the following equation:

$$DGC = \frac{(\text{Final Length})^{0.33} - (\text{Initial Length})^{0.33}}{\text{Number of Days}} \times 100\% \quad (\%\ \text{Length day}^{-1})$$

Statistical Analysis

Statistical analyses of treatment effects were performed using SPSS statistical software package. One-way ANOVAs (Sokal and Rohlf, 1969) were done along with descriptive statistics, regression analysis and Tukey's post hoc test ($P < 0.05$). Variances were tested for normality and homogeneity. Results are presented as mean ± standard error (SE).

Leaching results were averaged for 3 replicate samples for each diet at each time interval and given as mean ± SE.

5.3.1.4 Results

Larvae at 28 dph that were grown according to protocol G12, averaging WW of 253.4 ± 10.3 mg and SL of 21.5 ± 0.3 mm, were significantly larger than those grown in either of the treatments that received *Artemia*. G12A and P12A treatments sustained similar growth ($P > 0.05$) (WW of 218.2 ± 13.7 mg and 209.0 ± 12.0 mg respectively and SL of 20.2 ± 0.4 mm and 19.8 ± 0.4 mm respectively) (Figures 1 and 2).

Regression analysis showed a positive relationship ($r^2 = 0.9995$) between the number of days that rotifers were fed to each treatment and the final size of the larvae at 28 dph (Figure 1 and Figure 2). The size of larvae at 10 dph was adversely affected by introducing the MD earlier. Treatment G12, which started co-feeding with rotifers and MD at 9 dph was significantly ($P < 0.05$) bigger at 10 dph than all other treatments that received MD earlier.

During the period that P12A and G12A received *Artemia* the larvae in these treatments grew significantly ($P < 0.05$) better than G12 as indicated by the DGC values (Figure 4). However, following the discontinuation of *Artemia* inputs from 22 to 28 dph, G12, G7 and G3 all had better

growth than P12A and G12A, as indicated by the DGC values (Figure 4). Treatments G7, G12, G12A and P12A experienced reduced performance in growth by 28.1 ± 19.1%, 55.0 ± 16.4%, 40.2 ± 4.0% and 44.0 ± 9.0% respectively during the period directly after live feed was discontinued. The recovery in growth rate following the lag period post wean, for treatments G12 and G12A, was 381.7 ± 171.1% and 214.2 ± 36.3% respectively (Figure 4).

Juveniles on-grown until 36 dph following weaning treatments G12 and G12A, showed no significant difference in size between the treatments at 36.6 ± 4.1 mm and 37.7 ± 3.5 mm SL respectively and 0.71 ± 0.20 g and 0.75 ± 0.20 g WW respectively.

Treatment G12A showed significantly ($P < 0.05$) better survival than all other treatments except P12A. P12A and G12 were not significantly different from each other, however P12A showed significantly ($P < 0.05$) better survival than treatments G, G3 and G7. Treatment G12 was not significantly different from treatment G7, however it showed significantly ($P < 0.05$) better survival than treatments G and G3. Treatments G7 and G3 were not significantly different, but both treatments showed significantly ($P < 0.05$) better survival than treatment G, which had very few survivors to 28 dph. A regression analysis showed that survival in treatments G, G3, G7 and G12 related directly ($r^2 = 0.94$) to the exposure time to rotifers. Survival% of the initial stocking numbers surviving to 28 dph in treatments G12A, P12A, G12, G7 and G3 were 13.5 ± 1.3%, 8.6 ± 3.2%, 5.9 ± 0.9%, 2.5 ± 1.0% and 2.0 ± 0.7% respectively (Figure 5).

G12A showed the strongest resilience to stress but not significantly better than P12A and G12. The remaining treatments had significantly ($P < 0.05$) lower survival following stress. The larvae's resilience to stress showed a positive correlation ($P < 0.05$) to the length of time that they received rotifers and / or *Artemia* and mirrored the growth and survival data (Figure 5.).

MD leaching resulted in losses of 20.7 ± 0.6%, 15.7 ± 0.5% and 13.3 ± 0.3%, for Gemma Micro 150, Gemma Micro 300 and Gemma 0.3 respectively, and 11.7 ± 0.4%, 10.4 ± 0.4% and 9.3 ± 0.6% for Proton 2, Proton 3 and Proton 4 respectively, after 16 minutes in marine bore water (Figure 6).

The proximate analysis of the diets showed that the protein levels are not significantly ($P < 0.05$) different between MDs. However, the fat levels were significantly ($P < 0.05$) higher in Gemma Micro MD than Proton MD at 19.9% DW and 12.9% DW respectively (Table 1).

5.3.1.5 Discussion

The results of this experiment have shown that barramundi larvae cannot survive and grow adequately when fed exclusively on the currently available commercial MD from first feeding. This was expected as Gemma Micro is designed to replace *Artemia* feeding and not as a full replacement for live feed inputs. Fernández-Díaz and Yúfera, (1997) also found that gilthead sea bream larvae fed microcapsules from first feeding have limited growth. There is evidence to suggest that the premature introduction of MD, even when co-feeding, can inhibit their growth. When MD was introduced at 6 dph it resulted in a significant reduction in growth to 10 dph, when compared to the introduction of MD at 9 dph. It is suggested that live feed stimulates feeding and the development of the digestive system through their movement and by introducing exogenous digestive neuropeptides / enzymes to the larvae gut (Kolkovski et al, 1997; Hjelmeland et al. 1988; Kolkovski, 2001). This is supported by the present trial, where larval growth and survival improve proportionately to the exposure time to rotifers. However, a continuation of live feed inputs using *Artemia* showed no improvement in growth.

All the treatments in the current experiment underwent a short lag phase in growth rate post weaning followed by a growth compensation period (Tian and Qin, 2004). The significance of this phase related to the length of time that the larvae received live feed and the timing of weaning in relation

to the larvae's development. Kolkovski et al. (1995) found that gilthead seabream (*Sparus aurata*) larvae preferentially selected live feed over MD during the co-feeding period and became more difficult to wean as the duration of live feed input increased. In the current trial the discontinuation of rotifers at 13 dph, resulted in a greater drop in growth rate than when rotifers were discontinued earlier. However in this case the period of reduced growth resulting from earlier weaning was more prolonged and resulted in significantly smaller larvae at 28 dph. The continuation of live feed inputs using *Artemia* until 20 dph also reduced the impact of weaning on growth, maintaining high growth for the extended co-feeding period and then a proportionately less severe lag phase.

Evidently, there seems to be two weaning effects taking place. The more severe reduction in growth rate pre metamorphosis may be due to a combination of learned feeding behaviour and the larvae's gut physiology, being yet to form a functional stomach at this stage of development (Walford and Lam, 1993). Before the stomach develops the gut has a high pH where tryptic digestion takes place, which hydrolyses the proteins at only a low rate. The development of the stomach at metamorphosis allows the larvae to reduce its gut pH, which facilitates the more efficient peptic digestion (Walford and Lam, 1993; Moyano et al, 1996). Furthermore, live prey is thought to contribute a proportion of the enzymes to the undeveloped larvae gut, which assists in digestion (Walford and Lam, 1993; Person-Le Ruyet, 1989; Kolkovski, 2001). This may have contributed to a decreased ability to digest and assimilate the MD once the live feed inputs were discontinued (Person-Le Ruyet, 1989), resulting in a long period of low growth as in treatments G3 and G7, until the stomach is formed. The effect would be compounded when coupled with a reduced intake of nutrients caused by the learned feeding behaviour, as in treatment G12, which underwent the most severe drop in growth rate.

Where rotifers were discontinued and *Artemia* continued past metamorphosis, the larval growth rate remained high. However, providing *Artemia* also resulted in reduced subsequent growth, albeit less severe. Given the larvae's developmental stage, this is more likely to be solely attributed to a preference for live feed and subsequently a reduction in nutrient intake, rather than an inability to efficiently digest the MD (Canavate and Fernandez-Diaz, 1999; Cahu and Zambonino Infante, 2001; Kolkovski, 2001). Despite the differences in severity of the impact of weaning either before or after metamorphosis, there was no difference in overall larval growth to 36 dph in treatments G12 and G12A. This was the result of a greater compensation effect and increased recovery in growth rate following the earlier weaning at 13 dph. Therefore, apart from an increase in survival, the inclusion of *Artemia* in the weaning protocol has provided no obvious advantage, when co-fed with Gemma Micro.

The ideal time to complete weaning onto dry MD seems to be at the earliest stage when the larvae can easily digest the MD and when live feed inputs no longer provide growth and survival benefits to the larvae (Kolkovski et al. 1995; Canavate and Fernandez-Diaz, 1999; Cahu and Zambonino Infante, 2001). This seems to be between 18 and 22 dph for barramundi, as indicated by the increase in growth rates of larvae in treatments G12, G7 and G3, which were fed exclusively on MD during this period. This would suggest that the MD is being more efficiently digested at this stage and is also assumed to correspond with the formation of the stomach, which is thought to develop when barramundi larvae are around 12 mm (Walford and Lam, 1993). This coincides with a larval age of 18 to 22 dph in treatments G12, G12A and P12A. However, treatments G7 and G3 are significantly smaller at this stage, but undergo a similar increase in growth, which indicates that the formation of the stomach may be more closely related to larval age (degree days). Nonetheless, a continuation of live feed inputs until at least 18 dph may be of benefit, in order to minimize the loss of growth potential pre metamorphosis, due to an inability to adequately digest the MD.

The proximate analysis of both Gemma Micro MD and Proton MD (Table 3) show that they are similar in total protein levels. However, factors such as the binder used and protein source can

dictate the digestibility of the MD (Lindner et al, 1995; Lee et al. 1996; Partridge and Southgate, 1999; Kolkovski, 2000). The percentage of free amino acids may also vary between the two MDs. This coupled with the type of binder can make the diet more attractive or palatable by leaching a greater amount of amino acids into the water, thus leading to increased ingestion of one diet and subsequently improved growth and/or survival (Kolkovski et al. 2001; Partridge and Southgate, 1999). Furthermore, barramundi larvae in all treatments were observed to more actively feed upon the Gemma Micro MD with less waste evident on the bottom of the tanks.

Diets containing high energy levels are also thought to improve larval growth and survival (Izquierdo and Fernandez-Palacios, 2001; Cahu and Zambonino Infante, 2001; Koven et. al., 2001). Gemma Micro MD has 19.9% DW total fat content, which is 7.0% DW higher than the total fat levels found in Proton MD. Zambonino Infante and Cahu (1999) found that the best growth and survival in European sea bass larvae was obtained using a diet of 30% total lipid, while Brinkmeyer and Holt (1995) showed that red drum (*Sciaenops ocellatus*) larvae perform best on a diet containing 18% total lipid. This suggests that lipid requirements are species specific. However, both these species performed better on diets containing 18% or greater lipid levels. In the current trial, a growth reduction occurred concurrently with the discontinuation of highly lipid enriched *Artemia*. This may suggest that higher lipid levels along with lipid groups (i.e. phospholipids) (Coutteau et. al., 1997; Izquierdo and Fernandez-Palacios, 2001) more adequately supplied the growth requirements of the barramundi larvae and / or a preference for the live feed resulted in an overall reduction in nutritional intake (Kolkovski et al, 1997; Koven et. al., 2001). The results suggest that it is the latter, as those larvae that did not receive any *Artemia* were greater in size at 28 dph, and there was no difference in size between the two treatments following the lag phase in the growth pattern of G12A. However, significantly better survival was achieved in those treatments that received the higher lipid levels or n-3 HUFA levels from enriched *Artemia* and a further increase in survival was shown in treatments that received MD with higher lipid levels (Kanazawa et. al., 1982; Kanazawa, 1993; Salhi et al. 1994; Coutteau et. al., 1997). Therefore, in the current trial relatively higher lipid levels have promoted survival, rather than improving growth.

The resulting low survival of all treatments in this trial was attributed to the initial handling of the larvae and the late application of their first feeding, although the treatment affects were clearly defined. The results suggest that a period of live feed inputs equivalent to the length of the larval stage and the inclusion of lipid enriched *Artemia* into the protocol, coupled with a better adapted MD can give higher survival and stronger, more stress resilient larvae. We have shown that it is not necessary to include *Artemia* in the weaning protocol in order to achieve good growth while feeding Gemma Micro MD, however better survival was achieved in those treatments that received lipid enriched *Artemia*. These results contradict those previously reported for many marine fish species (Koven et al, 2001; Kolkovski, 2001; Izquierdo and Fernandez-Palacios, 2001), which state that *Artemia* is essential for larval growth. This fact is probably due to the improvement of commercial MD nutrition and physical properties.

This trial indicates that the protocol program as a whole affects growth, survival and larval rigor. Production quality relies on a combination of the MD and live-feed with regard to input period and placement in relation to the larvae's development. Therefore, a holistic approach is needed when developing both larval specific MDs and weaning protocols (Kolkovski, 2004 Phuket). Based on the results of this experiment it is suggested that weaning barramundi from rotifers (starting at 2 dph) onto an inert MD should be completed as early as 18 to 20 dph (at 28°C), following a co-feeding period using an appropriate MD, starting at 7 to 9 dph and a nutrient enriched live feed organism of an appropriate size, in order to maximize both growth and survival.

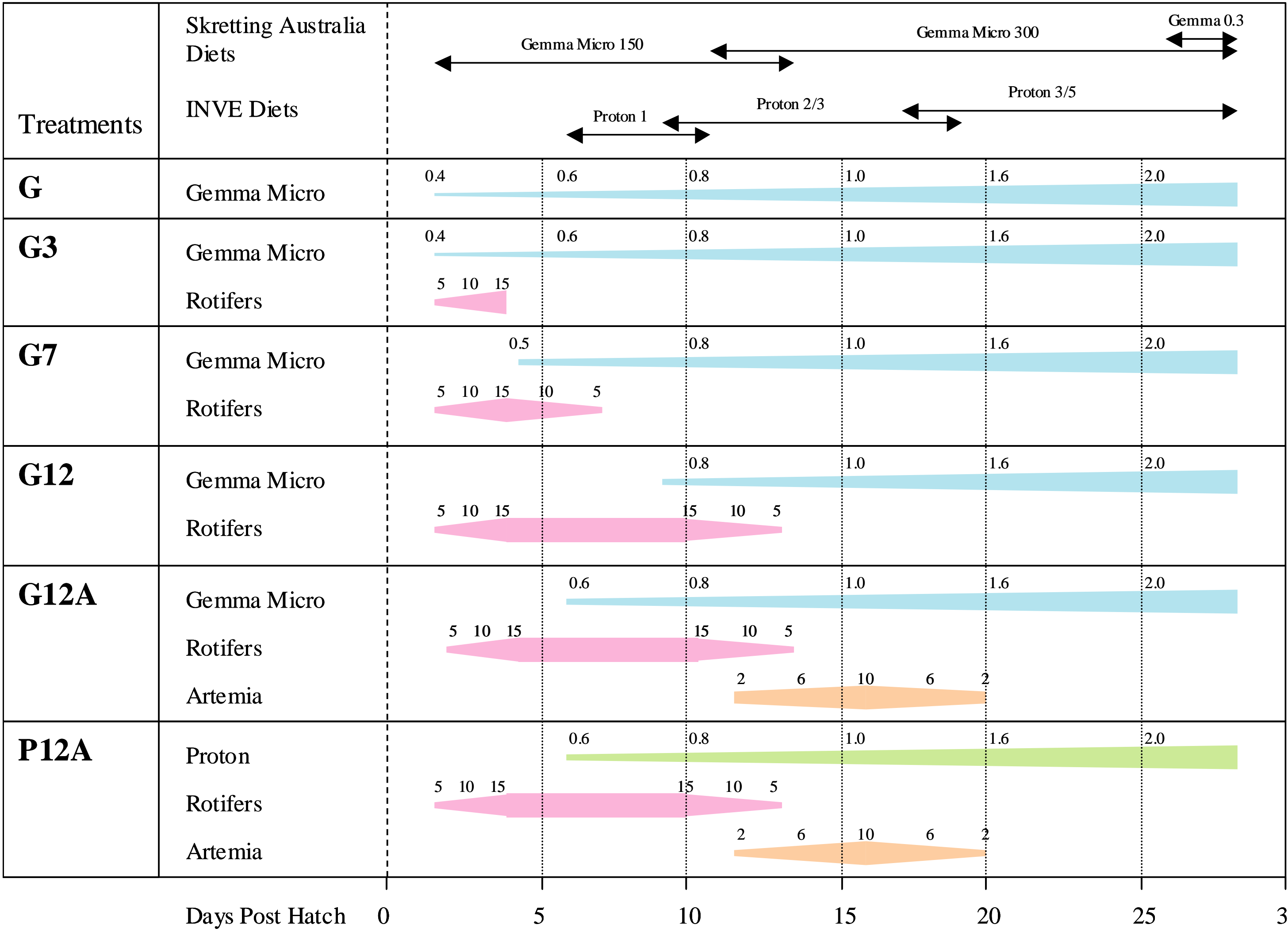

Figure 1. Weaning protocols, from 2 dph to 28 dph, are shown. Microdiet feeding rations are expressed as 'g (1000 larvae)$^{-1}$ day^{-1}', rotifers are expressed as 'number of rotifers ml^{-1}' continuously and *Artemia* are expressed as 'number of *Artemia* ml^{-1} day^{-1}'. Temporal microdiet size class distribution is shown for Skretting and INVE diets, with a 3-day overlap for each transitional period. Microdiet feeding regimes are common for all treatments, but starting at different times.

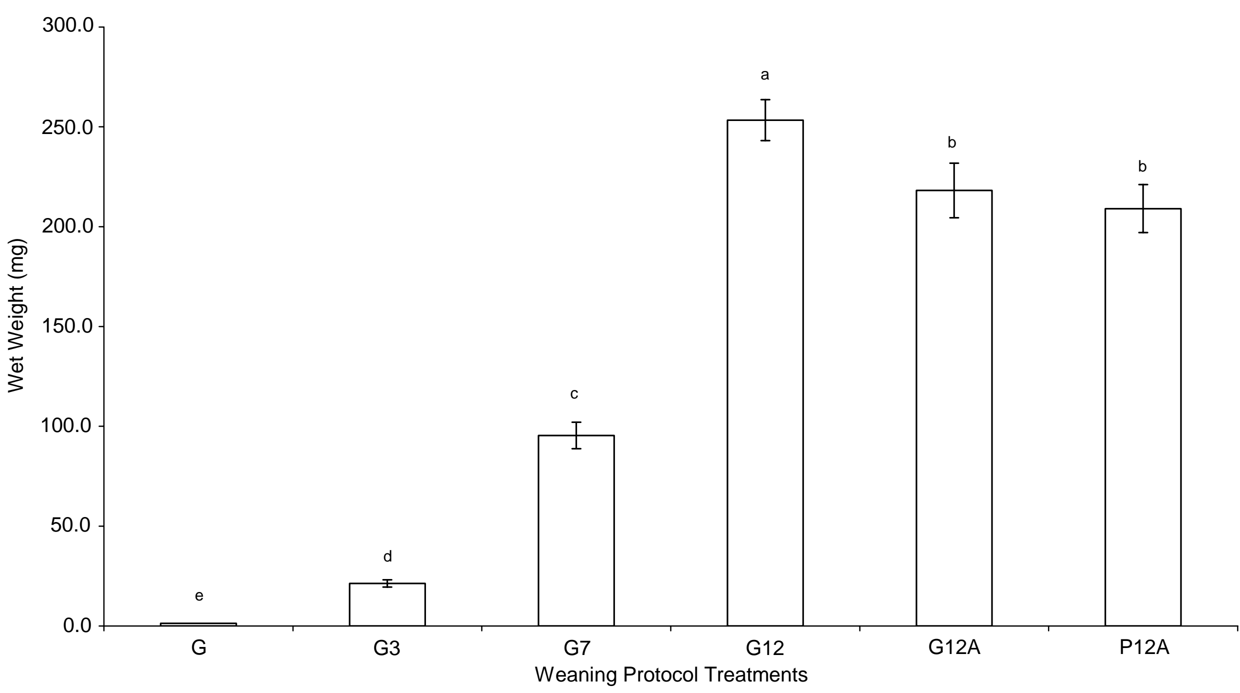

Figure 2. Final wet weight of larvae at 28 dph for each treatment. Ave ± SE, different lettering on columns denotes significant (P < 0.05) differences.

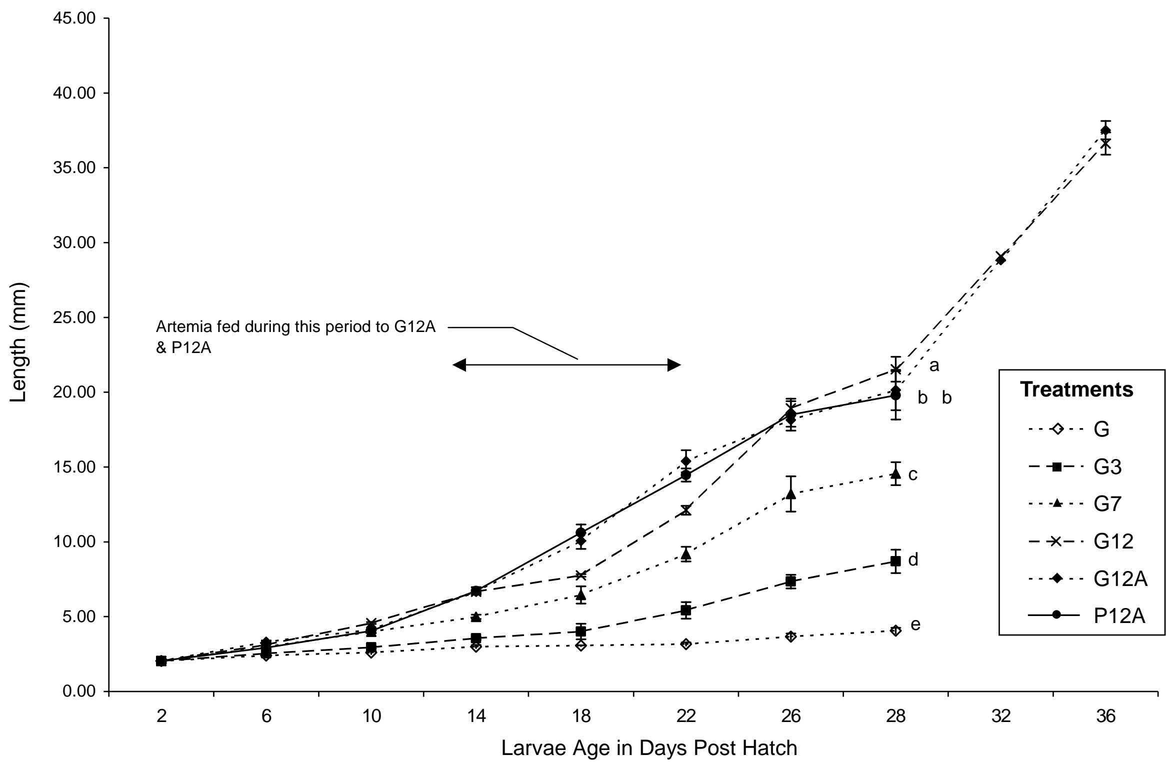

Figure 3. Length growth curve of larvae weaned according to six different protocols, from 2 days post hatch (dph) to 28 dph, lettering denotes significant (P < 0.05) differences at 28 dph, and treatments G12 and G12A from 28dph to 36 dph. Ave length (mm) ± SE.

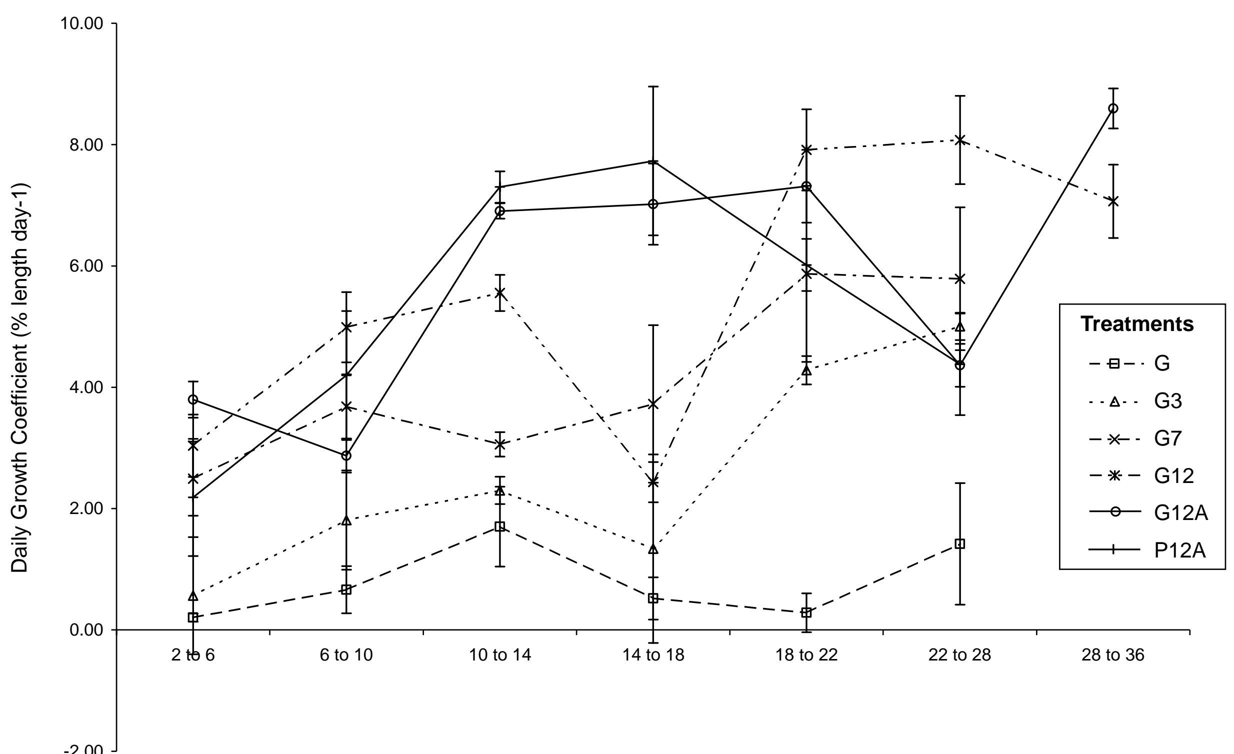

Figure 4. Daily Growth Coefficient (length mm) for periods between observation days.

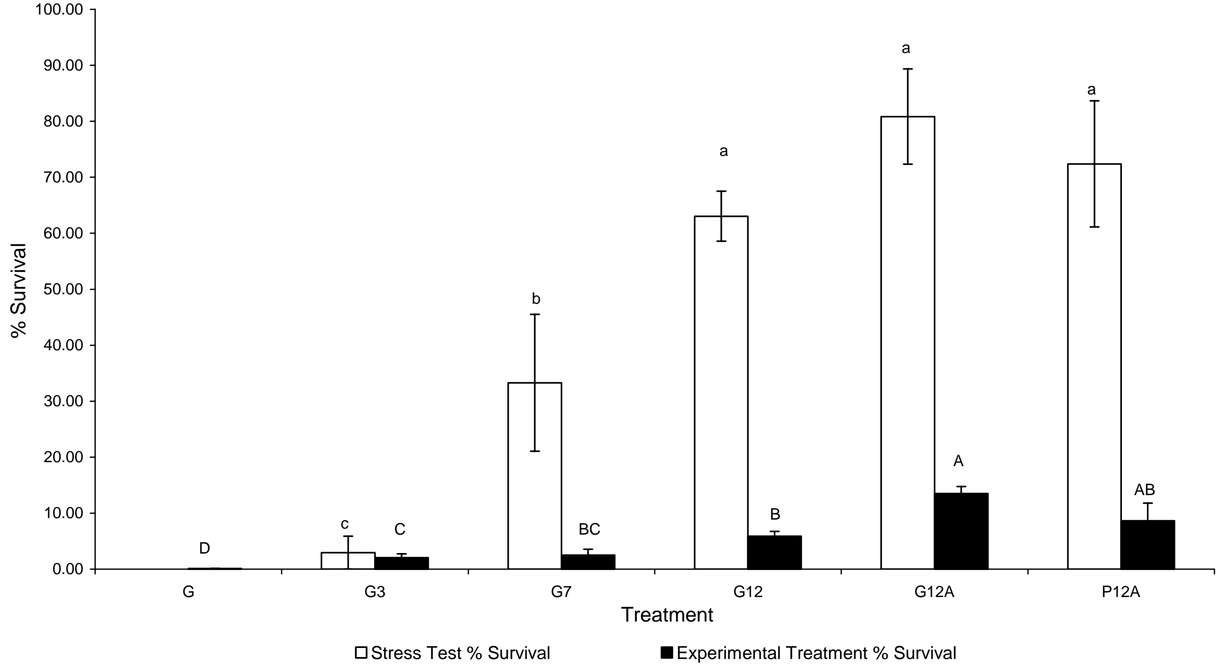

Figure 5. Average survival for replicate treatment tanks and average survival post stress for treatments, where differences in lower case lettering denotes significant ($P < 0.05$) differences between stress test survival results and differences in upper case lettering denotes significant ($P < 0.05$) differences between treatment survival results.

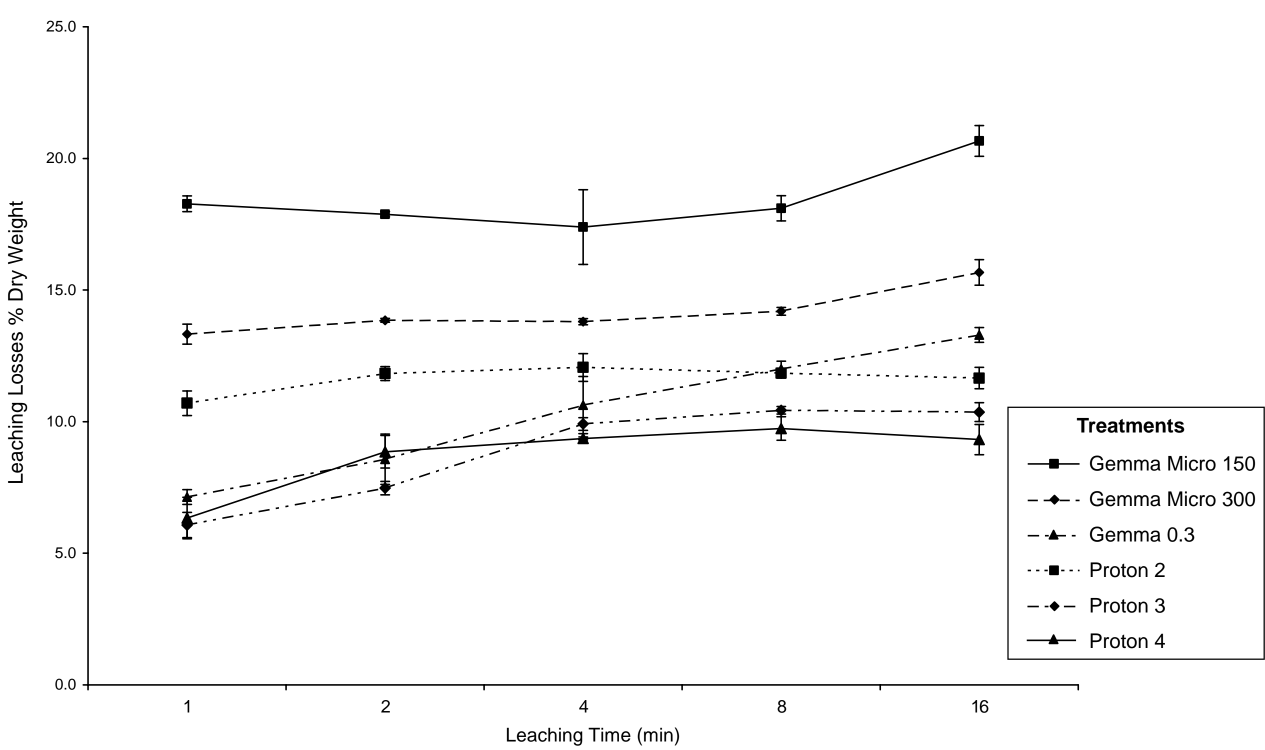

Figure 6. Leaching rates for micro-diets over time in marine bore water at 28°C.

Table 1. Proximate analysis of micro-diets.

Diet	H_2O % AR	Ash % DW	Protein (crude) % DW	Fat % DW
Gemma Micro MD	6.2	14.5	58.8	19.9
Proton MD	7.2	12.6	56.9	12.9

5.3.1.7 References

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5.3.2 The effect of various co-feeding and weaning regimes on growth and survival in barramundi (*Lates calcarifer*) larvae

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5.3.2.1 Abstract

Two commercial weaning microdiets (MDs), Gemma Micro (G) and Proton (P) were compared for barramundi (*Lates calcarifer* Bloch) larval growth and survival across three weaning protocols, until 33 days post hatch (dph). From mouth opening, rotifers were fed to larvae in all treatments. At 13 dph *Artemia* were introduced to two weaning treatments and continued into a period of co-feeding with MD for 8 days. Protocol 1 (treatments G-1 and P-1) started co-feeding with MD at 20 dph and protocol 2 (treatments G-2 and P-2) starts co-feeding with MD at 16 dph. Protocol 3 (treatments G-3 and P-3) continued rotifers as live feed until 30 dph and started co-feeding with MD at 13 dph.

Barramundi larvae completed metamorphosis successfully with high survival rates when co-fed algae-enriched rotifers and MD. However, larvae in treatment P-3 achieved a significantly (P < 0.05) lower final wet weight (WW) 103.6 ± 16.6 mg than larvae in all other treatments. Treatment P-1 resulted in larvae that were significantly (P < 0.05) bigger than those in treatments P-2 and G-2, (226.5 ± 19.6 mg, 201.1 ± 18.0 mg and 187.5 ± 10.5 mg WW respectively). Larvae resulting from treatments P-2 and G-2 were not significantly different from each other, but were significantly bigger than larvae resulting from treatments G-3 and G-1 (170.0 ± 22.8 mg and 164.1 ± 14.3 mg WW respectively).

Survival was significantly (P < 0.05) higher in Gemma treatments when compared to Proton treatments. The rates of cannibalism in the Proton treatments were significantly (P < 0.05) higher than the Gemma treatments, averaging 22.8 ± 0.9% and 9.2 ± 0.6% respectively. A bias towards better growth was evident in the Proton co-fed larvae due to the higher rate of cannibalism. Cannibalism events became significantly more apparent in all treatments when the larvae reached 8 - 10 mg wet weight and further increased after the withdrawal of live feed.

5.3.2.2 Introduction

Larval nutrition is a key element for the successful culture of marine fish. Live feed such as rotifers (*Brachionus* sp.) and brine shrimp (*Artemia*) are considered obligatory for successful culture (Baskerville-Bridges and King, 2000; Sorgeloos et al., 2001). However, complicating factors such as high levels of pathogenic bacteria can be associated with live feeds and can adversely affect fish larvae performance (Moretti et al., 1999; Makridis et al., 2000; Olsen et al., 2000; Olafsen, 2001). Moreover, high costs and labour requirements along with the highly variable nutritional value of live feeds (Cahu and Zambonino Infante, 2001; Langdon, 2003), and the unreliability of mass cultures associated with the production of live feeds illustrate the need to find viable alternatives (Baskerville-Bridges and King, 2000). However, live feeds continue to be essential for the production of marine fish larvae. They are thought to stimulate larval feeding and enzyme production by their movement and chemical release, and may also provide a source of exogenous enzymes to assist the larvae's undeveloped digestive capacity (Hart and Purser, 1996; Kolkovski et al. 1997; Cahu and Zambonino Infante, 2001; Kolkovski, 2001).

Although it seems essential to include live feeds during the larval weaning stages, minimizing or eliminating their use is economically advantageous for marine finfish hatcheries (Baskerville-Bridges and King, 2000). Person- le Ruyet et al. (1993) found that by starting the weaning process of European sea bass (*Dicentrachus labrax*) onto MD at 20 dph instead of 35 dph, a reduction of up to 80% of live prey costs during weaning (50% of the total cost of 3-month-old juveniles) could be achieved. Therefore in an effort to reduce the reliance on live feed it is of major benefit to wean larvae onto formulated diets at the earliest possible time. In the past early weaning has led to reduced growth and inferior quality larvae with an increased risk of skeletal deformities (Person-le Ruyet et al., 1993). However, Cahu and Zambonino Infante (2001) reported that larvae have the ability to digest and thrive on compound diets if the diet is well adapted. Therefore, if present commercial MD formulations are adequate, it may be possible to wean the larvae directly from rotifers onto MD by co-feeding , without loss of growth or survival, which could minimize or eliminate the use of *Artemia* (Cahu and Zambonino Infante, 2001).

Factors such as the type and nutritional quality of live feeds, timing of first introduction of the MD, duration and method of weaning from live feeds to MD can collectively effect larval growth and survival (Hart and Purser, 1996; Baskerville-Bridges and King, 2000; Cahu and Zambonino Infante, 2001). Weaning schedules vary from hatchery to hatchery and depend on the fish species. They may be rapid, at around the time of metamorphosis, or gradual over a period of several weeks

leading up to metamorphosis. The standard weaning protocol for pink snapper (*Pagrus auratus*), for example, begins when the larvae reach an average total length of 10 mm (Day 22-28) and is complete by Day 48-55 (at 20°C), following first feeding of rotifers and then *Artemia* nauplii (Partridge, 2002). In contrast, barramundi larval weaning is faster, usually co-feeding with MD starts when the larvae reach an average total length of 12-16 mm at around 20 dph and continues for 10-15 days (at 28-30°C, Darwin Aquaculture Centre, pers. comm.).

'Co-feeding' weaning protocols simultaneously using inert and live diets have also been developed to allow a fast and efficient change over period onto dry MD from live feed (Hart and Purser, 1996; Daniels and Hodson, 1999; Koven et al., 2001). This method has been found to achieve higher growth and survival than feeding either live feeds or MDs on their own (Kolkovski et al., 1995). It has been shown that early co-feeding of an acceptable MD can improve larval nutrition and condition the larvae to more readily accept MD when live feed is withdrawn (Canavate and Fernandez-Diaz, 1999; Cahu and Zambonino Infante, 2001; Kolkovski, 2001). However, commercial hatcheries usually develop 'in house' protocols that are rarely tested within statistically rigorous experimental designs. This creates the need to evaluate current and available formulations for commercial marine finfish hatcheries within a statistically rigorous framework.

The current trial investigates the effect of various weaning strategies on barramundi larvae growth and survival when co-feeding live and dry diets aimed at minimising the requirement of *Artemia*. The trial also compares larval growth and survival when fed two commercially available weaning diets and a further two diets post weaning.

5.3.2.3 Methods

Three weaning treatments compared the growth and survival of barramundi larvae. Two weaning protocols using rotifers and then *Artemia* for different periods were compared. The third treatment determined the effect of weaning the larvae without *Artemia* by prolonging the rotifer stage. Each weaning treatment was followed using two different MDs.

Four dry MDs were used within two MD treatments. MD treatments P-1, P-2 and P-3 used Proton MD followed by NRD (INVE, Belgium) (Figure 4), and MD treatment G-1, G-2 and G-3 used Gemma Micro MD followed by Skretting crumble (Nutreco / Skretting Australia) (Figure 4).

Proton MD and NRD were supplied in 4 and 2 nominal particle size ranges and as shown (Figure 4). The Gemma Micro MD and the Skretting diet were supplied in 2 nominal size ranges each (Figure 4). The Skretting diet was screened into size ranges that replicated those of the diets supplied by INVE. During the transition from one size range to the next the Skretting diets were mixed, in order to compensate for the larger number of size categories offered by INVE.

Twenty-four 270 L conical tanks (Kolkovski et al., 2004) were each stocked with 1,400 - 11 day post hatch (dph) barramundi larvae and fed according to the attached weaning protocols (Figure 4). Prior to stocking the larvae were reared using a 'green water' technique (Moretti, 1999) using *Nannochloropsis* sp. and rotifers. First feeding of *Artemia* nauplii occurred at day 13. Periodically, 5 random larvae from each tank were removed and sacrificed using iced water. These larvae were measured for standard length, weight and then dry weight after 48 h at 80°C. The final observations were averaged for 20 larvae from each tank.

Algae and rotifers were automatically supplied by a feeding system (Kolkovski et al., 2004), while enriched *Artemia* were manually supplied 3 times daily, and MD was administered at intervals of 30 min to 1 h. An average secchi depth of 68 ± 17 cm was maintained and reduced proportionately as the rotifer numbers were reduced according to the protocol (Figure 4). Marine bore water was

used at an average temperature of 26.6 ± 0.3°C, with an initial flow rate of 0.5 L min^{-1} increasing to 1.0 L min^{-1} as larval development progressed. The physiochemical parameters were maintained at pH 8.06 ± 0.02, DO 5.7 ± 0.1 ppm (mean ± SE), salinity 34.1‰ and a photoperiod of 12 h light / 12 h dark.

Artemia nauplii (GSL, INVE) were incubated and hatched in marine bore water at 28°C over a 24 h period, harvested and enriched for a further 24 h using Super Selco ®. Larval Daily Growth Coefficient (DGC) (Figure 2) was calculated using the following equation:

$$DGC = \frac{[(\text{Final weight})^{0.33} - (\text{Initial weight})^{0.33}]}{\text{Number of Days}} \times 100\% \quad (\%\text{weight gain day}^{-1})$$

Cannibalism was calculated as a percentage of the initial number of larvae that could not be accounted for other than mortalities due to handling at initiation of the trial and daily mortalities removed during the trial, (Table 1) and was calculated according to the following equation:

$$\text{Cannibalism} = \frac{L_I - L_S - L_F - M_R}{L_I} \times 100\%$$

Where, L_I was initial larvae numbers stocked, L_S was the number of larvae removed for observation during the trial and L_F was the final number of larvae in each tank and M_R was the number of mortalities removed during the trial.

Survival percentage was calculated as the fraction of the initial number of larvae that contributed to the final biomass (total average wet weight (g) of larvae per tank), not including larvae that were sampled during the trial for observations (Table 1).

Leaching rates were determined by adding approximately 1 g (weighed to 4 decimal places) of each diet into a beaker containing 500 ml of marine bore water at 28°C. The samples were stirred using an automatic overhead stirrer (IKA Labortechnik, RW20) with a 25 mm propeller type agitator set to 800 rpm, for the allotted time period less filtering time. The stirrer was set to keep the majority of MD particles suspended in the water column for the duration of the leaching process (Yufera et al, 2002). A preweighed filter paper (FP) or amount of MD was dried at 80°C for 48 h and then the DW% was determined as a percentage of the initial weight. This percentage was then used to calculate initial DW of each diet sample. The filtering process was timed and used to estimate total leaching time, so that the water was removed from the filter funnel at leaching times of 1, 2, 4, 8 and 16 min ± 5 sec. Deionised water was used to wash the sample at the end of the filtering process to remove salt deposits from the MD. The MD and pre weighed FP was then dried and the losses were determined using a calculated initial dry weight (DW_i) of the MD and FP and final dry weight (DW_f) of MD and FP, as follows:

$$\text{Leaching losses} = \frac{\text{Initial (MD. } DW_i + \text{FP. } DW_i) - \text{Final (MD. } DW_f + \text{FP. } DW_f) \text{ X } 100\%}{\text{Initial (MD. } DW_i)}$$

Proximate analyses of the diets were conducted by the Department of Agriculture Western Australia, following the methods specified by the AOAC (1990). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25. Phosphorus was determined spectrophotometrically using the vanadomolybdate method. Total ash contents were calculated gravimetrically following ignition of samples in a muffle furnace at 550°C. Crude fat content was determined gravimetrically following the extraction of the lipids using the method of Folch et al. (1957).

Statistical analyses of treatment effects were performed using SPSS statistical software package. One-way ANOVAs (Sokal and Rohlf, 1969) were done along with descriptive statistics, regression analysis and Tukey's post hoc test ($P < 0.05$). Variances were tested fo normality and homogeneity. Results are presented as mean ± standard error (SE).

Leaching results were averaged for 3 replicate samples for each diet at each time interval and given as mean ± SE.

5.3.2.4 Results

Protocols

The final average wet weight of larvae in treatment P-1 (INVE - protocol 1, Figure 4) were significantly ($p < 0.05$) bigger than those in treatments P-2 (INVE -protocol 2, Figure 4) and G-2 (Skretting - protocol 2, Figure 4), which were 226.5 ± 19.6 mg and 201.1 ± 18.0 mg and 187.5 ± 10.5 mg respectively. Larvae resulting from treatments P-2 and G-2 were not significantly different from each other, but were significantly bigger than larvae resulting from treatments G-3 (Skretting - protocol 3, Figure 4) and G-1 (Skretting - protocol 1, Figure 4) achieving final wet weights of 170.0 ± 22.8 mg and 164.1 ± 14.3 mg respectively, with no significant difference between them. Treatment P-3 (INVE - protocol 3, Figure 4) resulted in larvae of significantly ($p < 0.05$) lower final wet weight at 103.6 ± 16.6 mg than larvae in all other treatments. Barramundi larvae completed metamorphosis successfully with high survival rates when co-fed algae-enriched rotifers and both MDs tested (Table 1).

During the period 18 dph to 22 dph, all treatments receiving *Artemia* grew significantly ($P < 0.05$) better than the rotifer only treatments (G-3 and P-3) (Figure 2). However, larvae reared following protocols G-3 and G-1 converged in size after 22 dph, where G-1 were co-fed MD and reduced numbers of *Artemia*, resulting in no significant difference in final larval wet weight (Figure 1). From 29 dph to 33 dph the DGC for G-3 was notably better than G-2 and significantly better ($P < 0.05$) than G-1 (Figure 2). Treatment P-3 also experienced a reduction in DGC between 18 dph and 22 dph. However, this treatment maintained a slower growth rate than P-1 and P-2.

Diets

There were no significant differences in growth between diets within each weaning protocol until 22 dph. Following this point, G-3 had significantly ($P < 0.05$) better growth than P-3. Larvae weaned according to the P-3 protocol remained significantly ($P < 0.05$) smaller than all other treatments. The *Artemia* co-fed treatments showed no significant divergence until after 29 dph, however after *Artemia* were withdrawn at 29 dph the Skretting based protocol treatments indicated higher growth (DGC) than the INVE based protocol treatments. The DGC increased in all *Artemia* fed treatments following the withdrawal of live feed, moreover treatments P-1 and P-2 performed significantly better than treatments G-1 and G-2 during this period (Figure 2). This resulted in P-1 being significantly ($P < 0.05$) larger than P-2 and G-2, which were not significantly different from each other, but were significantly bigger than larvae in treatment G-1 (Figure 1).

Survival in all Gemma Micro/Skretting MD treatments (G-1, G-2 and G-3) averaging 82.7 ± 2.3%, were significantly higher than those in Proton/NRD MD treatments (P-1, P-2 and P-3) averaging 66.2 ± 3.3%, with no significant differences within MD treatments (Table 1). The number of larval mortalities attributed to cannibalism was significantly ($P < 0.05$) higher in those treatments co-fed on INVE diets compared to Skretting co-fed treatments, averaging 22.8 ± 1.8% and 9.1 ± 0.2% respectively, with no significant difference between any of the live feed weaning protocols within MD treatments (Table 1).

MD leaching resulted in losses of 20.7 ± 0.6%, 15.7 ± 0.5% and 13.3 ± 0.3%, for Gemma Micro 150, Gemma Micro 300 and Gemma 0.3 respectively, and 11.7 ± 0.4%, 10.4 ± 0.4% and 9.3 ± 0.6% for Proton 2, Proton 3 and Proton 4 respectively, after 16 minutes in marine bore water at a temperature of 28°C (Figure 3).

The results of the proximate analysis of the diets used in this trial are shown in Table 2. Protein content of both Gemma Micro and Proton MD were not significantly different. However, Gemma Micro had higher fat content than Proton, at 20.1% and 14.9% respectively. The Skretting diet had significantly lower protein and higher fat content than the NRD.

5.3.2.5 Discussion

Successful weaning of marine finfish larvae from live feeds onto dry inert MDs is primarily dependent on matching the live feed co-feeding protocol to the MDs performance characteristics. Early weaning relies on diet attributes such as attractiveness and palatability, digestibility due to diet composition and type, stability in water controlling leaching of nutrients and / or attractants, and larvae behaviour such as learned feeding preferences (Hart and Purser, 1996; Kolkovski et al., 1997; Rosenlund et al., 1997). The key to increasing the bioavailability of MD nutrients during the early life stages is intrinsically linked to the suitability of the MD to the larvae's physiological development (Alarcon et al., 1999). Walford and Lam (1993) have shown that during pre-metamorphosis, without a functional stomach, tryptic digestion occurs but this process hydrolyses the proteins at only a low rate. At this stage live prey is thought to assist digestion by contributing a proportion of the enzymes (Walford and Lam, 1993; Kolkovski, 2001), and / or stimulating an increase in endogenous production of enzymes within the larvae gut (Hjelmeland et al., 1988; Kolkovski, 2001). Further to this, live feed stimulates the feeding response of larvae, which leads to an increase in overall intake and consequently a higher assimilation of nutrients (Kolkovski et al., 1997).

In the current experiment there was no significant effect on growth up to 22 dph that can be attributed to MD. The differences in growth during this period are largely due to variations in live feed inputs. This indicates that MD was not being efficiently digested and assimilated and / or a preference for live feed existed that eclipsed any MD effect. Tandler and Kolkovski (1991), have found that gilthead seabream (*Sparus aurata*) eat live food (dry weight) at ten times the rate of MD during co-feeding. However, both Walford et al. (1991) and Kolkovski et al. (1997) have shown significant increases in MD ingestion and digestion when fed to barramundi and European sea bass (*Dicentrarchus labrax*) respectively, by co-feeding with live feed organisms, compared to feeding solely MD. The reduction in growth rate in treatments G-3 and P-3 at 18 dph coincides with a reduction in rotifer inputs and may be due to the combination of a learned feeding behaviour, an overall reduction in intake caused by the diminished feeding stimulus and the larvae's inefficient gut physiology at this stage of development (Person-Le Ruyet, 1989). Hence, the larvae's digestive capability may be reduced proportionately with the rotifers until their ability to complete hydrolysis of proteins into peptides and amino acids is enhanced with the development of the stomach and gastric gland secretion of pepsine (Person-Le Ruyet, 1989).

During the period 18 to 22 dph, barramundi larvae were shown to benefit significantly from receiving enriched *Artemia* nauplii. It was observed that *Artemia* stimulated larval feeding activity more than rotifers, which is thought to subsequently increase MD intake (Kolkovski et al., 1997). This may be an effect caused by the size of prey being offered. Fernandez-Diaz et al., (1994) showed that the preferred live and inert prey size of *Sparus aurata* larvae is a function of mouth size, showing preference for prey in the range of 0.1 to 0.8 times the mouth width. Barramundi larvae of 18 – 22 dph have a mouth gape of 1 – 1.5 mm and evidently prefer larger prey, as larvae

were observed attempting to eat other larvae half of their own total length. Therefore rotifers of a total length of 160-180 µm would be in the lower limits of the barramundi's prey size range and far less attractive as a food item than *Artemia* nauplii of 400 - 450 µm, and consequently a weaker stimulus for feeding. Additionally, the *Artemia* would provide more biomass to the larvae than the rotifers, per catch effort.

All treatments experienced an increase in growth rate after a short lag phase post wean, which was thought to be a compensatory effect, being the result of a previous reduction in nutrient intake, followed by the restoration of optimal nutrition (Tian and Qin, 2003). The rate of recovery reflects the magnitude of the reduction in nutritional intake (Tian and Qin, 2003) and therefore, the level of bioavailability of MD nutrients at the time of weaning (Hart and Purser, 1996; Kolkovski et al., 1997; Rosenlund et al., 1997). The reduction in nutritional intake was thought to be the result of a learned feeding preference for live feed organisms during the acclimation period onto MD (Kolkovski et al., 1997). Gilthead seabream (*Sparus* aurata) larvae, having been previously fed on live feed, have been found to preferentially select live feed over MD during the co-feeding period and become more difficult to wean onto MD the longer they receive live feed (Fernandez-Diaz et al., 1994; Kolkovski et al., 1995). Therefore, larvae weaned more quickly onto MD more readily accept the MD than older larvae more strongly conditioned to accept live feed (Canavate and Fernandez-Diaz, 1999; Cahu and Zambonino Infante, 2001). In the current experiment this was true for those larvae being weaned onto the Gemma Micro MD. However, a longer co-feeding period better suited the larvae being weaned onto the Proton MD.

The prolonged weaning protocol onto Gemma Micro MD was thought to provide a relative reduction in nutrient intake. Helland et al, 2003, have shown that lipid enriched *Artemia* metanauplii might enter a state of negative nitrogen balance and therefore be relatively deficient in protein, when fed to marine fish larvae. If the MD is meeting the lipid requirements of the larvae, the reduction in total ingested protein caused by a feeding preference for *Artemia* could be detrimental to growth. This is supported by the lower growth rate during the period when *Artemia* were co-fed, compared to feeding Gemma Micro MD alone, and an increased magnitude of the growth recovery period post wean. However, treatments receiving Proton MD benefited from prolonged *Artemia* inputs, which indicate a relatively lower nutritional value of Proton MD. This is further supported by the significantly smaller larvae resulting from treatment P-3, which shows a considerable reduction in nutrient intake during weaning, when compared to the G-3 treatment that was able to fully compensate in growth following the acclimation period (Tian and Qin, 2003).

The result of feeding an inadequate MD can delay or even prevent the onset of the maturation process (Cahu and Zambonino Infante, 2001). A functional stomach develops when barramundi larvae are around 12 mm (Walford and Lam, 1993), which coincides with a larval age of 26 dph and 28 dph in treatments G-3 and P-3 respectively, whereas the larvae in *Artemia* fed treatments reached this stage at 22-24 dph. Zambonino Infante et al. (1996) found that quantitatively restricting the feed intake of European sea bass to one quarter of a full ration delayed the enhancement of trypsin secretion causing the primary digestive features to persist. This may also account for the differences in MD performance post wean. Gemma Micro MD is possibly a more easily assimilated MD at this early stage and more readily facilitates the development of the digestive process (Zambonino Infante et al., 1996; Canavate and Fernandez-Diaz, 1999; Cahu and Zambonino Infante, 2001).

Factors such as the protein levels, protein source, percentage of free amino acids (FAA) and binder used can dictate the digestibility of the MD (Lee et al., 1996; Ronnestad et al., 1999). The total protein levels of Gemma Micro and Proton are similar, however the FAA content may vary between the two MDs, which in combination with the type of binder can make the diet more attractive or palatable by leaching a greater amount of attractants into the water, thus leading to increased

ingestion of one diet and subsequently better growth (Partridge and Southgate, 1999; Kolkovski, 2001). Losses due to leaching were found to be higher in the Gemma Micro MD compared to the Proton MD. Although not quantified, barramundi larvae in all treatments were observed to more actively feed upon the Gemma Micro and Skretting diets with less waste evident on the bottom of the tanks. This possibly attributes to a higher bioavailability of nutrients from Gemma Micro MD, leading to greater assimilation and subsequent better growth.

Cahu and Zambonino Infante (2001) have shown that larval growth is promoted by diets containing high energy levels, supplied as neutral lipid and phospholipid mixtures. Proximate analysis has shown Gemma Micro MD to be 5% higher in total lipid levels than the Proton MD. Optimal growth and survival in European sea bass larvae is obtained using a diet of 30% lipid (Zambonino Infante and Cahu, 1999), while Brinkmeyer and Holt (1995) found that a diet containing 18% lipids gave the best results for red drum (*Sciaenops ocellatus*) larvae. In the current trial, post 18 dph (until 29 dph) barramundi larvae showed better growth when fed Gemma Micro/Skretting diets. Higher lipid levels and / or the ratio of lipid groups (i.e. phospholipids) found in Gemma Micro MD may be a contributing factor, while n-3 HUFA enriched *Artemia* significantly promoted growth (Salhi et al., 1994; Coutteau et al., 1997).

A significant improvement in growth occurred in treatments P-1 and P-2 following the discontinuation of *Artemia*, although the corresponding Gemma Micro MD treatments had previously performed better. Cannibalism can be shown to increase at around this time, by an observed increase in cannibalistic behaviour and the number of mortalities being removed from culture with characteristic marks on their heads. Therefore, the apparent increased growth is more likely to be linked to the significantly higher rate of removal of smaller larvae from the population, rather than to a better performing MD. This may be attributed to the diets physical characteristics such as sinking rate or palatability and a subsequently lower ingestion rate and/or perhaps nutritional characteristics of the diet, causing higher growth variation within the tanks (Goldan et al., 1998). Although the rate of cannibalism was similar in all Proton MD fed treatments, the larvae in P-3 remained significantly smaller, while P-1 and P-2 were biased towards a larger average WW. This indicates a significantly lower nutritional intake in P-3, and that enriched *Artemia* inputs are essential when using this MD. Cannibalism is an unwanted side effect of a less suitable MD, especially in an aggressive piscivorous species such as barramundi (Goldan et al., 1998; Daniels and Hodson, 1999; Cahu and Zambonino Infante, 2001).

5.3.2.6 Figures and tables

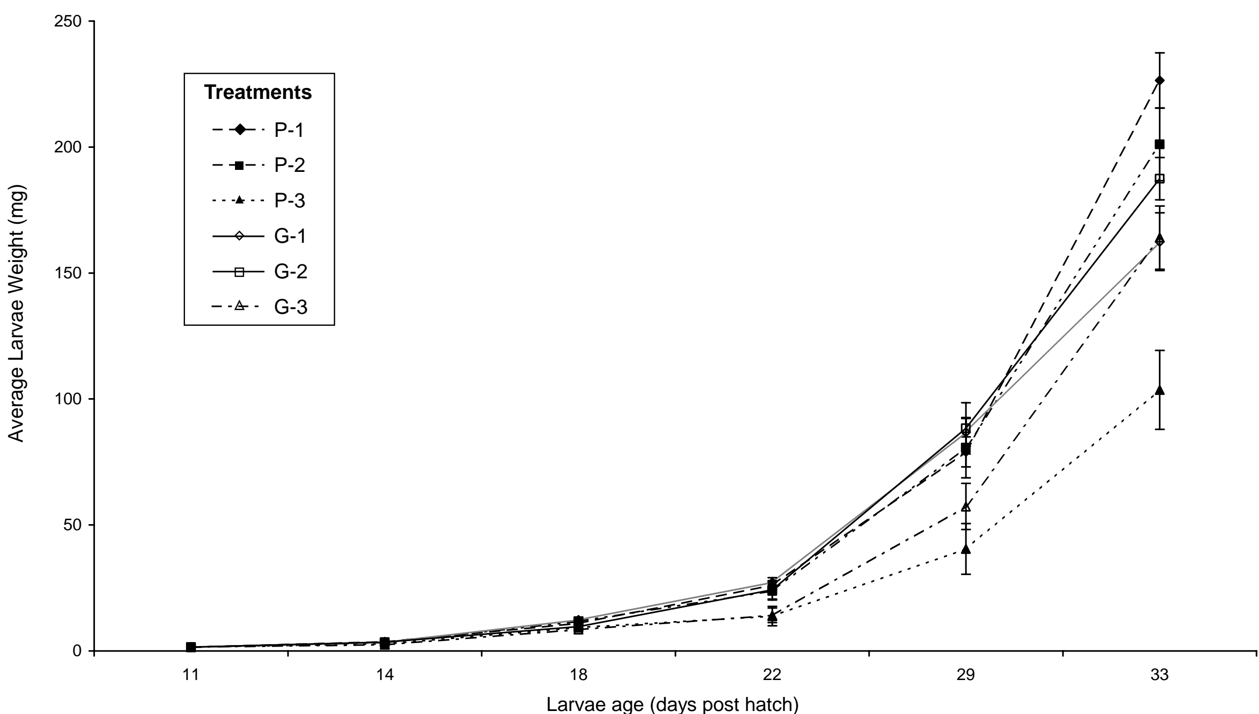

Figure 1. Average larvae weight for treatments at indicated larvae age.

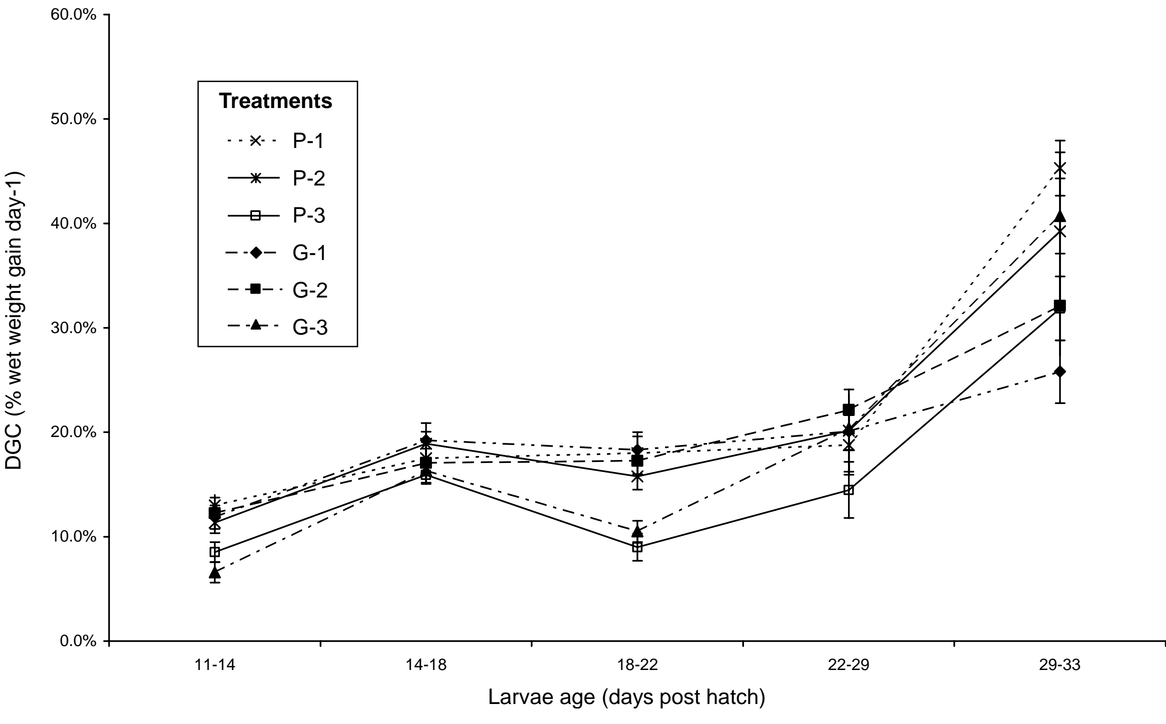

Figure 2. Average DGC between weigh events for each treatment over the weaning period.

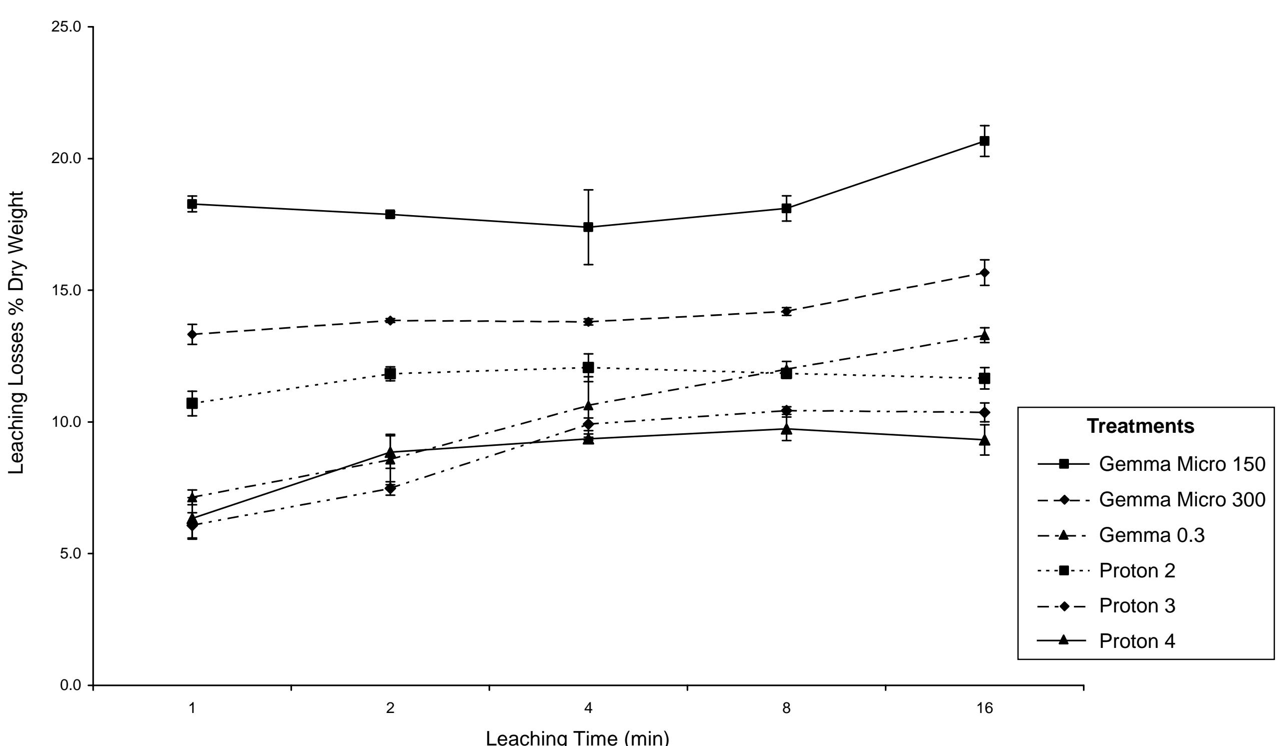

Figure 3. Leaching losses of less than 10 µm in micro diets over time in 28°C marine bore water of salinity 34.1%o and pH 8.1.

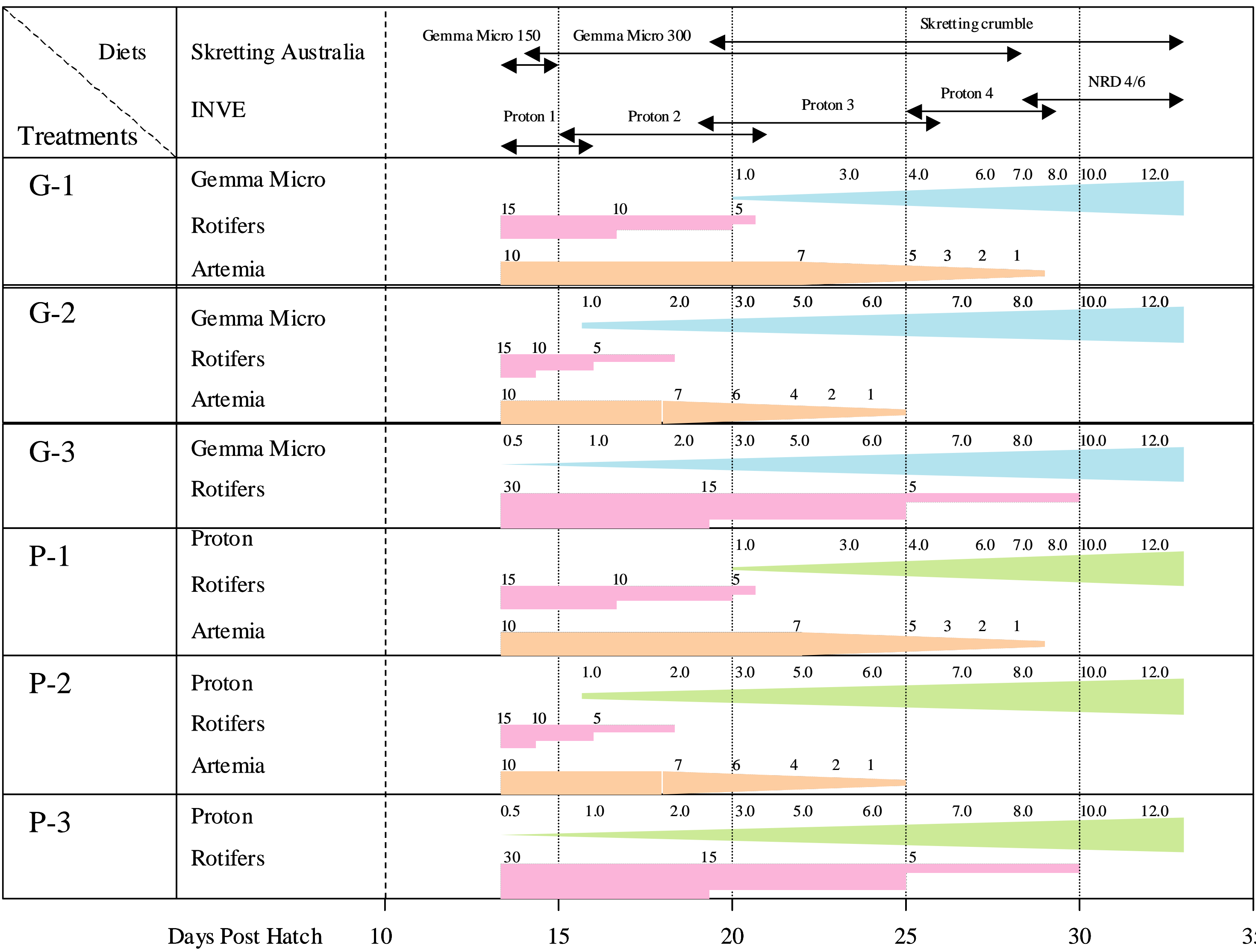

Figure 4. Weaning protocols, from 13 dph to 32 dph, are shown. Microdiet feeding rations are expressed as 'g (1000 larvae)$^{-1}$ day^{-1}', rotifers are expressed as 'number of rotifers ml^{-1}' continuously and *Artemia* are expressed as 'number of *Artemia* ml^{-1} day^{-1}'. Temporal microdiet size class distribution is shown for Skretting and INVE diets, with an overlap for each transitional period.

Table 1. Final weight and survival of larvae weaned according to three live feed protocols within two MD protocols. Letters in superscript denote significant differences for cannibalism and survival figures.

Weaning Protocol	Average Larvae Final Wet Weight (mg) ± S.E.	Cannibalism% ± S.E.	Survival% S.E.	Average Tank Final Wet biomass (g) ± S.E.
P-1	226.5 ± 19.6 [a]	20.0 ± 1.8 [a]	64.5 ± 5.3 [a]	204.5 ± 8.4
P-2	201.1 ± 18.0 [b]	26.2 ± 0.7 [a]	63.2 ± 7.0 [a]	177.9 ± 9.9
P-3	103.6 ± 16.6 [d]	22.1 ± 0.3 [a]	70.8 ± 15.4 [b]	102.8 ± 11.2
G-1	164.1 ± 14.3 [c]	9.6 ± 0.7 [b]	82.0 ± 3.4 [c]	186.5 ± 3.8

G-2	187.5 ± 10.5 [b]	9.3 ± 0.9 [b]	81.9 ± 8.0 [c]	214.9 ± 10.5
G-3	170.0 ± 22.8 [c]	8.8 ± 0.3 [b]	84.1 ± 9.8 [c]	193.2 ± 11.2

Table 2. Proximate analysis of microdiets.

Diet	H_2O % ar	Ash % ar	Protein % ar	Phosphorus % ar	Fat % ar
Gemma Micro	6.3	13.2	51.7	1.97	20.1
Skretting 0.6mm	7.3	10.4	47.4	1.57	17.6
Proton	6.7	9.6	51.9	1.43	14.9
NRD 5/8	6.1	12.2	53.0	1.60	13.7

5.3.2.7 References

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5.4 MICRODIET

5.4.1 Physical and chemical properties

5.4.1.1 Free amino acid leaching from a protein-walled microencapsulated diet for fish larvae

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5.4.1.1.1 Abstract

The leaching loss of free amino acids (FAA) from an experimental protein-walled microencapsulated diet (MC) for larval fish after immersion in water was measured and compared with leaching loss from a gelatin microbound diet (MB) containing the same dietary ingredients. The loss of FAA (as a percentage of total dietary FAA) was significantly higher in the MB diet compared to the MC diet. After 5 min of re-hydration, 22% of FAA were leached from the MB diet compared with 8% from the MC diet. After 60 min, 85% of FAA from the MB diet were leached into the water compared to 17% from the MC diet. The addition of free lysine to protein-walled microcapsules (MC-L) was also investigated to determine the suitability of MC for delivering specific FAA. Lysine was incorporated into the particles with an efficiency of 7.5% and accounted for approximately 60% of the total FAA in the diet. The loss of this amino acid from MC-L after 60 min of immersion was 1.4%. The loss of specific FAA from MB and MC diets was found to be negatively and positively correlated to the hydrophobic character (hydropathy index) of each FAA, respectively. These results support the use of protein-walled microcapsules as a vector for specific dietary amino acids using a macronutrient-balanced diet.

5.4.1.1.2 Introduction

The development of a formulated microdiet to replace live prey in the larval stages of marine fish culture remains one of the main challenges for developing marine finfish culture. When designing such diets, a balance must be found between a particle with satisfactory water stability and one that is readily digestible by the larvae with a poorly differentiated digestive tract. Special attention must be paid to retaining low molecular weight dietary ingredients such as amino acids, vitamins and minerals, which can be rapidly leached from diet particles prior to ingestion.

Yufera et al. (2000) and Koven et al. (2001) have reviewed the progress in the field of larval microdiets from a technological and nutritional point of view, respectively. As reported in these reviews, only limited growth and survival has been achieved when feeding formulated microdiets to first-feeding marine finfish larvae in the absence of live prey. The fact that comparable results have been obtained when artificial diets are supplemented with some live prey suggests that some nutritional factors may be lacking in the microdiets. Among the possible missing, limited or unbalanced compounds are free amino acids (FAA). FAA have three roles in the diet of fish larvae; as dietary components for growth, as sources of energy (Ronnestad et al. 1999) and as feed attractants (Kolkovski et al. 1997a,b). To support fish growth, the FAA must be retained in the food particle up to its ingestion by the larvae. To act as feed attractants, however, some FAA need to pass into the water to be detected by the larvae.

Several kinds of food particles (microbound particles, microcoated particles, protein-walled

microcapsules and liposomes) have been developed for small aquatic animals. The different manufacturing procedures of these diet types result in the differences in structure and properties (Kanazawa and Teshima, 1988; Jones et al. 1993; Watanabe and Kiron, 1994). Promising results have been obtained from feeding early larvae of several species by using different microdiets (Cahu et al. 1999; Fontagne et al. 2000; Lazo et al. 2000). Regarding microencapsulated diets, we have obtained relatively good results using a prototype of a protein-walled microencapsulated diet for feeding gilthead seabream (Fernandez-Dıaz et al. 1997; Yufera et al. 1999, 2000). A further step that is required in developing the formulation of this microdiet is an understanding of whether FAA is incorporated into the particle and whether the particle is able to deliver the FAA into the larvae's gut.

In order to determine the capacity of this protein-walled microcapsule to retain small molecules inside the shell, the present study has examined the leaching patterns of FAA from a completely formulated microencapsulated diet after rehydration. The results are compared with the leaching pattern of the same dietary ingredients in microbound particles. In addition, encapsulation efficiency and leaching rate in a protein-walled microencapsulated diet supplemented with lysine were also examined to determine the potential for this type of diet to deliver specific amino acids.

5.4.1.1.3 Materials and methods

The microencapsulated diets (MC and MC-L) were prepared by interfacial polymerisation of the dietary protein (Yufera et al. 1999). In the MC-L, krill hydrolysate was replaced with lysine (Table 1). The dietary ingredients were dispersed in a basic pH buffered Tris – HCl aqueous solution (10% w/v). Two parts of this solution were emulsified in five parts of a soy lecithin and cyclohexane solution (2% w/v) with a homogeniser (IKA, 45-mm 'ship propeller' stirrer) for 8 min at 1,000 rpm at room temperature (approximately 20°C). The cross-linking agent (1,3,5-benzenetricarboxylic acid chloride (trimesoyl chloride), dissolved in diethyl ether, was then added to the emulsion with continuous stirring and the reaction continued for 8 min further. The cross-linking agent represents 1/3 (w/w)of the total crude protein in the dry diet. The microcapsules formed were allowed to settle, and the cyclohexane – lecithin solution was decanted. After washing with cyclohexane, the microcapsules were dispersed in a gelatin solution (15% w/v) while stirring at the same pre-fixed speed for 3 min. Distilled water (approximately 2/3 of the total volume), with a temperature of approximately 38°C, was added while stirring. The capsules were then repeatedly washed with freshwater, then a pH 8 buffered saline solution in order to remove the debris.

The microbound diet (MB) was prepared by mixing the dietary ingredients with a gelatin binder according to Kolkovski et al. (1993). The dietary ingredients were the same as those used to produce the MC diet (Table 1). After preparation, all three diets were freeze dried and sieved into the range between 108 and 335 Am.

Leaching experiments were carried out in 500-ml beakers containing 250 ml of distilled water (20°C) continuously stirred at 60 rpm with a 45-mm ship propeller attached to a rotor (IKA). At 0 time, 500 mg of diet were added to the water. Water samples of 10 ml were removed at 1, 2, 5, 15, 30 and 60 min, using a 10-ml syringe. The water was double filtered through 0.25-Am filters (Millipore HV) and analysed for FAA using HPLC (Shimadzu with lichrosphere RP-8 select-B column by MERCK). FAA determination was based on the procedure described by Bidlingmeyer et al. (1987).

Table 1. Ingredient composition of the experimental diets (g kg^{-1} of dry diet).

Microdiet	MC	MB	MC-L

Casein[a]	500	500	500
Squid meal[b]	100	100	100
Krill hydrolysate (dry)[c]	120	120	–
Lysine[d]	–	–	120
Dextrin (type I)[e]	60	60	60
Fish lipids[f]	120	120	120
Soy lecithin[g]	30	30	30
Vitamin complex[h]	50	50	50
Vitamin C[i]	20	20	20

MC: microencapsulated diet; MB: microbounded diet; MC-L microencapsulated diet with lysine supplementation.
[a] ICN 901633.
[b] Rieber & Son, Norway.
[c] Specialty Marine Products, Vancouver, Canada.
[d] L-Lysine monohydrochloride, M.W. = 182.6, ICN 102218.
[e] ICN 101517.
[f] Kurios lipid emulsion, France.
[g] ICN 102147.
[h] Kurios polyvitamin fish complex, France.
[i] Showa-Denko, Shoko.

The loss of each specific FAA as well as of total FAA (except tryptophan) from each diet was calculated at each sampling time as a percentage of the total FAA in the respective diet. FAA retention, calculated as the difference between total leaching loss after 60 min and initial FAA dietary content, was expressed as the percentage of the total FAA in the diet. In order to know the capacity of each microdiet to retain hydrophilic molecules, the total leaching of each FAA in the three diets was plotted in relation to the corresponding hydropathy index using Kyte and Doolittle's (1982) scale. Hydropathy is a measure of the hydrophilic/hydrophobic character of an amino acid.

To quantify the dietary FAA composition, 15 mg of each diet was mixed with 30 ml of distilled water, homogenised (Ultra Turax homogeniser) for 15 min, and then sonicated for at least 10 min until the particles had completely disintegrated. The samples were kept 4°C during the disintegration process. The sampling procedure described above was carried out to determine the FAA content of these samples. The encapsulation efficiency of lysine in MC-L was calculated as the percentage of the total amount of lysine in the ingredient mixture retained in the diet particles on a dry weight basis. Two replicates per treatment were performed in all experiments.

Differences in leaching losses at different immersion times and between microdiets were analysed by means of one-way ANOVA followed by LSD multiple range test and Student's *t*-test for slope comparison using Statgraphic software package. The data, given as percentages, were arcsine-transformed prior to analysis. Differences were considered to be significant when $P < 0.05$.

5.4.1.1.4 Results

The total efficiency of encapsulation (mass of particles obtained from the total mass of ingredient mixture) of MC particles was 70% on a dry weight basis. Fig. 1 shows the FAA composition of the three different diets. Both standard diets (MC and MB) showed similar amino acid profiles. In both cases, the most abundant amino acids were arginine, alanine and glycine. Tyrosine was absent

in MB and threonine in MC diets. Lysine was accounted for 60% of the total FAA in the MC-L diet as compared to approximately 1% in the other two diets. All other amino acids were present in similar ratios to MC and MB. The amount of free lysine per unit weight of MC-L capsules was 7.18 Ag/mg. Taken into account that the amount of lysine in the ingredients mixture was 12% in a dry weight basis; the lysine encapsulation efficiency was 7.47%.

After rehydration, the amount of FAA leached from the particles (expressed as a percentage of total FAA in the diet) was significantly ($P < 0.001$) higher in the MB diet than in the two protein-walled microcapsules (MC and MC-L) (Fig. 2). After 5 min of immersion, 22%, 8% and 2% of FAA had leached into the water from MB, MC and MC-L diets, respectively. After 60 min, 85% of FAA from the MB diet had leached into the water as compared to only 17% and 15% from the MC and MC-L diets, respectively. The total FAA in the MC diet accounted for 0.42% of the particles' dry weight after the 60 in immersion. This figure was doubled when lysine was added to MC-L diet while only 0.07% remained in MB diet (Table 2).

A different pattern was found when comparing leaching losses of specific FAA from the MB with MC and MC-L diets. A significant ($P < 0.01$) negative, linear correlation ($r= 5.65$) was found in the MB diet between the leaching loss of specific FAA after 60 min and their corresponding hydropathy index (Fig. 3). In the MC and MC-L diets, a significant positive ($P < 0.01$), linear correlation ($r= 5.74$ and 5.27, respectively) was found between the loss of FAA and their hydropathy index (Fig. 3). No correlation between the molecular weight of FAA and their leaching loss was found for any of the diets tested ($P > 0.05$).

Table 2. Percentage of total FAA (average and SD) in dry weight basis before hydration and after 60 min of immersion in water in the three microdiets. MC: microencapsulated diet; MC-L microencapsulated diet with lysine supplementation. MB: microbounded diet.

Time (min)	MC	MC-L	MB
0	0.53 ± 0.01	1.06 ± 0.11	0.57 ± 0.09
60	0.42 ± 0.00	1.02 ± 0.01	0.07 ± 0.01

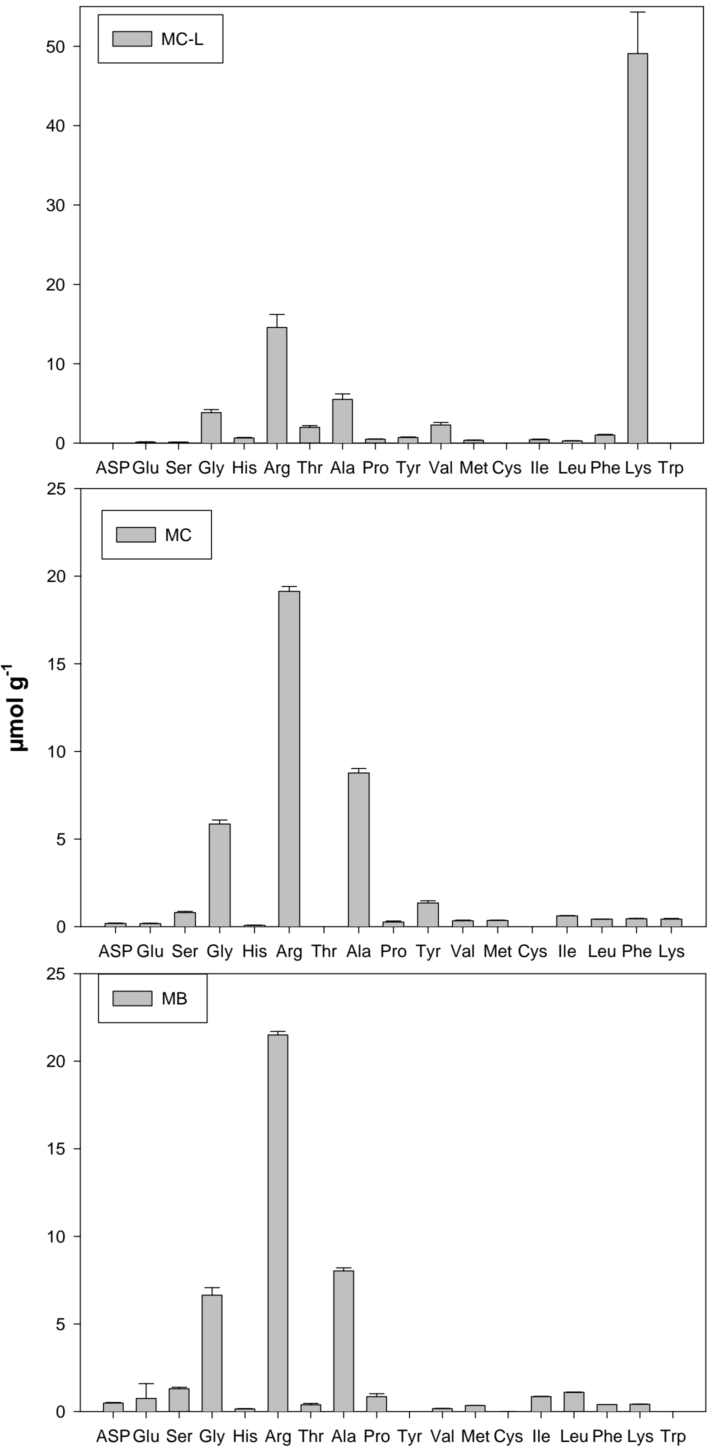

Figure 1. Free amino acid composition in the experimental microdiets. MC: microencapsulated diet; MC-L microencapsulated diet with lysine supplementation. MB: microbounded diet.

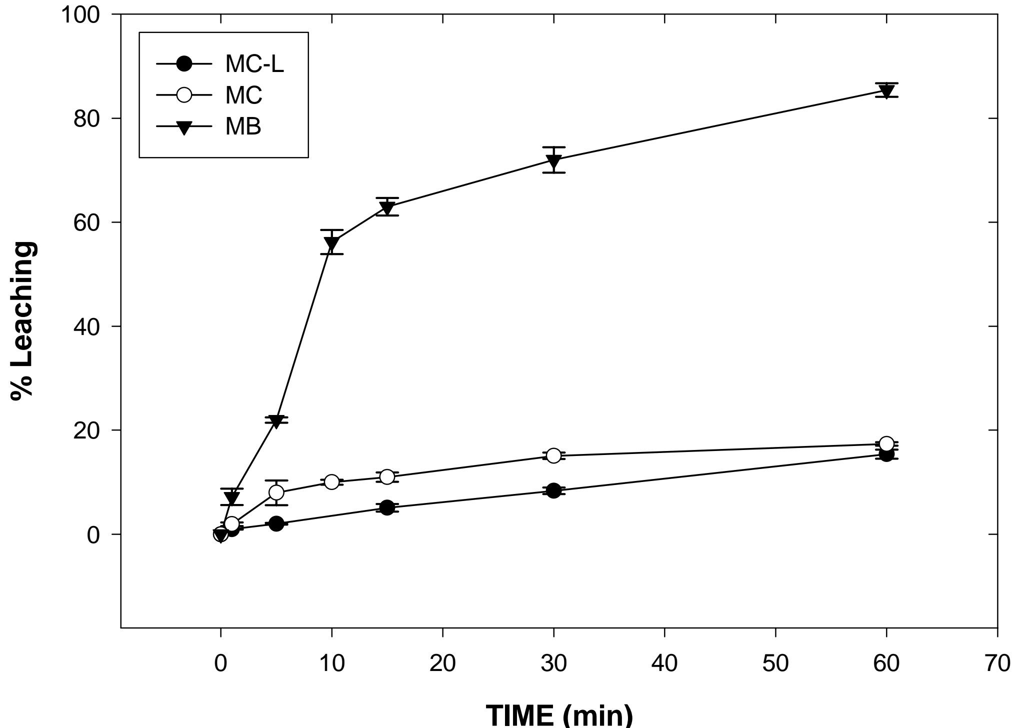

Figure 2. Leaching pattern of total free amino acids during 60 min of immersion in water. MC: microencapsulated diet; MC-L microencapsulated diet with lysine supplementation. MB: microbounded diet.

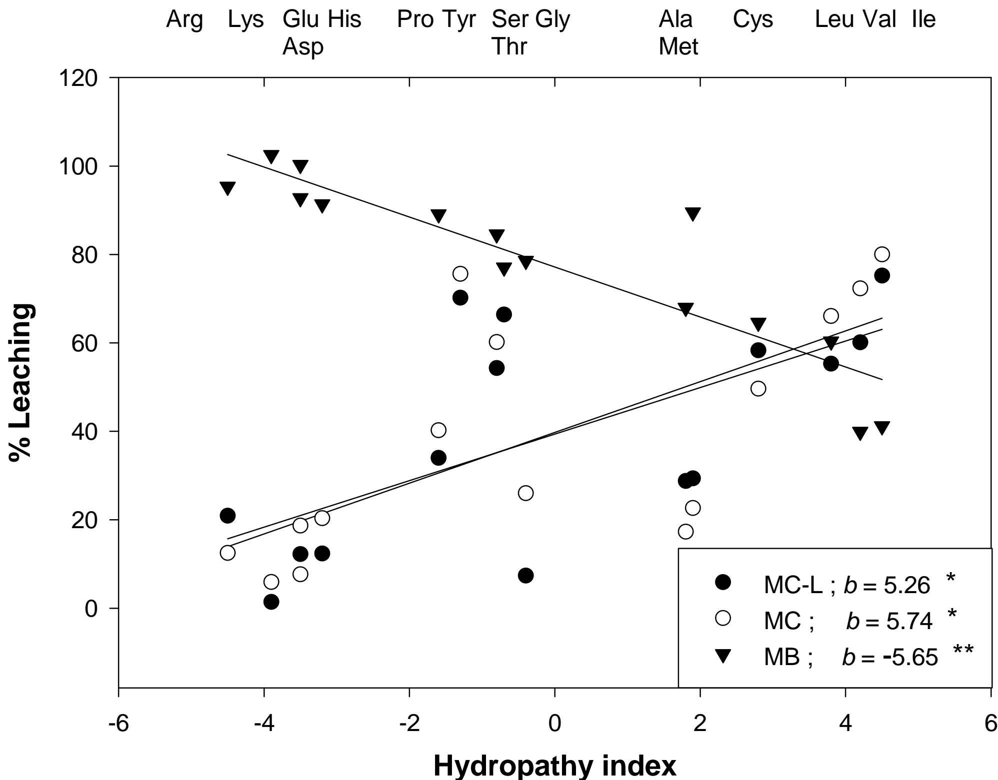

Figure 3. Percentage of FAA leached after 60 min of immersion in water in relation to the hydropathy index (Kyte and Doolittle, 1982). MC: microencapsulated diet; MC-L microencapsulated diet with lysine supplementation. MB: microbounded diet. Indicates the slopes of the linear regressions. Numbers with the same asterisk indicates no significant difference.

In spite of the differences found in relation to the hydropathy index, the leaching losses of specific FAA were almost always higher in MB than in MC and MC-L diets, except losses in valine and isoleucine. The highest losses of specific FAA after 60 min of immersion were of isoleucine from MC and MC-L diets (80% and 75%, respectively) and of lysine and glutamine from the MB diet (100%) (Fig. 3). Glycine and alanine, the most abundant FAA in the diets, showed a gradual leaching throughout the 60 min of immersion while most of the arginine leaching occurred during the first 15 min. Leaching rates of all these three predominant FAA were always much higher in MB than in the two microencapsulated diets (Figs. 4 – 6). Total amount of lysine leached after 60 min was higher in MC-L diet, but when comparing the percentages of the initial amount, the higher loss was found in MB. Although the majority of lysine leached from MC-L over the 60 min period was lost in the first few minutes, the overall loss of lysine in this diet was only 1.4% (Fig. 7).

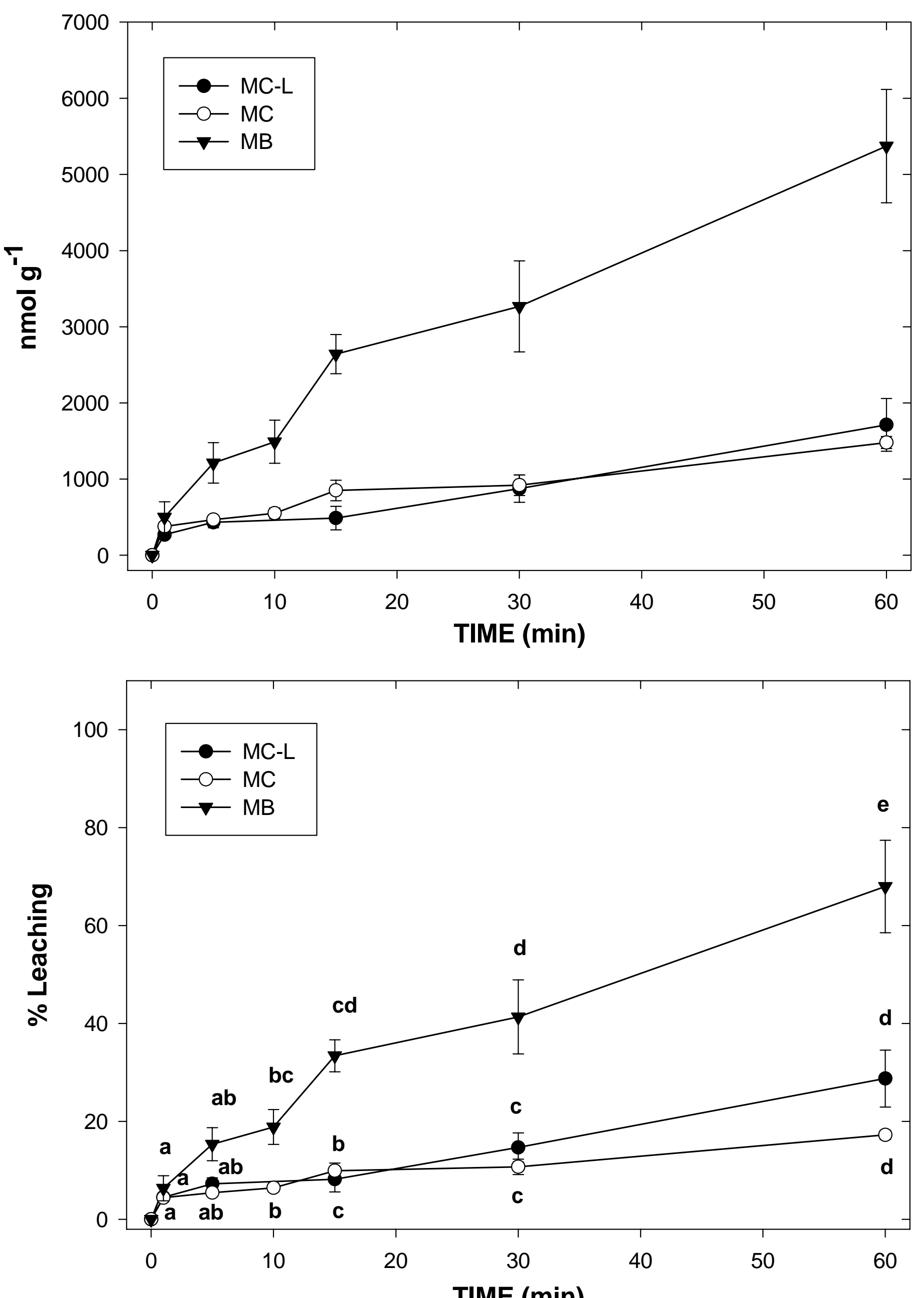

Figure 4. Leaching pattern of alanine in the experimental microdiets during 60 min of immersion in water. MC: microencapsulated diet; MC-L microencapsulated diet with lysine supplementation. MB: microbounded diet. In each diet, time point values with the same superscript are not significantly different ($P < 0.05$).

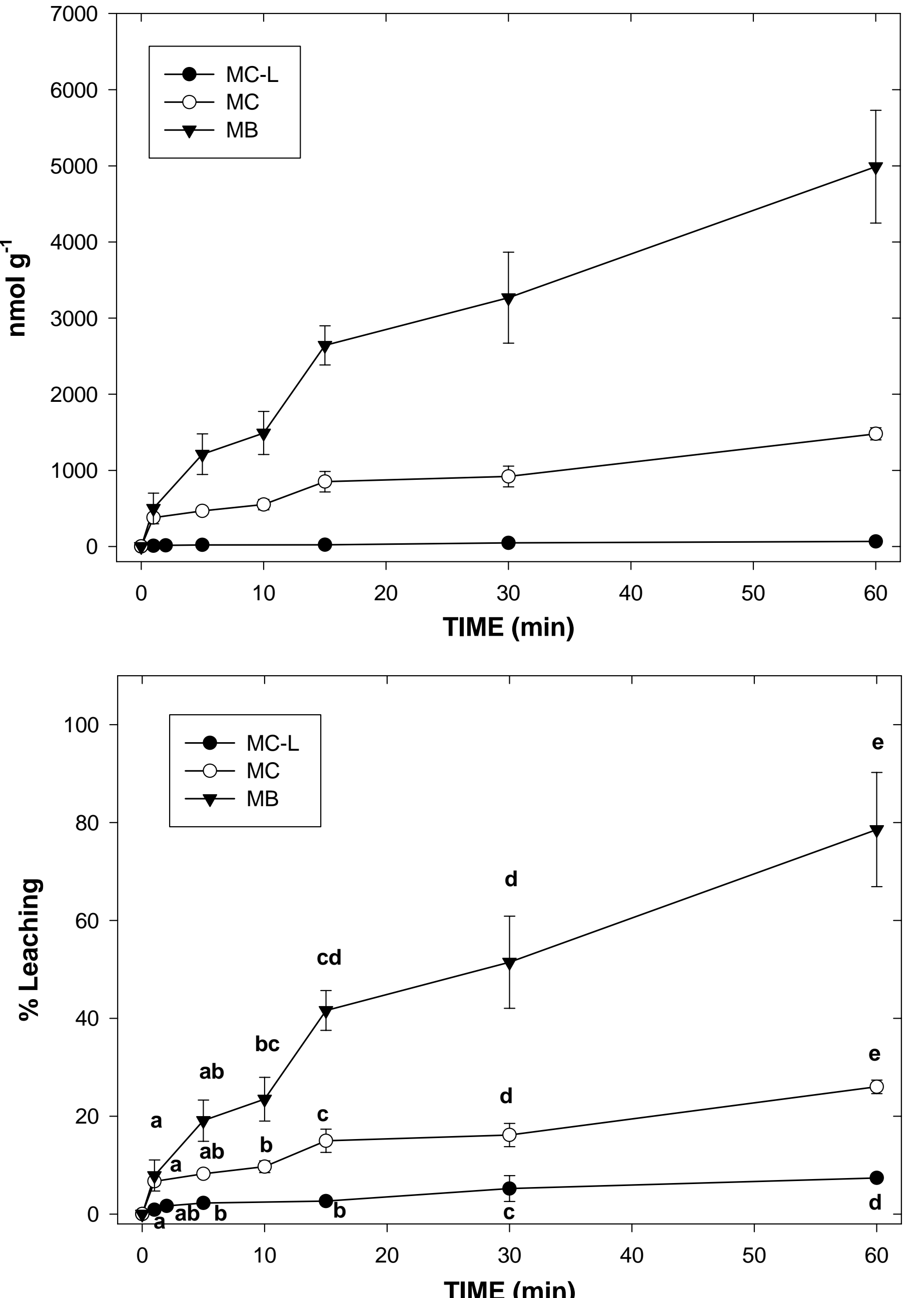

Figure 5. Leaching pattern of glycine in the experimental microdiets during 60 min of immersion in water. MC: microencapsulated diet; MC-L microencapsulated diet with lysine supplementation. MB: microbounded diet. In each diet, time point values with the same superscript are not significantly different (P < 0.05).

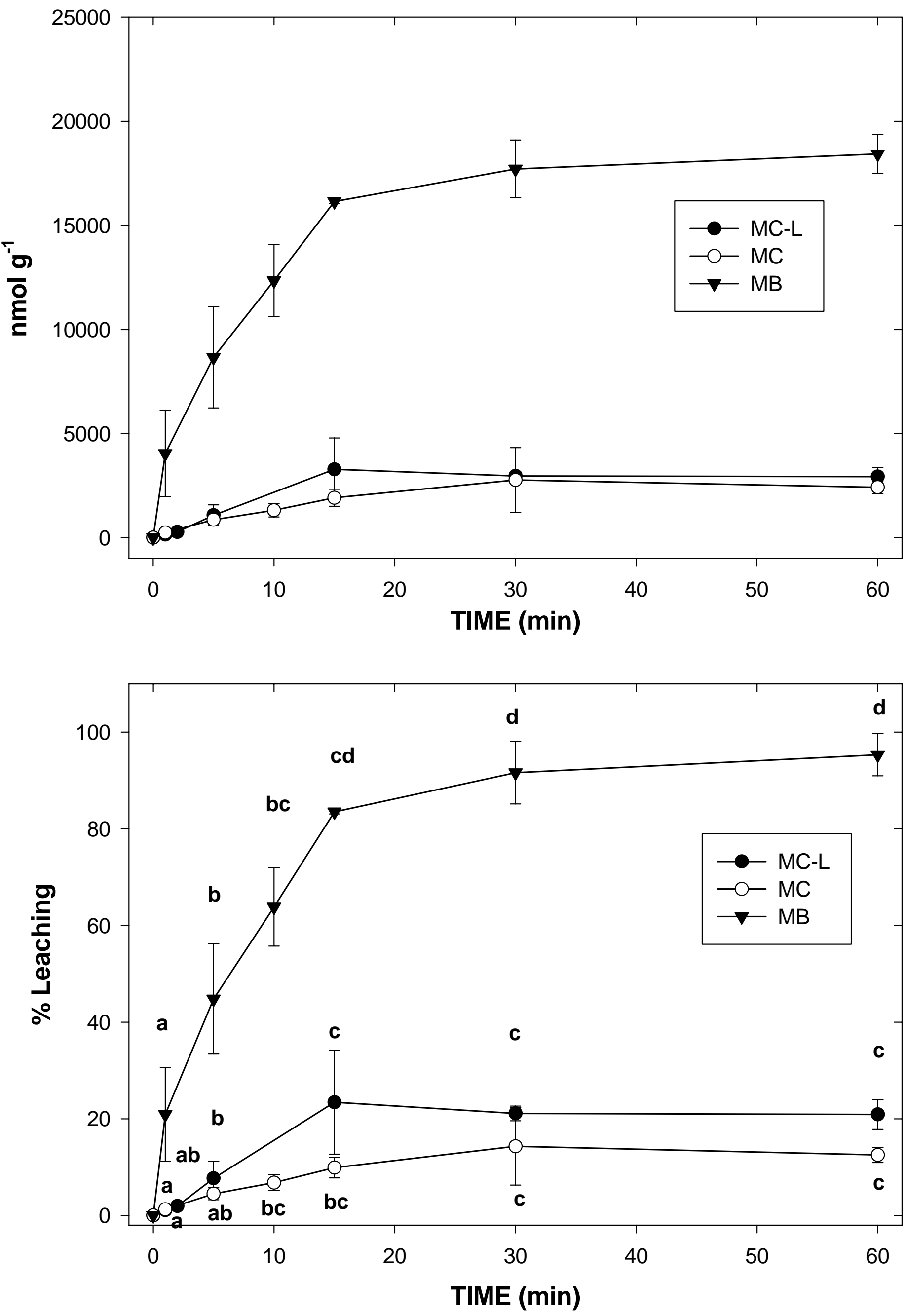

Figure 6. Leaching pattern of arginine in the experimental microdiets during 60 min of immersion in water. MC: microencapsulated diet; MC-L microencapsulated diet with lysine supplementation. MB: microbounded diet. In each diet, time point values with the same superscript are not significantly different ($P < 0.05$).

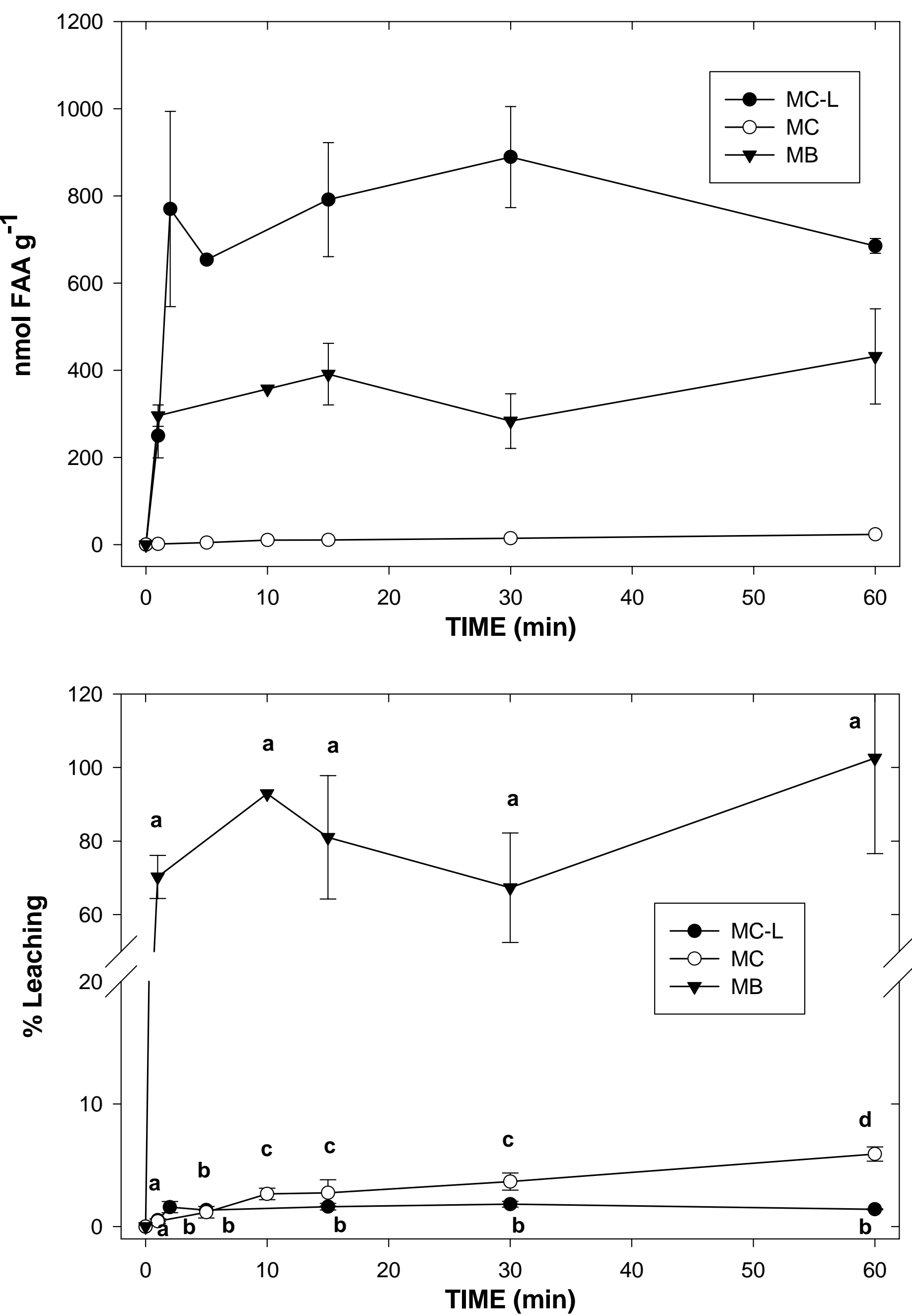

Figure 7. Leaching pattern of lysine in the experimental microdiets during 60 min of immersion in water. MC: microencapsulated diet; MC-L microencapsulated diet with lysine supplementation. MB: microbounded diet. In each diet, time point values with the same superscript are not significantly different (P < 0.05).

5.4.1.1.5 Discussion

The protection of micronutrients against leaching losses before the particle has been ingested has been an important objective in developing microdiets for small aquatic animals (Villamar and Langdon, 1993; Lopez-Alvarado et al. 1994; Ozkizilcik and Chu, 1996; Marchetti et al. 1999; Baskerville-Bridges and Kling 2000; Onal and Langdon, 2000). Lopez-Alvarado et al. (1994) examined the leaching rate of crystalline amino acids incorporated in different types of microdiets. They found the best retention in lipid-walled capsules (approximately 10% loss in 1 h) and the highest losses in microbound particles (>80% in a few minutes). Losses of FAA in protein-walled microcapsules were approximately 40% after 2 min of immersion in water. Similarly, Ozkizilcik and

Chu (1996) reported free lysine losses of 80% in protein-walled microcapsules and approximately 25% in complex protein microcapsules containing lipid-walled microcapsules after 60 min of rehydration. Lipid-walled encapsulation appears to be an effective way of retaining amino acids and has been used experimentally in enriching *Artemia* nauplii (Ozkizilcik and Chu, 1994; Tonheim et al. 2000) and in supplementing fish larvae microdiets (Ozkizilcik and Chu, 1996; Koven et al. 2001). Liposomes alone, however, are unable to provide a complete larvae diet as they are unable to incorporate all of the major nutrients in the required proportions. Baskerville-Bridges and Kling (2000) also observed high amino acid leaching (60% within 1 min of immersion in water) in different microbounded and microcoated diets for marine larval fish. Although the protein-walled microcapsules in the above-mentioned studies were not highly effective in retaining FAA, many parameters involved in the manufacturing process of protein-walled microcapsules can be modified to affect the final characteristics of the particles (Yu fera et al. 1996, 2000). The prototype feed tested in the present study (PT2; Yufera et al. 1999) significantly improved the retention of FAA to a level (approximately 83% in 60 min) similar to that reported for lipid-walled microcapsules by Lopez-Alvarado et al. (1994) and Ozkizilcik and Chu (1996). The use of protein-walled microcapsules has the benefit over lipid-walled microcapsules in that they allow a complete diet with the appropriate proportion of macronutrients to be encapsulated within a single particle. We found clearly different correlations between the leaching rate of each amino acid and hydropathy index in microbound and microencapsulated diets. Lopez-Alvarado et al. (1994) also found that either non-polar and charged amino acids showed different leaching rates from microbound and lipid-walled microcapsules. It seems therefore that the interaction between FAA polar proprieties and the particle chemical characteristic is one of the main factors involved in their retention capacity.

Nevertheless, comparison with results reported in other studies is only indicative because in addition to differences in the detection methods and in the intrinsic characteristic of particles, leaching rates depend on many factors such as water pH, solute concentrations, temperature and particle diameter.

The encapsulation efficiency of lysine was low (7.5%), although no intention to improve the encapsulation procedure was made in this study. The low incorporation of lysine may be due to the washing procedure during encapsulation where the majority of it may have been washed away. Another possibility is its incorporation as polypeptides or to the dietary proteins by the cross-linking reaction. Lopez-Alvarado et al. (1994) suggested that some FAA could become linked to protein by the polymerisation process. As observed in the leaching rates, the incorporation efficiency could also be a function of the hydropathy index taken into account the low pH used during the microencapsulation process. A more detailed analysis of the encapsulation process is required to detect the fate of non-encapsulated amino acids.

Although there is little information regarding the appropriate quantity of FAA to be included in microdiets for larval fish, the FAA content of live prey organisms may provide an indication of suitable levels. Dabrowski and Rusiecki (1983) reported a FAA content of 0.7% for freshwater rotifers *Brachionus* sp. and 2.8% for *Artemia* nauplii. Frolov et al. (1991) found that the amount of FAA in mixohaline rotifer *Brachionus plicatilis* ranged between 4% and 7% of dry matter depending on the salinity in which the rotifers were cultured. Mie et al. (1997) reported FAA values of 1– 2% in *B. plicatilis* and 0.6% in *Artemia* nauplii. The FAA content of the diets in the current study were somewhat lower than those reported above but of similar magnitude.

There have been few studies investigating the supplementation of FAA into microdiets for marine finfish larvae. Lopez-Alvarado and Kanazawa (1995) achieved the best growth and survival when 5% of the dry diet was replaced by crystalline amino acids during co-feeding experiments with *Pagrus major* larvae. These trials were, however, conducted using microbound diets, which had

been subjected to high leaching of FAA. Providing excessive amounts of FAA in a larval diet may result in an amino acid imbalance in the larvae due to a slower absorption of protein-bound amino acids than FAA (Ronnestad et al. 2000).

In addition to crystalline lysine, the potential sources of FAA in our diets were krill hydrolysate and squid meal. As no hydrolysate was supplied in the MC-L, we can assume that the FAA, except lysine, were mostly provided by the squid meal. FAA may play an important role as feeding attractants in microdiets. Kolkovski et al. (1997b) found that of the metabolites secreted from *Artemia* nauplii, arginine, glycine, alanine and the sodium salt of betaine act as strong chemical stimulants. The addition of these substances to larval rearing tanks increased the ingestion rates of microdiets by gilthead seabream larvae. These three amino acids were the most abundant in the MB and MC diets and after 60 min of immersion, leaching losses of these FAA from the MC diet were 12%, 26% and 17%, respectively (Fig. 7). The abundance of these FAA in the MC diet and the gradual leaching of glycine and alanine during a long time period could explain the excellent acceptance and ingestion of this diet by first-feeding gilthead seabream larvae (Yufera et al. 1995).

In summary, results obtained in the present study indicate that the protein cross-linking method enables effective entrapment of FAA within a complete diet particle, while allowing a limited amount of FAA to be released to the water to act as feed attractants. More research is required to determine the appropriate FAA level (in capsules and water) and a profile needed by marine fish larvae as well as on methods to incorporate this required profile into a complete formulated microdiet.

5.4.1.1.6 Acknowledgements

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5.4.1.2 Comparison of sinking and leaching rates of 11 commercially available microdiets

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5.4.1.2.1 Abstract

The larviculture of marine fish is currently dependent upon live feeds (rotifers and *Artemia*), which increase the cost and complexity of fish production, and contribute up to 50% of a hatchery's operating cost. This reliance on live food via the development of artificial microdiets is the focus of the majority of research within the larvae culture, as improved microdiets will increase larval culture profitability. Sinking and leaching rates are important factors when considering a microdiet. High sinking rates limit the length of time a diet remains in suspension and is available to the larvae, thereby affecting its efficiency. High leaching rates reduce the nutritional value of the microdiet, however some leaching is desirable to stimulate feeding. Knowledge of sinking and leaching rates of microdiets can be used to direct tank design, water flow and aeration, optimise ingestion, minimise wastage and prevent water quality deterioration. The present study evaluated settling and leaching rates of 11 commercially available microdiets, in an attempt to offer additional information other than what is provided by manufacturers. Results generally indicate that, the smaller the particle size, the slower the sinking rate and greater the leaching rate and vice versa. However variations exist due to other dietary factors such as the mean size, size range and binder composition.

5.4.1.2.2 Introduction

The larviculture of marine fish is currently dependent upon live feeds, mainly rotifers and *Artemia* (Rosenlund et al. 1997). The production of live feed increases the cost and complexity of juvenile fish production, contributing up to 50% of a hatchery's operating cost, via additional infrastructure, labour and energy to mass culture (Rosenlund et al. 1997; Southgate et al. 2000; Koven et al. 2001). Live feeds lack sufficient essential fatty acids thereby requiring enrichment, are subject to crashes and most importantly *Artemia* supply is uncertain in the future (Southgate et al. 2000).

Reducing the reliance on live food can increase larval culture profitability (Southgate et al. 2000).

Therefore much of the larval feeds research conducted within the aquaculture industry is focused on the development of artificial microdiets. Microdiets have the advantage of improved nutritional profiles that can match the developmental stages of the larvae while reducing costs (Southgate et al. 2000). However, the formulation of microdiets is not easy to achieve (Cahu and Zambonino, 2001). Microdiets must have a high acceptability, be easily digested by larvae with immature digestive systems, meet larval nutritional needs and be supplied so that food deprivation does not occur (Le Ruyet et al. 1993). Therefore, manufacturers must consider the dietary composition (i.e. protein, lipids, fibre, ash, vitamins, and minerals), physical properties (colour, buoyancy, leaching, and size) and biological properties (digestibility, nutritional value, and taste). Currently there are many different commercially available microdiets. Knowledge of dietary composition, physical and biological properties of each commercially available diet will ensure the most appropriate microdiet be fed to a given species.

Early stage larvae have limited swimming ability and microparticles must be caught while falling through the water column (Cahu and Zambonino, 2001). Larval feeding can be improved by using natural or chemical attractants and by improving visual detection with stained diets and correct lighting levels (Le Ruyet et al. 1993). Feed colour and movement can stimulate a feeding response; with stimulated larvae taking 60% more microdiet than unstimulated larvae (Kolkovski et al. 1997). Chemical stimulus is important for orientating larvae to prey, while vision becomes important for ingestion when close to prey (Kolkovski et al. 1997). Kolkovski et al. (1997) found the compound betaine and the L-isomers of the free amino acids arginine, glycine and alanine to be attractants to marine species.

Sinking and leaching rates are important factors to consider when choosing a microdiet. According to Baskerville-Bridges and Kling (2000), the analysis of microdiets within the water column should include both settling and leaching rates. Settling rate limits the length of time a diet remains in suspension and available to the larvae (Baskerville-Bridges and Kling 2000). Knowledge of sinking and leaching rates of microdiets can be used to direct tank design, water flow and aeration, to optimise ingestion, minimise wastage and prevent water quality deterioration (Le Ruyet et al. 1993). High leaching rates can reduce the nutritional value, although some leaching is desirable to stimulate feeding (Baskerville-Bridges and Kling 2000).

In this study, we investigated the sinking and leaching rates of 11 commercially available microdiets i.e. Proton 4, 3, and 2 (INVE Belgium), Micro Gemma 150, 300 and Gemma 0.3, (Skretting), NRD 4/6, 5/8 (INVE), Kinko 0, and 1, (Higashi Maru), and Grow Best L3 . This study is being performed in tandem with experiments conducted by Dr. Sagiv Kolkovski of Department of Fisheries Western Australia, relating to the leaching of free amino acids from microdiets Kolkovski et al. (2004).

5.4.1.2.3 Materials and methods

Microdiet Properties

Table 1. Size, cost and composition comparison of the 11 commercially available microdiets used within this experiment, as determined by various manufacturers.

Microdiet Brand	Size	Cost	Analysis Composition
Micro Gemma 150	50-200 µm	Not available	Fishmeal, lecithin, carboxymethylcellulose, betaine, vitamins, minerals. Protein 55%, lipids 14%, fibre 5%, ash 13.5%, phosphorus 2%, Vit A 40,000IU/kg, Vit D3 3,000IU/kg, Vit E 400IU/kg
Micro Gemma 300	250-400 µm	Not available	Fishmeal, lecithin, carboxymethylcellulose, betaine, vitamins, minerals. Protein 55%, lipids 14%, fibre 5%, ash 13.5%, phosphorus 2%, Vit A 40,000IU/kg, Vit D3 3,000IU/kg, Vit E 400IU/kg
Gemma 0.3	300 µm	Not available	Fishmeal, lecithin, carboxymethylcellulose, betaine, vitamins, minerals. Protein 55%, lipids 14%, fibre 5%, ash 13.5%, phosphorus 2%, Vit A 40,000IU/kg, Vit D3 3,000IU/kg, Vit E 400IU/kg
Proton 2	100–300 µm	$46/kg	Crude protein 55%, crude lipids 14%, crude ash 12%, crude fibre 1%, phosphorus1.5%, Vit A 30,000IU/kg, Vit D3 2,500IU/kg, Vit C 2000IU/kg, Vit E 700mg/kg, DHA/EPA 2, n-3 HUFA 30mg/g/dwt
Proton 3	200-400 µm	$46/kg	Crude protein 55%, crude lipids 14%, crude ash 12%, crude fibre 1%, phosphorus1.5%, Vit A 30,000IU/kg, Vit D3 2,500IU/kg, Vit C 2000IU/kg, Vit E 700mg/kg, DHA/EPA 2, n-3 HUFA 30mg/g/dwt
Proton 4	300-500 µm	$46/kg	Crude protein 55%, crude lipids 14%, crude ash 12%, crude fibre 1%, phosphorus1.5%, Vit A 30,000IU/kg, Vit D3 2,500IU/kg, Vit C 2000IU/kg, Vit E 700mg/kg, DHA/EPA 2, n-3 HUFA 30mg/g/dwt

Kinko 0	<210 µm	Not available	Not available (manufacturer's information printed in Japanese)	
Kinko 1	210-800 µm	Not available	Not available (manufacturer's information printed in Japanese)	
NRD 4/6	400-800 µm	$52/3kg	Protein >55%, lipid >9%, fibre <1.9%, moisture <8%	Marine proteins, cereal meal, plant proteins, fish oils, algae, lecithin, yeast, antioxidant
NRD 5/8	500-800 µm	$130/10kg	Protein >55%, lipid >9%, fibre <1.9%, moisture <8%	Marine proteins, cereal meal, plant proteins, fish oils, algae, lecithin, yeast, antioxidant
Growbest L3	Not available	Not available	Not available	Not available

Experimental Procedure

A clear acrylic cylinder, 2 m tall and 145 mm in diameter, was supported vertically. Marine bore water (32 ppt, 21°C) filtered to 100 µm, was directed into the top of the cylinder at a rate of 1 l/min. (Photo 1).

Photo 1. Experimental test vessel, a 2 m tall, 145 mm in diameter cylinder used to determine the sinking rates of 11 commercially available microdiets.

Two gram of diet, weighed out to 4 decimal places, was administered from a 50 ml container. Just prior to administration, 20 ml of seawater was scooped up into the container with the diet, and swirled for five seconds to facilitate a smooth distribution of the diet. The container was quickly rinsed in the cylinder water to ensure all diet was added to the test apparatus.

Two levels were marked on the upper end of the cylinder, showing the difference in volume when two litres is removed, thus giving an upper and a lower level. The lower level is 132 cm above the collection chamber. When the water (marine bore water, salinity 32 ppt, temperature 20±1C°) was aligned with the lower level, the diet was placed into the top of the water column. Water continued to enter at 1 l/min, so that after two minutes the water is at the upper level. When the water reached the upper level, the drain is opened and 2 L of the water collected in a 20 L container (Photo 2).

Photo 2. Indication of the upper and lower levels, which contains exactly two litres.

The content of the 20 L container was then emptied into a 4 L container. The 20 L container was rinsed into the 4 L container to ensure all residues were collected. The diet was retrieved from the water by filtering through an 11 µm filter (Whatman 98) using a vacuum flask. The filter paper (pre labelled and weighed) was wrapped and placed on a pre-labelled and weighed paper cup. Each diet was replicated 3 times; diets were tested in random order.

Dry weights were obtained by placing diets in an oven at 70°C for 24 h. Dry weights of all 11 diets; filter papers and paper cups were obtained. The difference between the dry weight and sample weight was subtracted from the sample weights. Samples were taken every 2 min for 12 min. After the 12th min sample, the entire water column was collected to determine the remaining amount of diet still in suspension. Therefore in total 7 samples were collected (i.e. 2, 4, 6, 8, 10, 12 min and End point).

Determination of Diet Size Composition

To ensure that all diets sizes correspond to manufacturers' claims, 20 g of each diet weighed out to 4 decimal places was screened through various sized filters and vibrated for a period of 15 min (i.e. 50,106, 150, 212, 300, 500, 710, 1000 and 2000 µm). This resulted in different sized particles accumulating above different screen sizes, which were then weighed to determine size composition of each diet. Allowing for a better understanding of each diet enabled a greater correlation between diet size and the sinking and leaching rates.

Statistical Analysis of Sinking Rates

All diet data up to the 12th min were plotted on an accumulative x y scatter graph. Sigma plot 8.0™ was used to plot the curve of best fit. Using the hyperbola equation:

$Y= (a-b)/(l+cx)^{1/d}$ accurately represented the curve of best fit for 10 of the 11 diets. The one exception was Gemma 150, the curve of best fit being accurately represented by the exponential growth equation $y=y_0+a*exp^{(b*x)+}c*exp^{(d*x)}$. Working from the equations to solve x when y was equal to 50% determined the time in minutes for 50% of the diet to settle.

This resulted in three replicates (i.e. time in minutes for 50% of the diet to settle) for each diet, with mean values and standard errors, graphed for comparison. One way analysis of variance (ANOVA)

followed by a Duncan's Multiple Range Statistical Test was performed on the time taken for 50% of the 11 diets to settle. Differences were considered significant when $P < 0.05$.

Statistical Analysis of Leaching Rates

The total amount of diet lost (i.e. leached) also underwent an ANOVA test, and Duncan's Multiple Range Statistical Test was performed to determine significant leaching differences of all 11 diets ($P < 0.05$).

5.4.1.2.4 Results

Diet Size Analysis

The diet size composition was plotted on an x y scatter graph for comparison with each other (Figure 1). Results indicated size differences between all 11 diets. Resulting from this it can be determined for each diet the mean diet size and total diet size range, which is valuable in the context of sinking and leaching rate analysis. Using SigmaPlot™ bell curves were generated for greater representation of total diet size ranges and mean diet sizes. The wavelength of each of the bell curves represents the total size range of each of the diets, whilst the peak of each bell curve represents the mean diet size. The amplitude is of no importance.

Comparison and Statistical Analysis of Sinking Rates

The acumulative diet return represents the raw data collected for the experimental trial. All raw data were plotted on an x y scatter graph for comparison with each other (Figure 2). Each diet had its curve of best fit plotted using the hyperbolic equation: $Y= (a-b)/(l+cx)^{1/d}$ except for Micro Gemma 150 which curve of best fit was best described by the exponential growth equation $y=y_0+a*\exp^{(b*x)+}c*\exp^{(d*x)}$. Results indicate specific sinking rates differences, based on the slope or curve of each of the 11 diets. Comparisons show there are two general types of curves, relating to sinking rates. The firstly general curve has a steep initial incline then develops into a plateau; whist the second general curve has a mild to shallow initial incline throughout. The first general curve is characterised by larger diets (Proton 4, and 3, Gemma 0.3, NRD 4/6 and 5/8, Kinko 1, and Grow Best L3), whilst the second general curve is characterised by smaller diets (MicroGemma 150, and 300, Proton 2, and Kinko 0)

Using SigmaPlot™ to solve x when y was equal to 50% determined the time in minutes for 50% of the diet to settle. The resulting times were then entered into SigmaStat™ and subjected to a Duncan's Multiple Range Test. There are significant levels of similarity ($P < 0.05$) between diets (Fig. 3). For example NRD 4/6 and Kinko 1 have significantly similar sinking rates, which is indicated by the letter 'B' above their corresponding graphs. Alternatively MicroGemma 300 and NRD 5/8, indicated by the letters 'F' and 'A' respectively have significant by dissimilar sinking rate.

Comparison and Statistical Analysis of Leaching Rates

The total amount of each diet leached was entered into SigmaStat™ and also subjected to a Duncan's Multiple Range Test. Due to the large standard errors obtained through experimental procedure, many diets were not significantly different (Fig. 4). For example the sinking rate of the Gemma 0.3 was not significantly different from the Kinko 0, Proton 4, NRD 4/6, and Grow Best L3, indicted by letters 'A', 'B', 'C', and 'D' above their corresponding figures (Fig, 3-4). In comparison, the sinking rates of the MicroGemma 300 and NRD 5/8, indicated by the letters 'G' and 'F' respectively were not significantly different.

Sinking and Leaching Trends Based on Diet Size

Both sinking and leaching rates were compiled onto one graph to indicate specific trends, as determined by the logarithmic curve of best fit (Fig. 5). Both sinking and leaching rates undergo logarithmic decay as diet size increases. Corresponding R^2 values for both sinking and leaching rates exponential curves of best fit, indicates a relative high R^2 value for the sinking rate data (0.706) and a relative low R^2 value for leaching rate data (0.289). This indicated that there is a greater affinity of the logarithmic curve of best fit for the sinking rates over leaching rates.

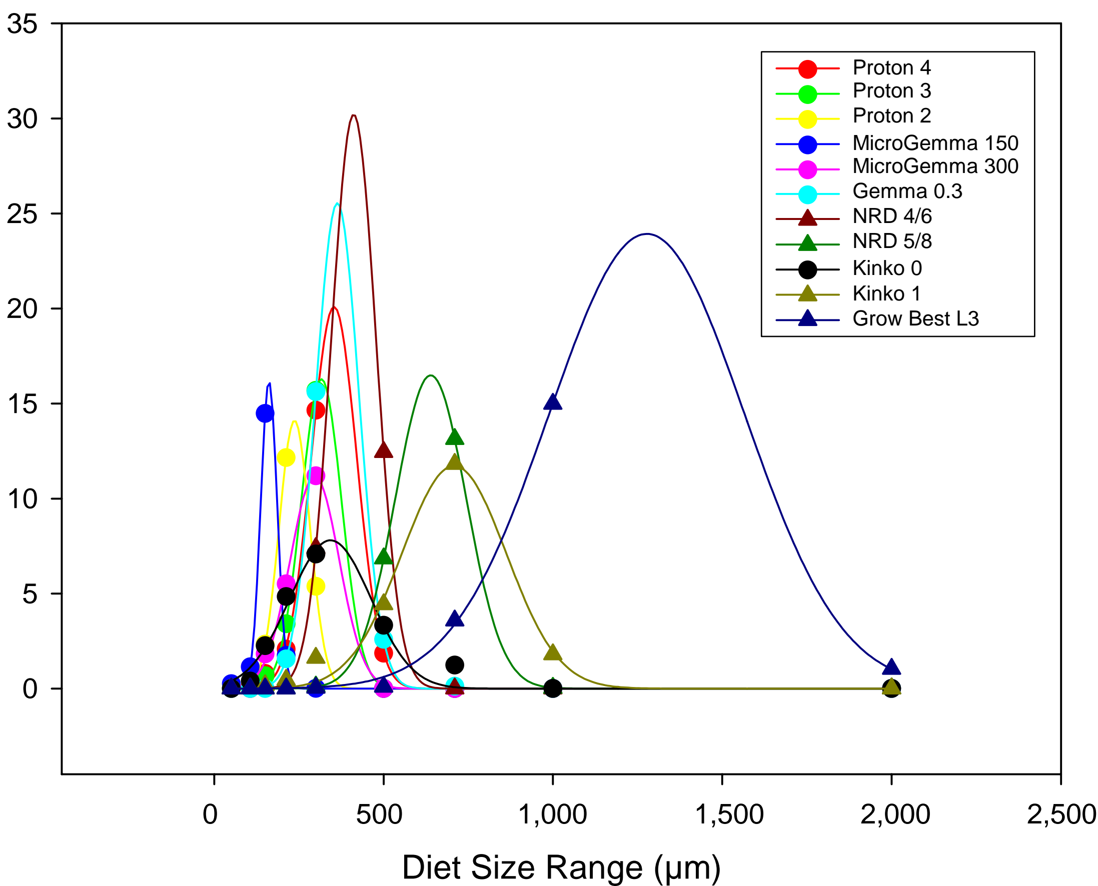

Figure 1. The size range composition of each diet, according to the raw data collected via filtering each diet through 50,106, 150, 212, 300, 500, 710, 1000 and 2000 µm screens.

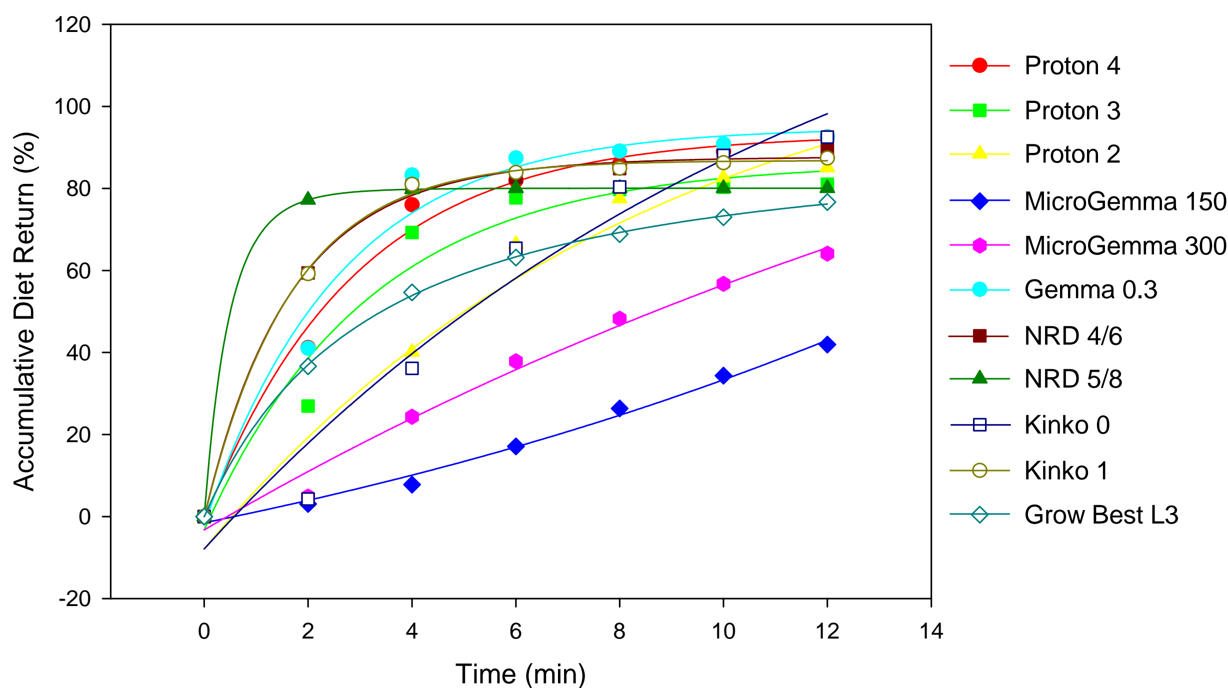

Figure 2. Accumulative raw data of diet return throughout the experiment, with corresponding curves of best fit.

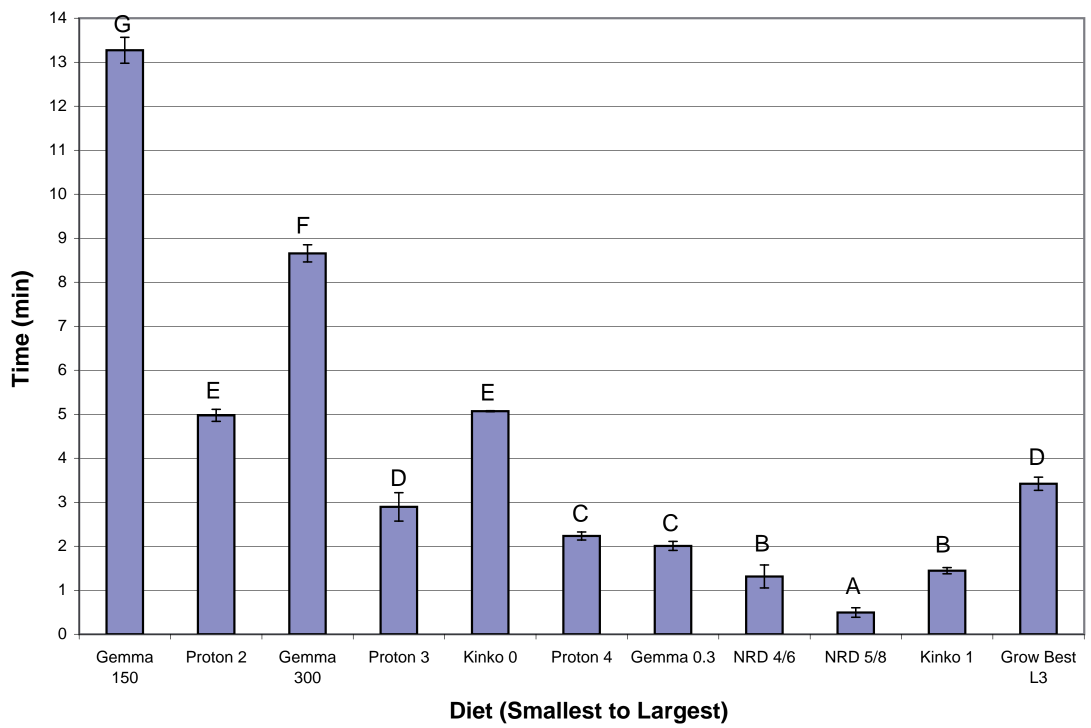

Figure 3. Mean time taken for 50% of each diet to settle, including standard errors. Letters indicate significant similar sinking rates, based on corresponding $P < 0.05$, determined via a Duncan's Multiple Range Test.

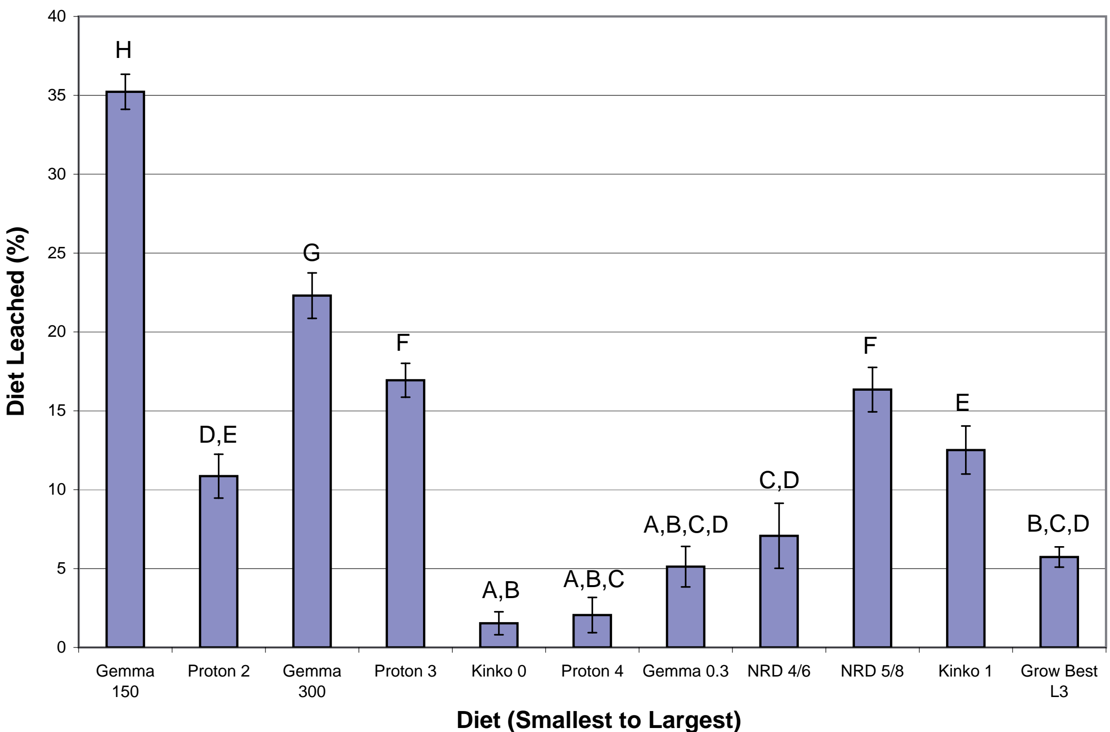

Figure 4. Total percentage of diet leached, including standard errors. Similar letters no significant difference in leaching rates, based on corresponding $P < 0.05$, determined via a Duncan's Multiple Range Test (n=3).

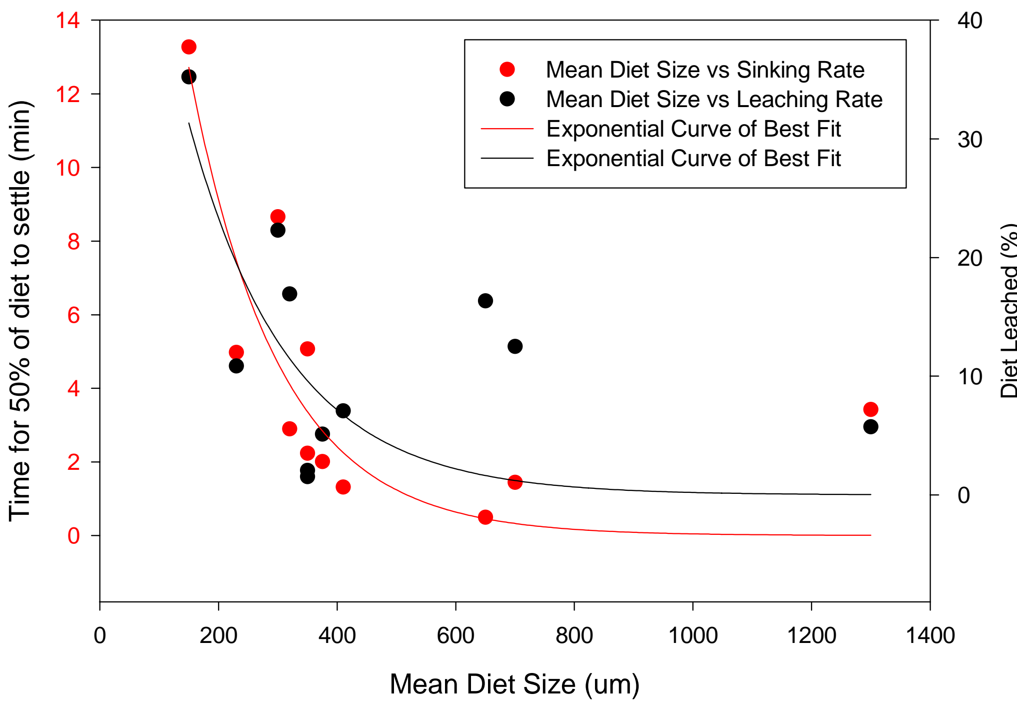

Figure 5. Logarithmic trends of mean diet size versus the time taken for 50% of each diet to settle, and total percentage of diet leached.

5.4.1.2.5 Discussion

Diet Size Analysis

Generally the mean diet sizes and ranges corresponded highly to the manufacturers' specifications, however a few diets, specifically NRD 4/6, Kinko 0, and Kinko 1 exceeded the size ranges claimed by manufacturers. NRD 4/6 was found to have a size range exceeding the manufacturers (INVE) specifications of 400 – 600 µm, specifically a size range of 250 – 550 µm (Figure 1). Possibly this diet may have been broken up during transport and handling, as diet sizes are smaller than manufacturers specifications. Kinko 0 and 1 are claimed to be >210 µm and 210 to 800 µm in size respectively, whilst results indicate sizes ranged from 50 to 600 µm and 250 to 1100 µm in size respectively (Figure 1). This may indicate the manufacturer (Higushimi Maru), has underestimated the sizes of their diets, as it is highly unlikely that diets have become bigger through transport and handling, unless moisture affected.

Sinking Rate Analysis

Generally, most of the data indicate the smaller the particle size of a diet the slower the sinking rate, as indicated by the logarithmic line of best fit (Figure 5), and its correspondingly high R^2 value (0.706). Larger diets (Proton 4, and 3, Gemma 0.3, NRD 4/6 and 5/8, Kinko 1, and Grow Best L3) tend to have a sharp initial incline then plateau, whilst smaller diets (MicroGemma 150, and 300, Proton 2, and Kinko 0), have a mild to shallow incline throughout. This can be attributed to the surface area to volume ratio. Large sized diets have smaller surface area volume ratio, thereby causing diets to move through the water column with less surface friction per unit of diet weight, resulting in a faster sinking diet. Smaller particles have a larger surface to volume ratio than larger particles, thereby causing diets to move through the water column with more surface friction per unit of diet weight, resulting in a slower sinking diet (Alldredge and Gotschalk, 1988).

Stroke's Law of particle settlement accurately estimated the sinking rates, given the radius and density of the particle, and the known density and viscosity of the water (Alldredge and Gotschalk, 1988). As the density and viscosity of the water column did not differ throughout our experiment, if two similar sized diets sank at different rates, it can be concluded that the faster sinking diet was more dense than the slower sinking diet.

Other possible explanations exist to explain differences between diets of similar size sinking faster. Diets do not have an exact diet size, but are represented as a mean diet size with a given size range. Therefore, diets with a higher proportion of smaller particles will have a slower sinking rate, distorting the appearance of a size-sinking rates trend. For Example Proton 4 and Kinko 0 both have mean diet sizes of 350 µm, whilst having size ranges of 200 – 500 and 50 – 600 µm respectively (Figure 1). Kinko 0 contains a percentage of smaller particles compared to Proton 4, therefore one would expect Kinko 0 to take longer to settle even though both have the same mean diet size. This idea is supported by the results obtained in Figure 2, in which Proton 4 has a steeper initial incline compared to Kinko 0 and Figure 3 in which statistical analysis indicates that the two diets are significantly dissimilar.

In the same way that diets with dissimilar sized means and ranges will differ, diets with similar means and ranges show a high level of similarity. For example Proton 4 and Gemma 0.3 both have similar mean diet sizes 350 and 375 µm respectively, and identical size range of 200 – 500 µm (Figure 1). Both of these diets have very similar initial inclines (Fig. 2), and statistical analysis indicates that they are not significantly different.

Leaching Rate Analysis

Leaching rates differed significantly between all 11 microdiets. General trends indicate that leaching rates decrease as diet sizes become larger as indicated by the logarithmic line of best fit (Figure 5). However as the corresponding R^2 value is relatively low (0.289), the confidence of the non-linear regression line becomes reduced. (Therefore, our conclusions are limited due to the unexpected and conflicted results.)

However, the results generally indicate that the smaller the diet size the greater the amount of leaching, as surface area to volume ratio increased. For example Micro Gemma 150, Micro Gemma 300 and Gemma 0.3 which are all manufactured by Skretting, have significantly different size ranges (100 - 225µm, 125 - 450µm and 200 - 500 µm respectively), indicating that leaching increased as diet size decreased (Figure 4). This is supported by the statistical analysis, which indicates a significant difference in ($P < 0.05$). It is also supported by Kolkovski et al. (2004), who found that after 16 minutes, Gemma 150, Gemma 300 and Gemma 0.3 leached 21.48% ± 0.002, 20.28% ± 0.003, and 12.70% ± 0.004 respectively.

This trend in increased rate of leaching with an increase in surface area to volume ratio, is considered to be beneficial to larval finfish. As smaller diets are fed to younger, less well-developed larvae, with less developed visual sensors, which are important in acquiring feed, using a sense of smell may be important. In nutritionally deficient larvae, increased chemical leaching stimulates a feeding response, thereby increasing the nutrition profile of larvae (Kolkovski et al. 1997). As larvae mature there is less reliance on leached chemical cues for the acquisition of feed, and a greater reliance on visual cues, but small larvae also require a nutritionally complete diet so it is important the leaching doesn't affect the nutritional quality of the diet. Therefore, it is important that as diets become larger, leaching rates decline, as high leaching rates can reduce a diets nutritional value (Baskerville-Bridges and Kling*,* 2000).

Other results indicate that the larger the diet the smaller the amount of leaching. For example NRD 4/6 and 5/8 have size ranges of 250 - 550 and 400 - 880 µm respectively. It is thereby expected that 4/6 would have greater leaching than 5/8, although this turned out not to be the case, with 5/8 leaching significantly more than 4/6 (Figure 4). This differs to results obtained by Kolkovski et al. (2004) who determined that after 16 min, 10.53% of 4/6 and 3.30% had leached. Differences in the leaching rates could be due to the differences in methodology. Specifically, in our experiment, diet was sampled every 2 minutes. The samples then stood for several minutes while the previous 2 minute interval samples were filtered in the vacuum flask. Diets that were small took much longer to filter, as a result, the samples of the smaller diets stood for longer before they were filtered. This would have increased the time available for leaching.

In contrast, Kolkovski et al. (2004) did not do interval sampling. Instead, they sampled after a period of time and then filtered the water to retain the diet. For example, the diet would be placed in the water and at 4 minutes would be filtered. Because they had only one sample at a time, samples were filtered much sooner, resulting in less time for leaching. Other possible explanations may result from different manufacturing processes, as Skretting manufactures both diets, it would be assumed that both contain identical dietary compositions, and therefore that leaching rates would differ based on diet size alone. In this case we would expect 4/6 to have leached more than 5/8.

Differences in dietary composition between diets may affect physical properties. For example, Micro Gemma 300 and Proton 3 have similar mean diet sizes (300 and 320 µm respectively) and similar size ranges (125 – 450 and 180 – 450 µm respectively). It could be assumed if both diets were manufactured with identical dietary compositions that both would have significantly similar leaching rates, however, this study found statistically dissimilar leaching rates (P value > 0.05).

This is supported by Kolkovski et al. (2004) who determined that after 16 min, 20.38% of Micro Gemma 300 and 11.01% of Proton 3 leached. The most likely reason for these differences would be the binder composition. The diet manufactures do not release specific details of the composition of binders, therefore we were unable to prove or disprove this theory.

Feeding Recommendations

It is suggested that different feeding methods be adopted for each diet to maximise ingestion whilst minimising waste. As NRD 5/8 was the quickest diet to settle, it is therefore recommended that instead of the total amount of diet being administered in one dose, that it be administered in five to ten aliquots, every 0.5 minutes. NRD 4/6 and Kinko 1 may be administered as aliquots approximately every 1.5 minutes, Proton 4 and Gemma 0.3 approximately every 2.0 minutes, Proton 3 and Grow Best L3 approximately every 3.0 minutes, Proton 2 and Kinko 0 approximately every 5.0 minutes, Gemma 300 approximately every 8.5 minutes, and finally Gemma 150 should be administered approximately every 13.0 minutes. These recommendations are based on results indicating the time taken for 50% of each diet to settle.

The size of the diet must be in relation to mouth gap and not the length of the larvae (Qin and Hillier, 2000). For example NRD 5/8 is fed to pink snapper (*Pagrus auratus*) larvae when they are approximately 15 mm total length (Partridge et al. 2002). Pink snapper larvae of this size have developed a swimming ability that allows them to quickly seize food. Thus larvae of this size may be able to utilise the quick sinking diets. The actual rate of addition of aliquots should be based on direct observation of the feeding response of the larvae.

5.4.1.2.6 Conclusion

Generally, the smaller the particle size, the slower the sinking rate and greater the leaching rate and vice versa. Settling rates are related to the mean size, size range and the density, whilst leaching rates are related to mean size, size range and binder composition. While the larger particles sink faster, they are designed for larger larvae with greater swimming ability and thus greater ability to access the faster sinking food. It may be that the faster sinking rates of the larger diets do not decrease the availability of the diet, because of the corresponding increase in swimming ability of the larger larvae. Too much leaching will decrease the nutritional value of a diet, whilst not enough leaching may reduce the feeding as it is known to stimulate a feeding response. In a commercial setting, with a great number of potential variables, staff should directly observe the feeding response of larvae, to ensure optimum ingestion while minimising wastage.

5.4.1.2.7 Acknowledgments

We would like to thank Dr Sagiv Kolkovski, John Curnow and Justin King, of the Department of Fisheries, Western Australia for their help with the experimental design and set up of the system. Data from this experiment are to be used by Dr. Kolkovski to complement his experiments on the leaching of free amino acids from microparticulate diets. Thanks to Dr. Jill Woodworth and Gavin Partridge for advice on statistical analysis, and the rest of the staff at the Aquaculture Development Unit (ADU).

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5.4.1.3 Delivering bioactive compounds to fish larvae using microencapsulated diets

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5.4.1.3.1 Abstract

The efficient delivery of nutrients and hormones has special relevance to the development of rearing technologies for fish larvae and juveniles. The main aim is to find an effective and measurable way to administer them into the body of small aquatic animals. In this study, three different compounds (hormones, amino acids, and vitamins) were incorporated into protein-walled microencapsulated diets. Specifically these microencapsulated diets were examined for (a) the kinetics of incorporation of estradiol in *Sparus aurata* larvae, (b) absorption and leaching patterns of the free amino acids (FAA), and (c) growth results and tissue incorporation of vitamins in relation to the supplementation of vitamin C in larvae of *S. aurata* and *Solea senegalensis*. The efficiency of inclusion was relatively low, but the capsules were able to retain enough of these compounds when immersed

in water and to deliver them into the digestive tract of the larvae. There are noticeable differences among the nominal amount of a given substance in the ingredient mixture, the actual amount in the microparticle and the amount delivered in the larval gut. It is therefore necessary to examine carefully whether the ingredient is reaching the digestive tract for achieving suitable conclusions in nutritional studies. These results indicate the applicability of these microencapsulated particles in nutritional studies of small aquatic animals.

5.4.1.3.2 Introduction

Nutrient supplements, hormones and therapeutic agents have special relevance in the development of rearing technologies for fish larvae and juveniles. The main question to solve when working with these substances has been to find an effective way to deliver them into the body of small aquatic animals. The most obvious method seems to be the incorporation of such compounds in the food and to administer them with the regular feed as proven in research on juveniles. However, it has become increasingly clear that the feeding and dietary needs, both quantitatively and qualitatively, of growing larvae are distinctly different from those of juveniles and adult fish. In general, this stems from different feeding behaviour and diet preferences but also from the fact that during the first weeks of life, the digestive tract is still developing and is changing its function. This results in insufficient digestion and assimilation of nutrients provided in dry microdiets. It is, therefore, necessary that food particles are stable in water but digestible by the poorly developed gut of early larval fish.

The microencapsulated food particles manufactured by polymerisation of dietary protein meet such prerequisites (Yufera et al. 2000) and offer the possibility of controlling the leaching of molecules (such as amino acids and vitamins) while preventing leaching of important soluble nutrients (Jones et al. 1987). Therefore, these microencapsulated particles can be used as a delivery vehicle that can carry different specific substances.

The inclusion of a new compound with a specific function in the food particle is obviously searching for a positive response in larval growth and development. This requires a capsule that retains nutrients and other encapsulated compounds until the capsule is ingested by the larvae and releases its content completely in the larval gut. Nevertheless, there are many potential causes of lack of the expected response (Table 1). Such constraints may occur during microparticle preparation, during water immersion, during digestion, and finally, during growth experiments with larvae. Checking all these steps and possibilities, summarised in Table 1, is laborious and the level and degree in which each compound can be examined will vary in each case depending on the characteristics of the substance, experimental facilities and previous knowledge. In any case, possibilities of a given substance to be analyzed are restricted to its determination in the food particle, in the rearing water and in the larval tissues.

Table 1. Summary of main causes that could prevent a positive response in larvae of a given fish species when testing a new ingredient in the microdiet formulation.

Particle manufacturation	ingredient not included ingredient included but altered
Water residence period	ingredient lost by leaching particle not ingested by – poor availability in water column – bad taste
Ingestion and assimilation	particle not digested particle digested but ingredient destroyed
Rearing experiment	inadequate ingredient concentration deficiency in other nutrient no response is achieved with such ingredient

The objective of this study was to use ‘‘state-of-the-art’’ encapsulation technology to encapsulate the complex mixture of nutrients required in a suitable larval diet. We have evaluated the use of encapsulated diets as a carrier for three different substances: steroid hormones, free amino acids and vitamins. These three examples were examined at different levels. Specifically, we (1) evaluated the use of encapsulated diets as a carrier for estradiol and the kinetics of incorporation of this hormone in fish larvae, (2) we analyzed the incorporation and losses by leaching of free lysine in microencapsulated diets, (3) we evaluated the use of encapsulated diets as a carrier for vitamin C and its biological value in fish larvae. The aim of this heterogeneous series of experiments is to illustrate possibilities and constraints of protein-walled microcapsules in developing a complete microdiet for live prey substitution in the rearing of larval fish. The other aim is to point out a variety of problems and gaps that need to be solved when testing a complete formulation in feeding experiments with larval fish.

5.4.1.3.3 Materials and methods

The microencapsulated diet was manufactured by means of a procedure based on cross-linking of dietary protein (Yufera et al. 1999). Basically, the procedure consists of the emulsification of a dietary aqueous solution in cyclohexane and then the addition of the cross-linking agent (trimesoyl chloride). The microcapsules formed were allowed to settle and the cyclohexane was removed, and then the paste of capsules was dispersed with gelatine and repeatedly washed in order to remove debris. Finally, the microcapsules were freeze-dried and stored. When immersed in water, the rehydrated capsules showed a rounded shape (Fig. 1) and exhibited a long permanence in the water column. The composition of the experimental diets is shown in Table 2. The basal formulation (diet #1) corresponds to prototype PT2 in the work of Yufera et al. (2000). Three different compounds were tested: 17h-estradiol, lysine, and ascorbic acid and as explained above, each compound has been examined at different level.

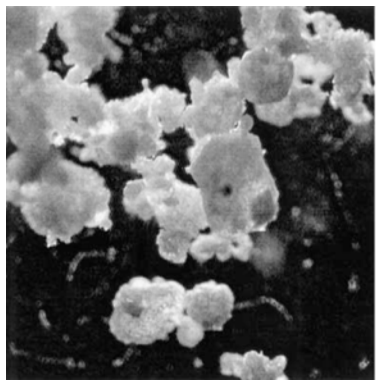
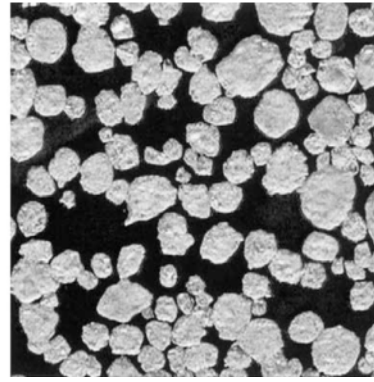

Figure 1. Microphotographs showing the protein-walled microcapsules before and after rehydration.

Table 2. Diet ingredients in the different experimental microcapsules (g kg $^{-1}$).

Batch	1	2	3	4	5
Casein	500	450	500	500	500
Cephalopod meal	100	100	100	100	100
Krill hydrolysate (dry)	120	120	–	120	120
Dextrin (type I)	60	60	60	60	60
Fish oil	120	–	120	120	120
Copepod oil	–	120	–	–	–
Soy lecithin	30	30	30	30	30
Vitamin complex[a]	50	50	50	40	–
Premix C[b]	20	–	20	–	–
Vitamin C (mono/ polyph-)	–	20	–	30	70
Lysine	–	–	120	–	–
Estradiol	–	50	–	–	–

Batch 1: basic diet; Batch 2: diet with 17h-estradiol (Sigma E8875); Batch 3: diet with Lysine (L-Lysine monohydrochoride, M.W. = 182.6; ICN 102218); Batch 4: diet with 3% vitamin C (ascorbyl-2-mono-or polyphosphate; Showa-Denko, Shoko); Batch 5: diet with 7% vitamin C (ascorbyl-2-mono-or polyphosphate; Showa-Denko, Shoko).

[a] Kurios, Polivitamin fish complex (mg/ml complex): vitamin B1 3.75; vitamin B2 1.5; vitamin B6 1.25; vitamin B511.25; vitamin B311.25; methionine 1; choline 10; vitamin B12 0.01; vitamin C 5; vitamin A (10 000 UI ml ^{1}); vitamin D3 (7500 UI ml ^{1}); vitamin E 7.5; vitamin K3 0.75.

[b] Dibaq (80 mg vitamin C/g premix).

Two marine fish species of high importance in the Mediterranean aquaculture, the gilthead seabream (*Sparus aurata* L.) and the Senegal sole (*Solea senegalensis* Kaup), were used in the experiments with larvae.

Assays with 17h-estradiol (E2) were carried out on 33-day-old *S. aurata* larvae. The larvae were fed for 4 h with capsules which included estradiol (MC-E2; diet #2; Table 2). Then, the inert diet

was replaced by live food (*Artemia* nauplii) in order to eliminate the estradiol from the digestive tract. Samples were taken at times 0, 1, 2.5, 4 (just before adding live food), 6, 8, 24, 28 and 52 h. The E2 concentration in larval tissue was determined by RIA according to the methods described by Ottobre et al. (1989) following extraction with ethyl ether. Larvae were sonicated in 1 N NaOH prior to the extraction (Feist and Schreck, 1990). Standard and [2,4,6,7,16,17-^{3}H] estradiol (141 Ci mmol $^{-1}$) were purchased from ICN Pharmaceutical (Costa Mesa, CA, USA) and New England Nuclear Life Science Products (Boston, MA, USA), respectively. The E2 antiserum was provided by Dr. R.L. Butcher (West Virginia University, WV, USA). The characteristics of the antiserum have been reported previously (Butcher et al. 1974).

Assays for evaluating free lysine leaching were carried out by immersing particles with lysine (MC-L, diet #3) in distilled water, and then water samples (two replicates per sample) were taken at 1, 2, 5, 15, 30 and 60 min. The water was filtered through 0.25-Am filters (Millipore HV) twice and analyzed for free amino acids (FAA) using HPLC (Shimadzu with Lichrosphere RP-8 select-B column by MERCK). FAA determination was based on the work of Bidlingmeyer et al. (1987). Retention efficiency was expressed as the percentage of the total FAA amount in the diet. To determine the total amount of FAA in MC-L diet (diet #3) and control diet (MC, diet #1) diet, 15 mg of each diet was mixed with 30 ml distilled water, homogenated (Ultra Turax homogenizer) for 15 min and then sonicated for another 10 min. After that, the same procedure as in the water sample was followed.

To examine the incorporation of vitamin C, two products were used: ascorbyl-2-monophosphate (AMP), with 41% of ascorbic acid (AA) equivalent, and ascorbyl-2-polyphosphate (APP) with 35% AA equivalent. Microcapsules were prepared by adding 3% or 7% (w/w, diets #4 and #5, respectively; Table 2) of the source of vitamin C, AMP or APP. Ascorbyl phosphates in the experimental diets were analyzed by HPLC (ShowaDenko, 1989). Ascorbic acid concentrations in fish tissues were determined colorimetrically using the dinitrophenylhydrazine (Dabrowski and Hinterleitner, 1989).

1.0 g of ground diet was weighed and 10 – 50 volumes were added of extracting solvent (1.0% metaphosphoric acid solution) depending on the expected concentrations, and then homogenized on the ice for 1 min. The homogenate was then centrifuged at 500 g for 10 min. The supernatant was taken and filtered with a syringe filter (0.45 Am, Whatman, Clifton, NJ, USA) before injection to HPLC. The prepared sample was analyzed immediately. The HPLC system consisted of a Beckman 506A solvent delivery system equipped with a 20-Al injection loop connected to a 4.6-150-mm NH2 column (Shodex J-411, Showa-Denko, Shoko, Tokyo, Japan) packed with aminopropyl-bonded silica gel (5 Am), and a UV detector (Programmable detector module 166, Beckman Instruments, San Ramon, CA, USA) set at 257 nm. The mobile phase was 0.07 M KH2PO4. Flow rate was set at 0.7 ml/min, and the retention time for the ascorbyl phosphate was 6.2 min. Vitamin E (a-tocopherol) in feeds (rotifers, *Artemia* nauplii, diets #1 and #4, respectively) and fish tissues (*S.senegalensis*) were also analyzed using HPLC coupled to fluorescence detection according to Cort et al. (1983) and Zaspel and Csallany (1983) with modification by Moreau et al. (1999).

The experiments to detect a response in relation to ascorbic acid (AMP) supplementation in fish larvae were performed with larvae of *S. aurata* and *S. senegalensis*. Experiments with *S.aurata* were carried out on 8-day-old (DAH) larvae throughout day 25, using six different feeding regimes: (A) control larvae fed with rotifers (10 rot ml $^{-1}$) and *Artemia* nauplii (1 nauplius ml^{-1}) from 15 DAH; (B) larvae fed exclusively with microcapsules without AMP; (C) larvae fed exclusively with microcapsules with AMP; (D) larvae fed with microcapsules without AMP+ 1 rotifer ml^{-1}; (E) larvae fed with microcapsules with AMP+ 1 rotifer ml^{-1}; and (F) larvae fed on 1 rotifer ml^{-1}. Larvae were reared in 300-l tanks with flow-through water circulation. Experiments with *S. senegalensis* were carried out with 13 DAH larvae. Three different feeding regimes were used

until day 29: (A) control larvae fed with *Artemia* nauplii (2 nauplii ml^{-1}); (B) larvae fed exclusively with microcapsules without AMP; and (C) larvae fed exclusively with microcapsules including AMP. Larvae were reared in 40-l tanks provided with a water recirculation system, at 20°C. Two replicates of each treatment were performed.

Larvae for analyses were sampled periodically during the experimental period and anaesthetised with 200 ppm of ethyl-4-amino-benzoate. Body dry mass was determined by drying samples of about 15 – 40 larvae at 85°C to constant weight. Growth rate (G) was calculated as the slope of the exponential regression of larval body dry mass on time. Larval density in the rearing tanks was estimated on days 8 and 25 in *S. aurata* by counting ten 250 ml samples, and on days 13 and 20 in *S. senegalensis* by counting all larvae in the tanks. Total biomass production has been estimated from the total increase in population dry weight per volume unit during the experimental time.

Data from the different treatments were compared using one-way ANOVA followed by Newman – Keuls multiple range. Growth rates were compared using Student's t-test for slope comparison. Before the statistical analyses, the dry weight data were log-transformed and percentage values were arcsine transformed.

5.4.1.3.4 Results

Hormones

Fig. 2 shows the kinetics of incorporation of 17h-estradiol in the larval tissue. No E2 was detected in larvae before the feeding experiment. E2 content significantly increased during the first hour of feeding on diet #2 up to 67 pg mg^{-1}. Larvae showed a high value in the following 3 h during which they continued feeding on diet #2. Most of the E2 was eliminated in 2 h following the MC-E2 replacement by live food, and the E2 content in the larval tissue decreased to 5 pg mg^{-1} approximately. This remaining content remained in the larval tissues for at least 2 days.

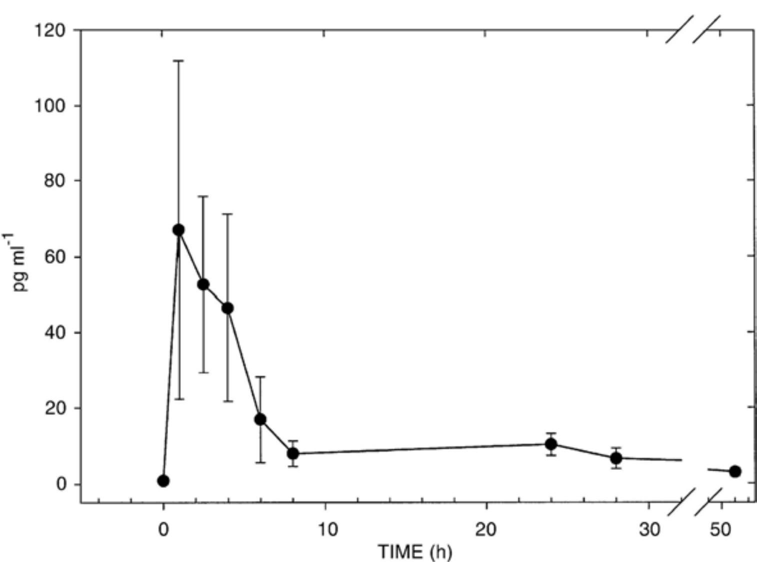

Figure 2. Uptake and release kinetics of 17-estradiol in *S. aurata* larvae fed during 4 h on microcapsules, which included that hormone (MC-E2, diet #2).

Amino acids

The analysis of the microcapsules showed that MC-L diet (diet #3) contained a high amount of free lysine (49.08 nmol mg^{-1}) which accounted for 60% of the total FAA composition in the food particles (Fig. 3). The rest of free amino acids composition comes from the diet ingredients and was similar in diets #1 and #3 although not identical. In the control diet (diet #1), lysine represented

only the 1.1% of total FAA. Total amount of FAA, including the lysine, in MC-L and MC diets were 81.33 and 38.19 nmol mg^{-1}, respectively. Taking into account that the amount of lysine in the ingredients mixture was 12% of total weight matter, the efficiency of encapsulation of lysine was 7.47%.

The leaching experiment showed that 1.5% of the initial amount of lysine included in the MC-L diet was found in the water after 2 min of immersion in water. This amount was kept constant ($P < 0.05$) during the remaining 58 min in water immersion (Fig. 4). Therefore, the MC-L diet retained 98.5% of the free lysine after a 60-min rehydration.

Vitamin C

Results of vitamin C analyses in the microencapsulated diets are shown in Table 3. The amount of vitamin C incorporated after manufacturation of the microcapsules was low, but proportional to the initial amounts supplied in the ingredient mixture (ca. 3%), while no ascorbate was detected in the control diet with premix C (diet #1). On the other hand, the live food showed values of the same order of magnitude as those observed in the formulated diets.

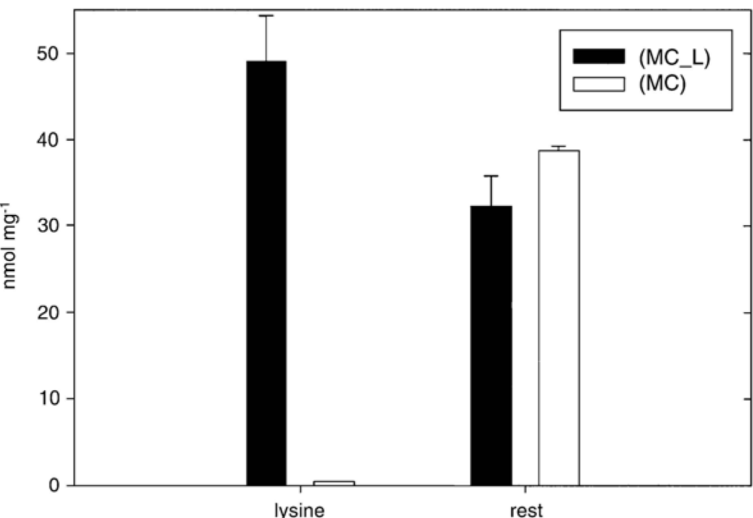

Figure 3. Amount of free lysine in comparison with the rest of free amino acids in microencapsulated diets with (MC-L, diet #3) and without (MC, diet #1) supplementation of lysine.

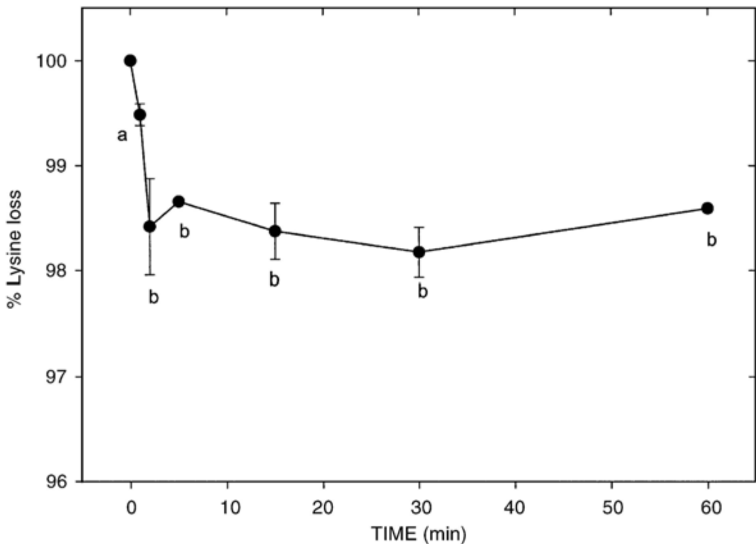

Figure 4. Leaching pattern of free lysine from microencapsulated diet (MC-L, diet #3) after immersion in water. Values with same superscript letter are not significantly different ($P < 0.05$).

Growth performance

Growth experiments with *S. aurata* showed that larvae grew faster and exhibited higher survival in the control tanks (regime A) and in those tanks receiving MC supplemented with a small dose of rotifers (regimes D and E). The larvae fed only a small dose of rotifers showed the poorest survival (Table 4). However, the variability between replicates made the comparison among treatments difficult. The total biomass produced during the experiment showed a clearer figure in the growth pattern (Fig. 5). Thus, when comparing the presence/absence of AMP in capsules, no differences in growth performance were observed. Regarding the total ascorbate content in the larval tissues (Table 5), the control larvae (regime A) showed higher content than in the other treatments. The larvae fed on regimes D and E showed also higher ascorbic acid content than the larvae fed on microcapsules alone (regimes B and C). The supplementation of AMP to the capsules showed no effect in the corresponding content of total ascorbate in larval tissues. Nevertheless, the values of the ratio of dehydroascorbate/total ascorbate (DAA/TAA ratio) were always lower in those treatments with AMP supplementation than in the corresponding treatments without AMP supplementation (Table 5).

Table 3. Amount of ascorbyl-monophosphate (AMP) and ascorbyl-polyphosphate (APP) determined in microencapsulated diets and live prey Diet Vitamin C concentration (Agg^{-1} dry weight) (Agg^{-1} wet weight)

Control diet (diet #1)	Not detected	Not detected
AMP, 3% (diet #4)	273.8	89.8
AMP, 7% (diet #5)	576.8	164.8
APP, 3% (diet #4)	369.8	106.7
APP, 7% (diet #5)	616.3	202.1
Rotifers	1381.7	165.8
Artemia nauplii	448.7	67.3

Concentration in dry diets expressed as ascorbyl-monophosphate equivalent. Values have been converted on the basis of 67% moisture in the microencapsulated diet, 88% in rotifers and 85% in *Artemia* nauplii.

Table 4. Growth rates (G) and survival (S; mean and standard error) of *S. aurata* and *S. senegalensis* under the different feeding regimes.

Regime	A (LP)	B (MC)	C (MC_ AMP)	D (MC + 1R)	E (MC_ AMP + 1R)	F (1R)
S. aurata						
G	0.098a	0.049b	0.070b	0.074ab	0.092a	0.080ab
S	99.0 (1.0)a	48.5 (10.5)ab	38.0 (15.0)ab	70.5 (29.5)ab	63.5 (36.5)ab	2.0 (0.5)b
S.senegalensis						
G	0.129a	0.059b	0.050b			
S	100 (0)a	65.2 (2.2)b	64.4 (8.4)b			

LP: live prey; MC: microcapsules without ascorbate; MCAMP: microcapsules with ascorbate; 1R: 1 rotifer ml^{-1}. Values with same superscript letter are not significantly different ($P < 0.05$).

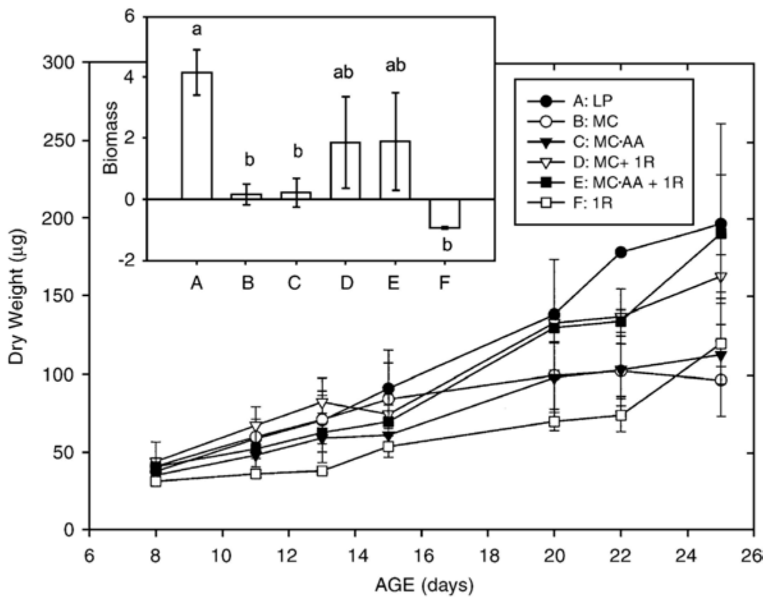

Figure 5. Growth curves and biomass production (g/g of initial dry matter) of *S.aurata* larvae fed different feeding regimes.

Table 5. Ascorbic acid content (Total ascorbate; Ag AA/g wet weight) and DAA/TAA ratio (between brackets) in larvae of *S. aurata* and *S. senegalensis* fed on different feeding regimes

Regime Age (DAH)	A (LP)	B (MC)	C (MC_AMP)	D (MC + 1R)	E (MC_AMP + 1R)
S. aurata					
8	65.0 F1.95 (77.8)	–	–	–	–
15	104.1 F5.56 (55.6)	35.6 F0.70 (84.8)	35.8 F2.07 (74.9)	48.9 F1.04 (58.3)	55.4 F2.16 (56.5)
22	116.8 F0.20 (68.3)	42.5 F1.45 (105.4)	34.3 F1.21 (94.8)	61.8 F2.12 (66.5)	49.5 F0.47 (61.0)
S. senegalensis					
29	70.0 F0.8 (89.2)	51.2 F7.0 (90.3)	45.5 F10.6 (90.2)		

Sample of day 8 on *S. aurata* was pooled among all treatments. LP: live prey (rotifers and *Artemia* nauplii); MC: microcapsules without ascorbate; MC-AMP: microcapsules with ascorbate. 1R: 1 rotifer ml^{-1}.

Experiments with *S. senegalensis* showed that larvae grew when fed exclusively with inert diet from 13 DAH onwards (regimes B and C) but at a lower rate than when fed on live prey (regime A) (Fig. 6, Table 4). The addition of AMP did not affect growth and survival and thus total biomass production in larvae fed on microcapsules (Fig. 6). Total ascorbate content in the larval tissues at the end of the experiment was higher in larvae fed with live food, while no difference was observed between the larvae fed on microencapsulated diet in the presence or absence of AMP in the diet. In this case, no difference was found in the DAA/TAA ratio among the three feeding regimes (Table 5). Live prey showed the highest concentration of vitamin E while the lowest content was observed in the microcapsules supplemented with ascorbic acid (Table 6). Larvae fed with live prey showed a higher vitamin E concentration in their tissues than larvae fed with microcapsules but no significant differences were observed between larvae fed on microcapsules with or without vitamin C supplementation.

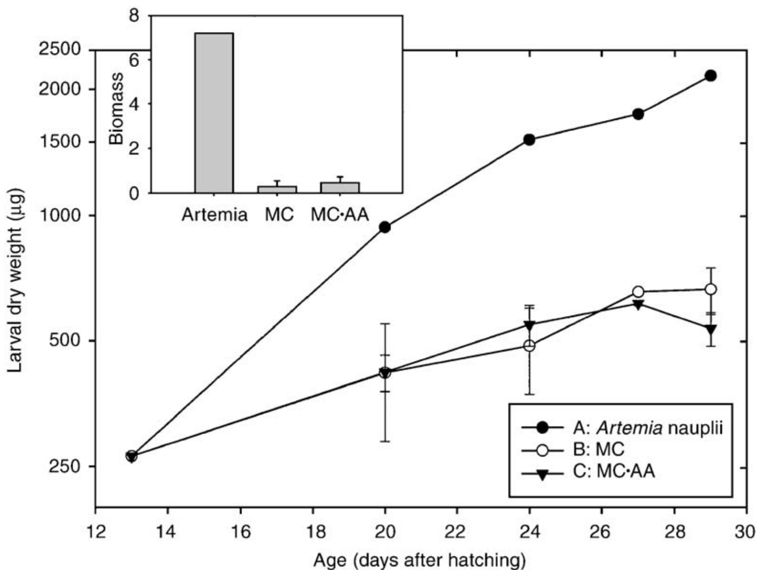

Figure 6. Growth curves and biomass production (g/g of initial dry matter) of *S. senegalensis* larvae fed different feeding regimes.

Table 6. Concentration of a-tocopherol in the live feeds, microencapsulated diets (Agg^{-1} dry matter) and *S. senegalensis* larvae.

Diet a-Tocopherol (Agg^{-1} wet weight)	22.1 F0.8	
Diets Fish		–
Brachionus plicatilis		
Artemia nauplii (regime A)	20.9 F2.0	7.3 F1.8a
MC (regime B)	3.5 F0.1*	3.8 F0.6b
MC_AMP (regime C)	1.7 F0.3*	4.3 F1.6ab

Fish were transferred to dry diets on day 13 and sampled on day 29. Means followed by the same letter are significantly different (P < 0.05).

* Values on microencapsulated diets recalculated on the basis of 67% moisture after rehydration.

5.4.1.3.5 Discussion

In the present study, we tested the microencapsulation technology as developed for larval fish with a basal diet formulation (PT2; Yufera et al. 2000) with no intent to improve encapsulation efficiency of tested compounds. Most technological processes developed in microencapsulation refer to only one substance or type of substances with the same chemical properties (Alexakis et al. 1995; Hildebrand and Tack, 2000; Uddin et al. 2001). The examples examined in this study illustrate different aspects of encapsulating some specific compounds together with a complete diet. Prepared diets include different substances varying in chemical and physical properties, therefore, the modifications in the encapsulation procedure addressed to enhance the encapsulation efficiency of a given compound can affect other diet ingredients.

In the case of estradiol, although the encapsulation efficiency and leaching pattern after immersion in water were not determined, its effective inclusion has been evidenced by the analysis of larval tissues. The kinetics of total amount into the larval body follow the classic pattern of incorporation of a substance via oral. The gut content with estradiol is released in 1– 2 h after replacing the diet by live prey; this is the gut passage time in *S. aurata* larvae with continuous feeding (Yufera et al. 1995). The remaining values (3.5 Agg^{-1} approximately) indicated the amount incorporated into the tissues that decreased progressively in the following 2 days.

Analysis for free amino acids in the capsules showed that lysine is incorporated although at a low efficiency while the analysis of water demonstrated that this compound is effectively retained after rehydration sufficiently long to be eaten by the larvae. The amount of other free amino acids was quite similar in microencapsulated diets with and without lysine supplementation. Therefore, the addition of crystalline lysine in the ingredients mixture practically duplicates the total FAA amount in the microencapsulated diet. There are several studies on the entrapment of free amino acid in microdiets for fish larvae (Lopez-Alvarado et al. 1994; Ozkizilcik and Chu, 1996; Baskerville-Bridges and Kling, 2000). The best retention, similar to that found in the present study, was obtained with lipid-walled microspheres (Lopez-Alvarado et al. 1994; Ozkizilcik and Chu, 1996). Nevertheless, the leaching rate strongly depends on the experimental conditions and comparison among studies is only indicative. Yufera et al. (2002) in a more complete study on the free amino acid leaching of this type of protein-walled microcapsule found that losses in water of each specific amino acid are highly correlated with the hydropathy index. No analyses in larval tissue were done and no larval physiological response has been examined in this case. Labelled FAA are necessary to discriminate whether a given amino acid is assimilated by the larvae (Ronnestad et al. 2000).

Experiments with vitamin C showed that both ascorbyl monophosphate and polyphosphate can be incorporated into the microcapsules. This incorporation occurred in proportion to the amount supplied in the initial mixture of ingredients, but in a low percentage in relation to the total amount supplied. Analysis of larval tissues in *S. aurata* showed an interesting relationship when the DHAA/TAA ratio was measured. Lower values are caused firstly by the presence of live food and secondly by the presence of AMP in the capsules. The fact that 22 DAH control larvae show similar or even a little higher DAA/TAA ratio than larvae receiving microcapsules plus rotifers could be due to the much lower TAA content in *Artemia* nauplii than in rotifers (Table 3). These results indicate that at least part of the supplemented ascorbate has been retained until the ingestion of the particles. According to Dabrowski (1990), higher values of the DAA/TAA ratio would indicate a deficiency in vitamin C.

The experimental results gathered to detect physiological responses in larvae to the ascorbate supplementation were not conclusive and although some effect on growth rate can be assumed in *S. aurata*, no differences in total biomass production were observed in either *S. aurataor S. senegalensis*. In this case, incorporation of nutrients in capsules after manufacture and incorporation in tissues and growth response have been examined. However, we did not address the leaching pattern into

the water before ingestion. Leaching of water soluble vitamins is high, and different protecting procedures have been studied. Marchetti et al. (1999) found a loss of only 14% ascorbic acid after 60 min of water immersion of fish feeds using fat-coated vitamins. Uddin et al. (2001) reported the retention capacity of ascorbic acid depending on the encapsulation method for human foods. These authors reported that unprotected ascorbic acid dissolves in water in 20 s and the best retention (50% in 60 min of water immersion at 30°C) was obtained with carnauba wax microspheres. The lack of a clear growth response in the present study with larval fish could therefore be due to an insufficient dosage in the capsules. In addition, the lack of other nutrients such as vitamin E may prevent the demonstration of the ascorbic acid effect. Another possibility is that vitamin C was not the limiting factor for growth in the present rearing conditions. This could be the case in *S. senegalensis* larvae in which the DHAA/TAA ratio was the same under all feeding regimes. No toxic effect seems to be involved because larvae of both fish species were able to continue growing during at least 2 weeks of feeding on microencapsulated diets. Loss of tocopherol acetate (vitamin mixture) from diets must have been considerable during microcapsule manufacturing with polar solvent as only a-tocopherol was found after processing. The level found in the diets was several times less than the level found in live prey. This amount corresponds to the quantities of a-tocopherol found in rotifers enriched with fish oil (49.3 Agg^{-1}) but much higher than in rotifers fed frozen algae (12 – 15 Agg^{-1}) (Zheng et al. 1997). The amount of tocopherol found in fish larvae indicates severe deficiency if comparison with salmon juveniles holds true (Hamre et al. 1997). As indicated in first feeding salmon, vitamin E deficiency significantly accelerates mortality due to the lack or deficiency of ascorbic acid. In conclusion, vitamin C protection against vitamin E deficiency in a dose-dependent manner may be at play in the present experiment with larval fish microencapsulated diets.

All three examples yield only preliminary results but they highlighted some methodological aspects that merit special attention. There are noticeable differences among the nominal amount of a given ingredient in the ingredient mixture, the actual amount in the microparticle and the amount delivered in the larval gut. It is therefore necessary to examine carefully whether the ingredient is reaching the digestive tract for achieving suitable conclusions in nutritional studies. Other different compounds with potential interesting roles in developing larval fish, such as enzymes, neuropentides, immunostimulants, antigens, etc. (Cahu and Zambonino Infante, 2000; Kolkovski, 2000) will require similar protocols to evaluate their incorporation into the microdiet as well as the corresponding physiological response in larvae. It is interesting to remark that this physiological response could vary among species. Encapsulation efficiency of each given substance depends evidently on the intrinsic physical and chemical characteristics of both the substance and the particles, but also on the manufacturating procedure. On the other hand, the leaching rate during water immersion depends largely on environmental conditions such as temperature, pH, particle diameter and solute concentration. Some parameters during manufacture can be modified to improve the final result regarding an individual substance but special attention should be paid because the changes could affect other substances.

Recent studies on microdiets for larval fish showed progressive advances in the possibilities and results for complete live prey replacement (Cahu et al. 1999; Fontagne et al. 2000; Lazo et al. 2000; Yufera et al. 2000). Such advances are possible because of the progress in both microparticulation technology and larval nutrition. The capsules of polymerisation of dietary protein can include very different compounds and deliver them into the larval gut. They could allow the encapsulation of a complete diet with the appropriate proportion of macro-and micro-nutrients in a single particle. To advance in such direction, more research effort should be addressed towards the adjustments in manufacturation to improve the encapsulation efficiency in each particular case and to reach the desired level that allows testing larval response properly.

5.4.1.3.6 Acknowledgements

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5.4.1.4 Food microparticles for larval fish prepared by internal gelation

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5.4.1.4.1 Abstract

A novel experimental microdiet for larval fish was prepared using a modification of the internal gelation microencapsulation procedure. In these microspheres, a complete formulated diet is entrapped in a matrix of Ca-alginate. When compared with our previous methodology of polymerisation of dietary protein, this procedure has the advantage of using more environmentally friendly reagents at a lower cost: vegetable oil and acetic acid vs. cyclohexane and trimesic acid. In addition, it allows a more balanced formulation because no high proportion of pure protein is required. These features represent an advantage for a potential upscaling of this microdiet to pilot and industrial level. The present study describes the preparation procedure and the basic characteristics of these food particles. In addition, the microdiet was tested with fish larvae. Growth and developmental performance of *Sparus aurata* larvae obtained at 15 days post hatching (dph) fed this microdiet were similar to those previously obtained with microcapsules prepared by crosslinking of dietary protein. *Solea senegalensis* larvae were able to reach 400 µg dry weight when fed exclusively on alginate microdiet from 7 dph up to 30 dph.

5.4.1.4.2 Introduction

The search for a formulated diet able to support growth and development of larval fish from first feeding has a clear aim, to reduce to a minimum the need for live organisms (microalgae, rotifers and *Artemia*) in the hatcheries. Although the first attempts were made in the 1980's, promising growth results have been reported only recently, due to advances in both larval nutrition and microencapsulation technologies (Cahu and Zambonino-Infante, 2001; Langdon, 2003). A primary requisite for mass producing a compound diet for the industry is obviously the reasonable cost of ingredients. Our previous work in microparticulated diets showed that protein crosslinked microcapsules (protein microdiet) are a good tool for research, but high cost and potentially toxic compounds are required in their preparation, namely cyclohexane and trimesic acid trichloride (Yúfera et al. 1999). This constraint could prevent potential scaling-up to a pilot scale. We have therefore explored the procedure of internal gelation with alginate in preparing food microparticles. This process is based on transformation from a sodium alginate solution to gelled calcium alginate. The most common method of producing Ca-alginate microbeads has been external gelation. In this method, the particles are obtained by dropping a Na-alginate solution over a solution of $CaCl_2$, where the gelation reaction occurs from the surface inwards, producing particles with a shell entrapping the core material. The droplet method produces beads above 200 µm with a relatively hard shell that can be inappropriate for the first days of feeding of many fish species. In internal gelation, the source of calcium is already in the initial alginate solution and pH-lowering induces delivery of Ca ions and the consequent gelation reaction. In these microspheres, the core material is entrapped in a sponge-like matrix of Ca-alginate (Poncelet et al. 1992; Chan et al. 2002). The aim of the present paper is to describe our results in designing the food microparticle of Ca-alginate (alginate microdiet) and testing this microdiet in early stages of marine fish larvae.

5.4.1.4.3 Material and methods

Microdiet preparation procedure

The microcapsules were prepared following a modification of the method of internal gelation described by Poncelet et al. (1992). The dietary ingredients (10% w/v) plus calcium citrate (1% w/v)

were dispersed in a sodium alginate solution (1.5% w/v). Two parts of this solution were emulsified in five parts of a soy lecithin and vegetable oil solution (2% w/v) with a homogeniser (Ika Ltd, 45mm ‘ship propeller’ stirrer) for 10 min at 1000 rpm at room temperature (approximately 20°C). A solution of acetic acid and vegetable oil (1/1) was then added to the emulsion with continuous stirring and the reaction continued for a further 10 min. The acetic solution represented 4% (v/v) of the total oil volume. The formed microparticles were allowed to settle, and the supernatant oil removed by pouring. The microparticles were then dispersed in a calcium chloride solution (0.5% w/v) (0.035mM) for 5 min and then in a Tween 80 solution (1%) for 5 min. The capsules were then washed with freshwater in order to remove debris and freeze-dried. The ingredient compositions of different microdiets used in particle characterisation and experiments with larvae are shown in Table 1.

Physical and chemical characterisation of particles

All experiments for particle characterisation and larval feeding were carried out using a pool of at least three batches of the corresponding particle type. Characterisation analyses were carried out with microparticles of type MA (Table 1). Size and shape of particles were examined by image analysis system after 120 min of rehydration with seawater. This was time enough to obtain a constant volume. The morphologic analysis was performed using the available free software UTHSCSA ImageTool (University of Texas Health Science Center, San Antonio, TX, and available at http://ddsdx.uthscsa.edu/). The measurements analysed in each particle were the following: a) Feret diameter, computed as $\sqrt{(4.Area/PI)}$, that is the diameter of a circle having the same area as the object; b) Compactness, computed as $\sqrt{(4.Area/PI)}$/Major axis length, that is an index of the sphericity of the particle; c) Roundness, computed as (4.PI.Area)/Perimeter^2, that is an index of the smoothness of the particle surface. Results are given as mean and SD of at least 50 particles. A detailed explanation of these measurements can be found in the ImageTool’s manual (free available at http://ddsdx.uthscsa.edu/).

Size frequency distribution of rehydrated particles was elaborated from the Feret diameter of 130 microcapsules. For a more detailed characterisation, the properties were examined for three diameter classes: <200 µm, 200-400 µm and >400 µm. The particles of each class were separate by sieving the dry microparticles. These three classes were considered taking into account the diameter distribution obtained and the particle size preferences of fish larvae during the first weeks. Swelling degree was calculated as Dt/Di, Dt being the average particle diameter at time t of rehydration and Vi the average diameter of dry particle. Samples of 30 particles were measured periodically during 120 min of immersion in seawater. A two-step saturation model with the formula $Y = a\,(1-e^{-b \cdot X}) + c\,(1-e^{-d \cdot X})$ was used to explain the increase in particle diameter vs. rehydration time.

Dry weight of microparticles was estimated in triplicate by immersing 5 mg of dry particles in 50 ml of water and counting the number of particles in four 1 ml subsamples. Carbon and nitrogen content was examined in three 1 mg subsamples using an elemental analyser (Thermoquest, mod. Flash 1112), using sulphanilamide as standard.

Feeding experiments with larval fish

The microdiet was tested with larvae of *Sparus aurata* and *Solea senegalensis*, two species with different feeding habits and in which we already checked the protein microdiet (Yúfera et al. 1999; 2003). The larvae of both species were reared in 300-l tanks at 19.5°C temperature and 33 ‰ salinity following the rearing methodology explained in Yúfera et al. (2000). Three replicates per treatment were performed in both species using different egg batches.

Three feeding regimes were tested with *S. aurata*: A) Control treatment with rotifers from first feeding at day 4 post-hatching (dph) up to the end of the experiment (15 dph). B) rotifers (10

individuals ml^{-1}) from 4 dph (first feeding) to 8 dph, and alginate microcapsules (MA) from 8 dph onwards. C) MA from 4 dph (first feeding) to the end of the experiment (15 dph).

The feeding regimes tested with *S. senegalensis* were: A) Control treatment with rotifers (10 individuals ml^{-1}) from 3 dph (first feeding) to 6 dph and *Artemia* nauplii (2 individuals ml^{-1}) from 6 dph up to the end of the experiment (30 dph). B) rotifers (10 individuals ml^{-1}) from 3 dph to 6 dph and microcapsules containing 55% protein (MA-55) from 6 dph onwards. C) Rotifers (10 individuals ml^{-1}) from 3 dph to 6 dph and microparticles containing 62% protein (MA-62) from 6 dph onwards. The compositions of MA-55 and MA-62 are shown in Table 1. The final crude protein content (N x 6.25) was established *a posteriori* from the elemental analyses.

In all cases, the microdiet was added six times a day with an automatic feeder. Larvae for analyses were sampled periodically during the experimental period and anaesthetised with 200 mg l^{-1} of ethyl-4-amino-benzoate. Body dry mass was determined by drying samples of about 15-40 larvae at 85°C to constant weight. Growth rate (G) was calculated as the slope of the exponential regression of larval body dry mass against time. In the experiment with *S. senegalensis,* the eye migration status (Fernández-Díaz et al. 2001) was examined periodically. Larval density in the rearing tanks was estimated at the beginning of the experiment by counting ten 250 ml samples and at the end by counting the remaining larvae.

Statistical analyses

Data from the different treatments were compared using one-way ANOVA followed by Newman-Keuls multiple range. Growth rates were compared using Student-t test for slope comparison. Before the statistical analyses, the dry weight data were log-transformed and percentage values were arcsine transformed.

5.4.1.4.4 Results

Physical and chemical properties

The preparation procedure gives round and well-formed particles (Fig 1).

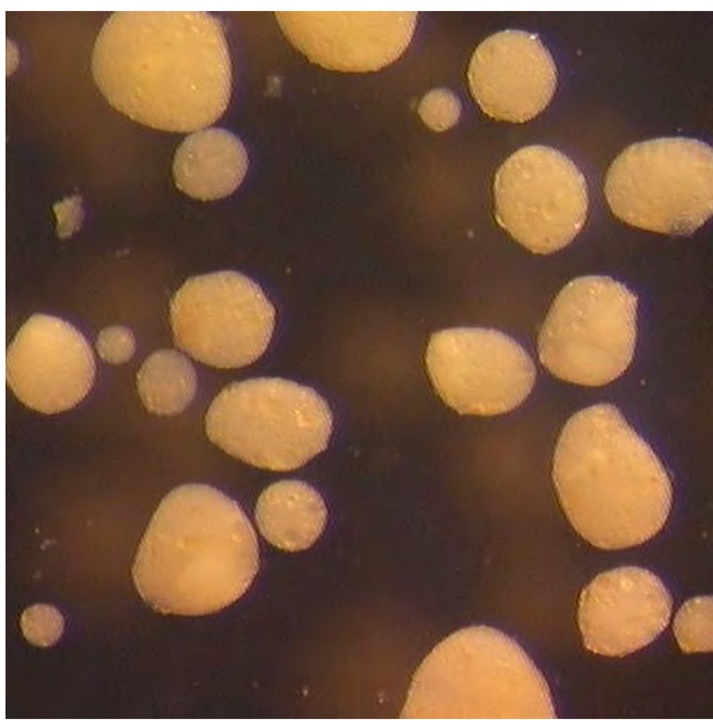

Figure 1. Photomicrograph showing the appearance of food microparticles prepared by internal gelation.

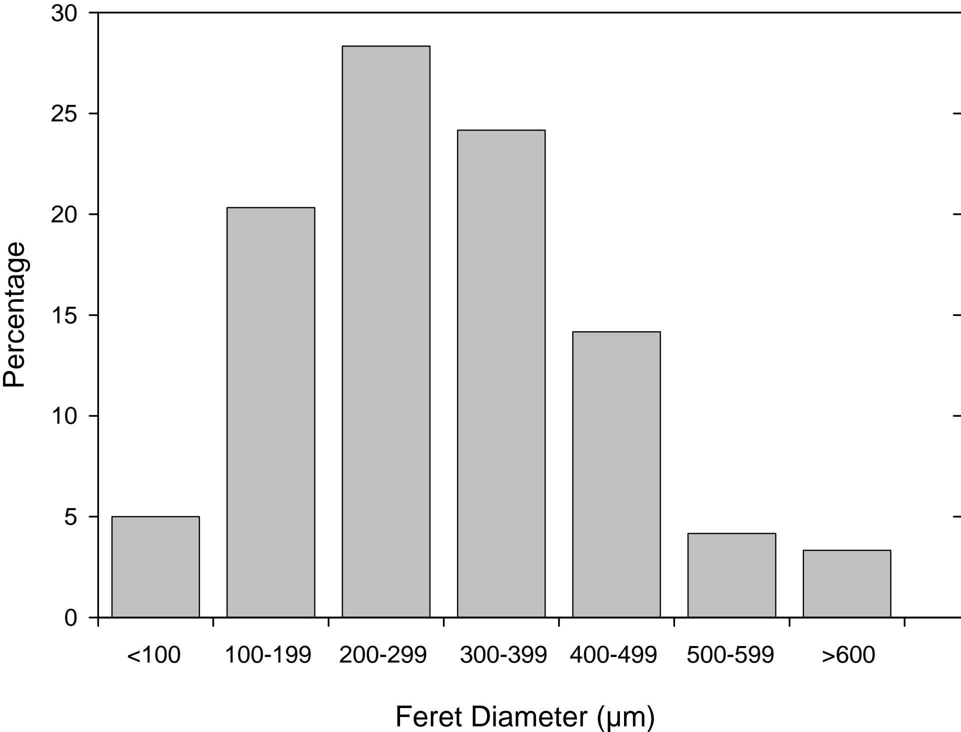

Figure 2. Frequency distribution of Feret diameters of MA.

The diameter distribution of rehydrated particles ranged from 75 to 685 µm (Fig. 2), with 90% between 100 and 499 µm, and 60% between 100 and 299 µm. After rehydration of the capsules, the diameter increased between in 15 and 100% depending on the particle size (Table 2), with greater swelling for particles belonging to the smaller size class.

The particle volume increased mainly during the first minute of rehydration and after 30 min the diameter remained practically unaltered (Fig. 3). Average dry weight of particles in the three size classes ranged from 0.64 to 1.74 µg. The total amount of carbon was approximately 51-52% of the dry mass and the total amount of nitrogen was approximately 8-9% (Table 2). The compactness index was above 0.8 in all cases and the roundness index decreased with particle diameter ($P < 0.05$).

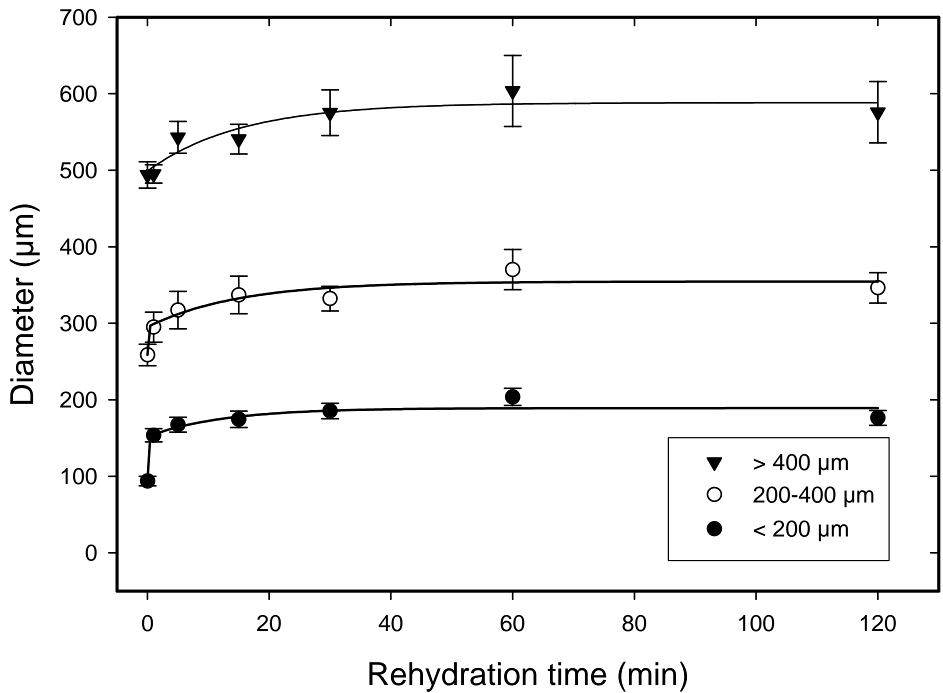

Figure 3. Changes of the microparticles diameter (mean ± SD of 30 particles) after rehydration in the three size classes. A) Diameter < 200 µm: $Y = 151.9 (1-e^{-959 \cdot X}) + 37.3 (1-e^{-0.080 \cdot X})$; r = 0.972. B) Diameter between 200 µm and 400 µm: $Y = 295.1 (1-e^{-2089 \cdot X}) + 59.4 (1-e^{-0.066 \cdot X})$; r = 0.931. C) Diameter > 400 µm: $Y = 499.0 (1-e^{-4577 \cdot X}) + 89.3 (1-e^{-0.065 \cdot X})$; r = 0.945.

Experiments with live fish larvae

Larvae of *S. aurata* ingested and broke down the capsules very well at first feeding. The larvae fed on MA from first feeding exhibited a poor growth. The larvae increased their dry weight from 26.0 µg at 4 dph to 35.6 µg dry weight at 15 dph, but with relatively high survival (57%). Larvae fed MA from 8 dph exhibited a slight lower, but not significantly different (P > 0.05) growth from that of the rotifer-fed treatment (Fig. 4; Table 3) and reached 87.6 µg dry weight. The survival was lower in larvae fed MA from 8 dph (68%) than in larvae fed rotifers (91%) (P < 0.05).

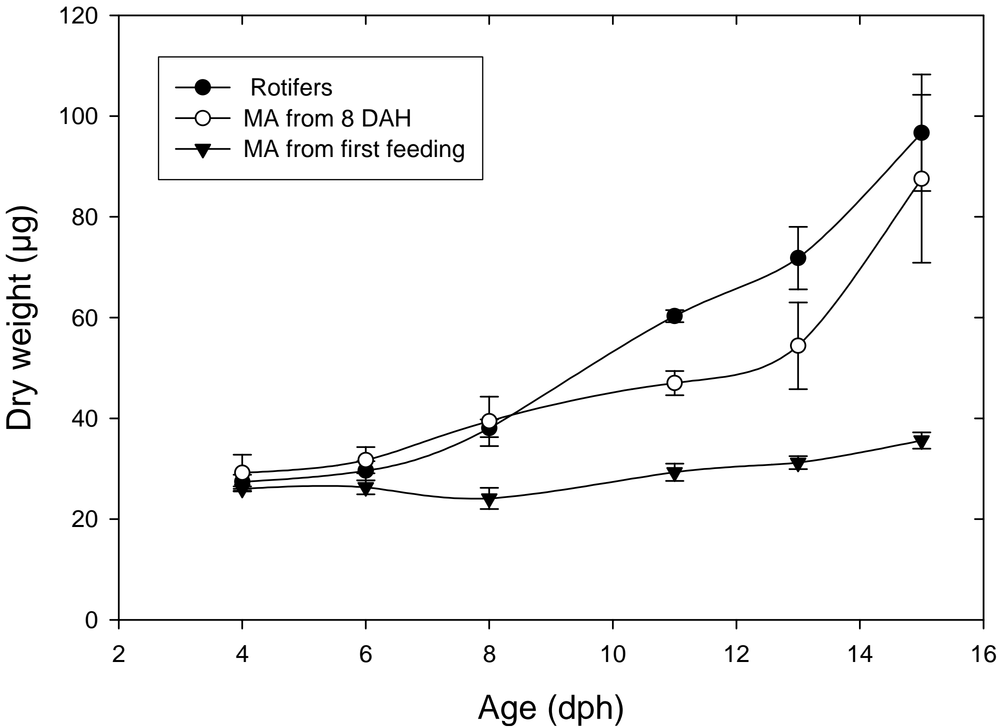

Figure 4. Larval growth of *Sparus aurata* larvae under the different feeding regimes. Values are mean ± SE of three tanks.

Larvae of *S. senegalensis* also ingested and broke down the microparticles very well from the start of the experiment. The larvae fed on microdiet alone from 6 dph continued growing and exhibited eye migration. The growth and survival were higher in *Artemia*-fed control treatment (Fig. 5, Table 3).

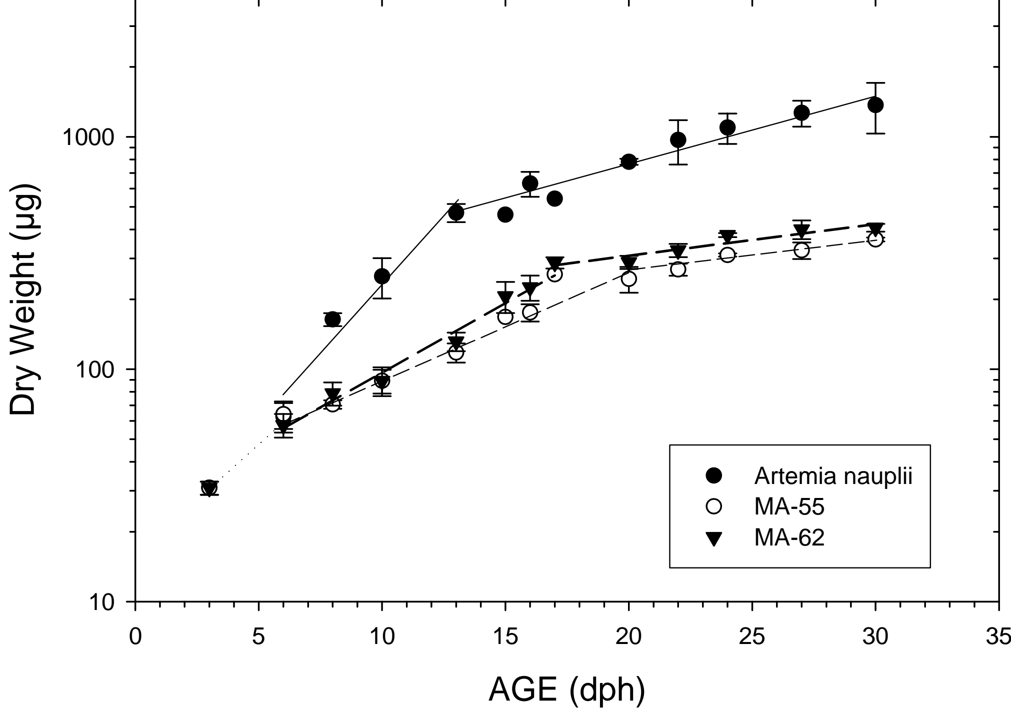

Figure 5. Larval growth of *Solea senegalensis* larvae under the different feeding regimes. Values are mean ± SE of three tanks.

Both groups of larvae fed on alginate microdiets with two different protein contents from 6 dph exhibited similar growth trends. Nevertheless, during the pre-metamorphic stage the growth rate was slightly higher ($P < 0.05$) in larvae fed on MA-62. Consequently, the final dry weight was also higher in larvae fed on MA-62 (406.6 ± 15.8 µg) than on MA-55 (365.0 ± 6.8 µg). Final survival was higher, but not significantly different, in larvae fed on MA-62 than on MA-55 (Table 3). Only part of the larvae fed on microdiet started eye migration at the same age as larvae fed on *Artemia* (Fig. 6a). At the end of the experiment (30 dph), the percentage of larvae having completed eye migration was 100% in larvae fed on *Artemia* nauplii, and 19 and 49% for groups fed on MA-55 and MA-62, respectively (Fig. 6b).

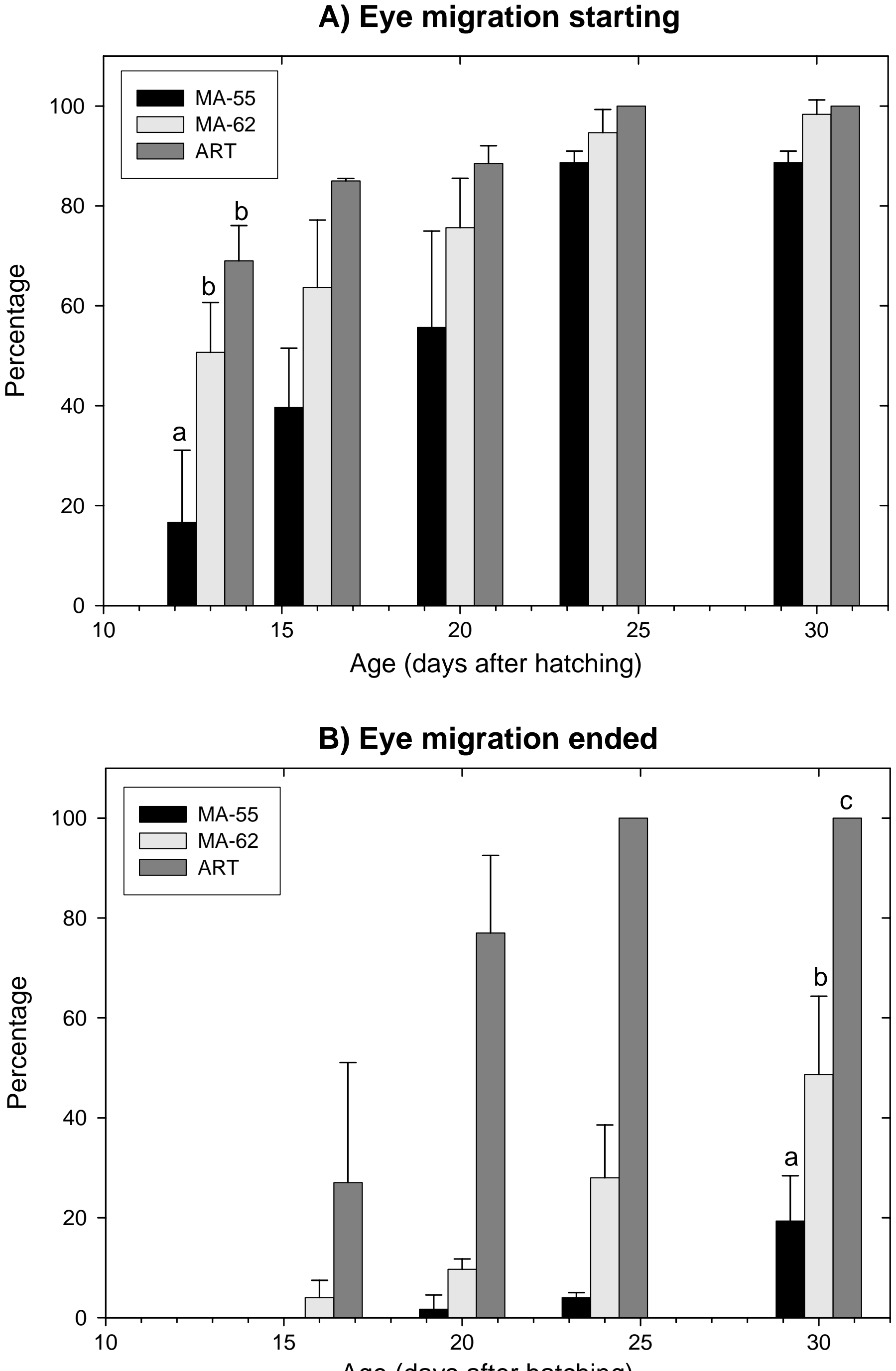

Figure 6. Percentages (mean ± SD of 3 tanks) of *Solea senegalensis* larvae starting (A) and finishing (B) eye migration under the different feeding regimes. For each case, same letters indicate no statistically significant difference.

5.4.1.4.5 Discussion

The methodology described in the present study for preparing the microdiet (Patent ES P200201435) includes two steps. Firstly, an emulsification and internal gelation to produce the microparticles. Secondly, a further hardening of the surface by gelling externally the remaining non-bound alginic monomers. Several basic conditions are required for food microparticles to be used during early larval stages of marine fish: structural stability after rehydration, good buoyancy, appropriate size and palatability to allow a good acceptance and rapid ingestion by larvae. Once ingested, the particles have to be easily broken down in the immature larval gut (Yúfera et al. 2000). All these conditions are satisfied by this alginate microdiet. The usefulness in supporting larval growth and development will depend on diet formulation and the capacity of the microdiet for delivering macro- and micro-nutrients within the gut lumen (Yúfera et al. 2003).

As alginate particles were obtained by emulsification of the dietary solution, the size distribution depends primarily on solute/water ratio of the dietary solution and stirring speed. At 1000 rpm, the diameter range of particles after rehydration resembles that of the plankton organisms to be replaced. Therefore, by adjusting stirring speed as well as sieving, it is possible to satisfy particle size preferences of larval fish during development. The mass and chemical composition (carbon and nitrogen) of the particles were also similar to those of the live prey used in larval rearing (Yúfera et al. 1997; Dhont and Van Stappen, 2003). Nevertheless, the re-hydrated particles above 400 µm had a relative low weight indicating a high amount of empty spaces inside the matrix. The shape of the particles is close to a sphere (compactness above 0.8) and the smoothness of the smaller particles is probably a consequence of higher incorporation of water after rehydration. Overall, all these characteristics indicate that alginate particles do not present any particular flaw to be accepted by the fish larvae.

When comparing the characteristics of alginate microcapsules with protein microcapsules, a remarkable difference is that a high proportion (> 30%) of pure casein is not required; a small amount of alginate (from 5 to 9%) is added instead. Without such a restriction, the alginate microdiet enables us to prepare more balanced formulations. Nevertheless, the main advantage is that the reagents used for the preparation of alginate microdiet (vegetable oil and acetic acid) are cheaper and more environmentally friendly than those used in the preparation of protein-walled microencapsulated diets (cyclohexane and trimesic acid trichloride). The main disadvantage is that Ca-alginate gel is a porous material unable to prevent efficiently the oxidation and degradation by micro-organisms of the encapsulated material in long-term storage. This constraint must be solved before attempting mass production of this methodology in order to obtain an adequate microbial stability and extend the shelf-life of ingredients sensitive to oxidation. Obviously, further hardening of surface will affect both digestibility and retention capacity of soluble compounds. This alginate microdiet exhibited a good break down in the developing guts of both fish species. Nevertheless, the capacity of retention of water soluble micronutrients before particle ingestion was not tested. The importance of this issue has been addressed in several studies designed to improve the amount of water-soluble nutrients delivered to the larval gut (López-Alvarado et al. 1994; Langdon, 2003; Yúfera et al. 2002; Önal and Langdon, 2004a, b).

Larvae of *S. aurata* fed on alginate microdiets showed similar growth patterns and survival as previously published for larvae fed on protein microdiets, although the growth was slightly better with the former microdiet (Yúfera et al. 2000). Comparable growth was also observed by Robin and Vincent (2003) (best result: 66 µg dry weight in 21 dph larvae) and Robin and Peron (2004) (best result: 54 µg dry weight in 17 dph larvae) in gilthead seabream larvae fed exclusively on a microparticulate diets from first feeding. The best growth in a sparid was reported by Kelly et al. (2000) working with *Sparus sarba*. These authors reported an increase from 45 µg at 7 dph to

120 µg at 15 dph for larvae fed on an alginate-based microdiet from first feeding. All these results indicate that microdiets prepared with different methodologies have supported to a certain extent larval growth and development from first feeding. A common characteristic of these microdiets is that food particles were well ingested and digested.

On the other hand, larval growth results with Senegal sole were better than in previous studies reported with the protein-walled microdiets (Fernández-Díaz et al. 2001; Yúfera et al. 2003). The alginate microdiet was able to maintain a continual growth and to support metamorphosis of Senegal sole larvae as a single food from 6 dph onwards. The change in the growth rate observed from 17 and 20 dph indicates the beginning of eye migration and the start of benthic behaviour. The same growth pattern was observed in *Artemia* nauplii fed larvae. Final weight and survival of larvae fed on the microdiets were approximately half the values obtained with the *Artemia* treatment. This is a good and promising result for larvae that are completely weaned at 30 dph. The microdiet containing 62% crude protein supported larvae with higher final weight and better developmental status than the microdiet with 55% crude protein. This means that a detectable improvement can be observed with a single change in macronutrient composition. Likewise, recent studies on larval nutrition in *S. senegalensis* (Aragão et al. 2004) and *S. aurata* (Robin and Vincent, 2003; Robin and Peron, 2004) reported detectable improvement when specific nutrients were properly supplied.

The results obtained in this study show that emulsification/internal gelation with alginate is an useful way to prepare experimental microdiets for larval marine fish. This microdiet is able to support growth and development in larvae of two species exhibiting different developmental history and behaviour (Sarasquete et al. 1995; Moyano et al. 1996; Martínez et al. 1999; Ribeiro et al. 1999a and b; Parra and Yúfera, 2001). Further studies and improvements on capsule performances to optimise the retention of soluble micronutrients maintaining the digestibility are still required. Nevertheless, as mentioned above, improvements in larval growth and survival depend to a great extent on advances in larval nutrition.

5.4.1.4.6 Acknowledgements

The Ministry of Science and Technology, Spain (MCYT Project AGL2000-0697-C02-01) supported this work. We thank E. García-Ramos Neto and J. A. Miquel for their helpful technical assistance. This work was conducted in collaboration with Dr Kolkovski, Department of Fisheries, Western Australia.

5.4.1.4.7 Tables

Table 1. Diet ingredients in the different experimental microcapsules (g kg^{-1}).

Type	MA	MA-55	MA-62
Casein [1]	260	-	-
Fish meal [2]	155	510	580
Squid meal [3]	145	100	100
Fish hydrolysate [4]	125	125	125
Oil emulsion [5]	135	65	65
Dextrin (type I) [6]	-	70	-
Soy lecithin [7]	30	40	40
Baker yeast	50	-	-
Vitamin C [8]	30	30	30
Vitamin E [9]	10	10	10
Sodium alginate [10]	60	50	50

[1] ICN 901633; [2] AgloNorse, Norway; [3] Rieber & Son, Norway; [4] CPSP 90, Soprepêche, France; [5] A1 DHA Selco, Inve; [6] ICN 101517; [7] ICN 102147; [8] Rovimix Stay C-35, Roche; [9] ICN 100555; [10] ICN 154724.

Table 2. Biometrical measurements after rehydration, dry weight and elemental composition of microparticles (Type MA in table 1) in relation to size class of dry particles. Values are mean ± SD.

Size class (µm)	<200	200-399	>400
Feret Diameter	199 ± 66	312 ± 145	526 ± 243
Compactness	0.895 ± 0.078 (a)	0.837 ± 0.077 (a)	0.833 ± 0.119 (a)
Roundness	0.787 ± 0.122 (a)	0.679 ± 0.146 (b)	0.598 ± 0.189 (c)
Swelling degree	1.97	1.24	1.16
Dry weight (µg)	0.64 ± 0.09	1.52 ± 0.24	1.74 ± 0.31
Carbon (%)	51.28 ± 0.10 (a)	51.86 ± 0.56 (a)	51.99 ± 0.30 (a)
Nitrogen (%)	7.96 ± 0.03 (a)	8.73 ± 0.27 (b)	9.11 ± 0.45 (b)

For each case, same letters indicate no statistically significant difference (Newman-Keuls range test; $P < 0.05$).

Table 3. Growth rates (d^{-1}) and survival at the end of the experiments (%; mean ± SD) obtained in larvae *of Sparus aurata* and *Solea senegalensis* with the different feeding regimes (Table 1).

Feeding regime	Growth rates		Final survival
Sparus aurata			
	From 4 to 15 dph	From 8 to 15 dph	
Rotifer	0.119 (a)	0.129 (b)	91.2 ± 2.6 (a)
MA	0.029 (b)	-	57.0 ± 0.1 (b)
Rotifer + MA	-	0.105 (b)	67.8 ± 10.0 (b)
Solea senegalensis			
	Before eye migration	After eye migration started	
Artemia	0.2778 (a)	0.0671 (a)	85.0 ± 9.0 (a)
MA-55	0.1054 (b)	0.0303 (b)	38.6 ± 5.0 (b)
MA-62	0.1404 (c)	0.0321 (b)	46.3 ± 0.6 (b)

For each species and each column, same letters indicate no statistically significant difference (Growth rates: Student-T test for slope comparison; Survivals: Newman-Keuls range test; $P < 0.05$).

5.4.1.4.8 References

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5.4.2 Nutritional properties

5.4.2.1 Early weaning of sole (*Solea senegalensis*) onto microencapsulated diets, with different levels of protein

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5.4.2.1.1 Abstract

Growth, survival and activities of the digestive enzymes trypsin, amylase, alkaline phosphatase, and pepsin in Senegalese sole, *Solea senegalensis*, were measured in weaned larvae. Sole larvae were weaned with a microencapsulated diet containing 10% (treatment M10) or 20% (treatment M20) of fish protein hydrolysate. Two experiments were performed with different co-feeding periods (3 and 12 days), both starting 17 DAH. The control group was fed on frozen enriched *Artemia* metanauplii. Growth was measured by taking the total length, wet weight, and dry weight of the larvae, and calculating the relative growth rate. Abdominal cavity homogenates were used for enzyme determination after the co-feeding period and at the end of each experiment (38-39 DAH). Larval growth was lower in the weaned groups, with no major differences between treatments M10 and M20 or treatments with different co-feeding periods. Mean survival in the control groups was 85.4 ± 2.4% (Experiment 1) and 99.1 ± 1.1% (Experiment 2), in comparison with the mean survival of treatments M10 and M20 that ranged between 46.9 ± 4.7% and 59.6 ± 20.5%. The specific activities of amylase, alkaline phosphatase (only in one of the two experiments), and pepsin were higher in the weaned groups, suggesting that larvae can alter their digestive capacity in response to a new diet. However, the segmental activities of all enzymes were higher in the control group, due to the higher growth attained by this group. These results lead to the conclusion that sole larvae are able to adapt their digestive system to a new formulated diet as soon as 17 DAH and their low growth and survival are not due to lack of digestive enzymes.

5.4.2.1.2 Introduction

Diversification of marine aquaculture species has been one of the main foci in the European industry for the last couple of decades. This need was especially emphasized after the market saturation for established species, such as sea bass, *Dicentrarchus labrax*, and seabream, *Sparus aurata*. A fish that attracted the interest of many aquaculturists and researchers, because of its high demand and market value is sole. It is a popular seafood, being consumed all over Western Europe. The sole species that attract most of the focus today and are considered as serious candidates to become one of the main products of European aquaculture are Dover sole (*Solea solea = Solea vulgaris*) and Senegalese sole, *Solea senegalensis* (Dinis and Reis, 1995; Dinis et al. 1999; Howell, 1997). *S. senegalensis* is found in temperate and sub-tropical climates in Mediterranean and Southern Atlantic waters. It represents 95% of the sole catch in the southern part of Portugal (Dinis and Reis, 1995), rendering it an important source for local economies. It is commonly cultured in the extensive earthen ponds along the south coasts of Portugal and Spain (Dinis et al. 1999).

Techniques for the cultivation of its different life stages are known, but further development at the commercial scale is essential for the expansion of production. A bottleneck in this development is the high mortality of *S. senegalensis* larvae taking place during and after the weaning process (Dinis and Reis, 1995). The common practice today is that larvae are weaned around 40 DAH. However, the formulation of an inert diet that allows an earlier weaning would reduce the dependence on the use of *Artemia*, decrease the production costs, and increase the predictability of production.

The acquisition and production of *Artemia* for the feeding of larval marine fish constitute a large

proportion of the larvae production costs (Callan et al. 2003). Also, the availability and price of *Artemia* vary widely from year to year, rendering the production an inconsistent process. Therefore, the formulation and production of a compound diet that can be ingested from the larvae soon after their hatching and meet their specific nutritional requirements could replace *Artemia* and accomplish the early weaning of larvae.

Many researchers tried to achieve this target of formulating a diet adequate to satisfy the nutritional requirements of marine larvae, but the majority of the diets did not satisfy all the requirements needed for its successful use in the commercial rearing of larvae (Fontagnz et al. 2000; Robin and Vincent, 2003). A factor that might constitute an obstacle in formulating the right diet is whether fish larvae have the digestive capacity to digest inert diets, and in particular if they are able to produce the required digestive enzymes. Opinions on this matter differ between researchers. Kolkovski et al. (1997a) and Kolkovski et al. (1997b) support that the ingestion, digestion, and assimilation of a compound diet by fish larvae is assisted by the utilisation of exogenous enzymes through the ingestion of live prey. On the contrary, Zambonino Infante and Cahu (1994a, b) support that larvae do produce adequate digestive enzyme levels and that lack of growth in larvae that fed on the formulated diet is not due to lack of endogenous enzyme activity. In the study of Ribeiro et al. (1999) on the development of digestive enzymes in larvae of *S. senegalensis*, it is reported that trypsin, amylase, alkaline phosphatase, and leucine-alanine peptidase are present at first feeding, and by the end of the first month of life, larvae acquire an adult mode of digestion. However, the ability of the digestive system of *S. senegalensis* larvae to digest a formulated diet is not yet known.

This study intends to contribute to the above discussion and focuses on two aims. Firstly, the investigation on the possibility of early weaning of sole larvae, focusing on the activities of digestive enzymes. Secondly, the acceptability of microencapsulated diets containing different levels of fish protein hydrolysate by the sole larvae, and their effect on growth, survival and digestive enzymes activity.

Senegalese sole

Life cycle

Senegalese sole generally spawn during spring, from March until June (Spanish-Frenh Atlantic coasts) over soft mud bottoms. Females mature during the third year of age, when total length is 32 cm and produce 509 oocytes/g fish (Dinis et al. 1999). The fertilised eggs hatch in less than 1 week and the newborn pelagic, bilaterally symmetrical larvae metamorphose into benthic-dwelling, asymmetric juveniles in about 2-3 weeks (Fig. 1). Eggs and larvae are planktonic. In the wild, young fish depend heavily on inter-tidal areas, estuaries, and shallow near-shore waters for food and shelter. Adults are found in near-shore coastal waters and make only limited migrations.

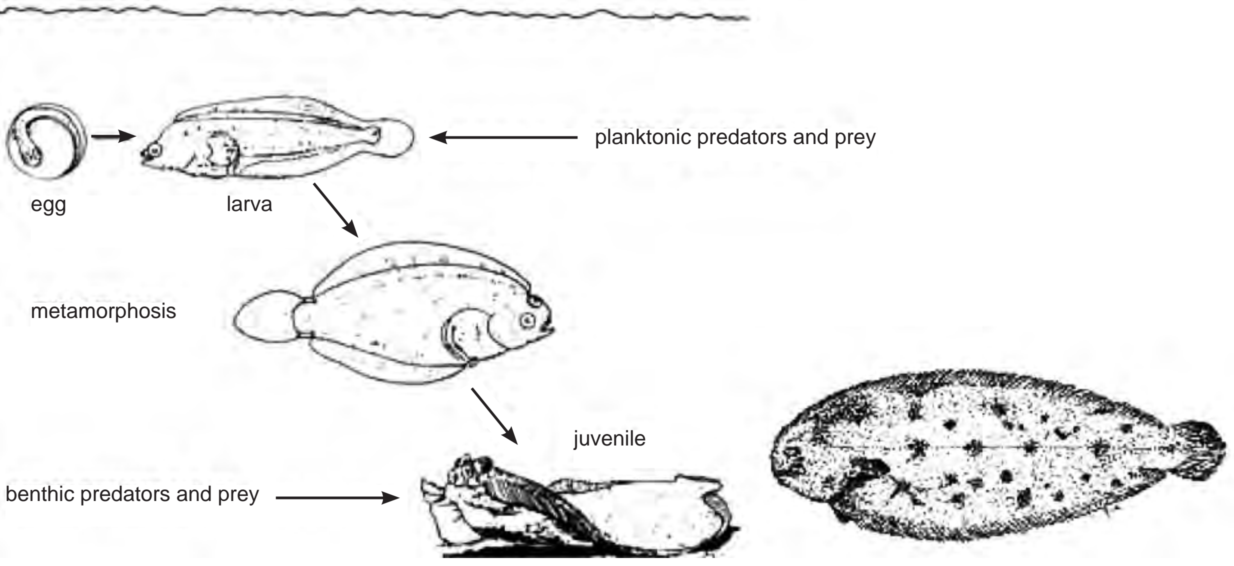

Figure 1. Typical life cycle of flatfish (Bone e.a., 1999).

Larviculture

Reproducing the life cycle of fish under controlled conditions is usually one of the main challenges in aquaculture. To achieve this is not an easy task, especially when little is known about the biology and ecology of the fish concerned. One of the main obstacles in doing so is the successful and consistent culture of the newly born larvae. It is well documented today that further expansion of aquaculture products is directly dependable on the consistent availability of high quality fingerlings for stocking grow-out production systems (Planas and Cunha, 1999; Sorgeloos et al. 1995).

The realised need for expanding and improving the production efficiency of fish larvae has led to some developments in the last two decades. These improvements concerned mainly three aspects of larviculture: disease status and microbial control, nutrient requirements and feeding, and rearing technology.

5.4.2.1.3 Nutrient requirements and feeding

The rearing of large numbers of larvae in relatively restricted areas requires good knowledge of their nutritional requirements in order to assist them meeting their high potential growth rates. A nutritionally balanced diet must contain all required nutrients in the proper proportion for the species of fish being fed. The main classes of nutrients included in a larvae diet are: protein (amino acids), lipids (fats), vitamins, minerals, and carotenoid pigments.

Protein

Marine fish larvae have high growth rates compared to adult fish. Therefore, the sum of protein deposition, turnover and catabolism necessary for their rapid growth requires high levels of amino acids (AA) (Ronnestad and Conceio, 2003). Protein in live food or formulated feeds is the primary source of amino acids to larvae. Prior the onset of first feeding, fish larvae acquire the amino acids (AA) required for their metabolism from free amino acids (FAA) found in the yolk-sack compartment (Ronnestad and Fyhn, 1993). After the reserves of the larva are depleted, FAA must come from exogenous supply (Fyhn, 1989). Atlantic halibut larvae found to derive 60% of their energy from FAA, while in cod 50-70% of energy dissipation was due to AA catabolism contributed by FAA (Fyhn, 1989; Ronnestad et al. 1999). In turbot (*Scophthalmus maximus*) approximately 70% of the FAA are utilised as an energy substrate, while the rest are polymerized into body proteins (Ronnestad et al. 1992). The use of AA by larvae includes the synthesis of functional body proteins (hormones, enzymes, and products of respiration), energy production, gluconeogenesis,

lipogenesis, and synthesis of other nitrogen-containing molecules (e.g. purines, pyrimidines) (Ronnestad and Conceicao, 2003).

20 principle AA have been found in fish tissues, categorised as essential (indispensable) AA (EAA), semi-essential AA, and nonessential AA (NEAA) (Table 1). The EAA must be supplied by the diet, because fish cannot synthesis them, whereas the NEAA can be synthesised from α-ketoacids. The semi-essential AA can only be synthesised from EAA (sometimes are grouped with EAA). Larvae fed feeds lacking in even one dietary EAA become inactive and present suppression in growth and appetite (Barrows and Hardy, 2001).

Table 1. Essential, semi-essential and non-essential nature of amino acids.

Essential	Non-essential and semi-essential
Arginine	Alanine
Histidine	Asparagine
Isoleucine	Aspartic acid
Leucine	Glutamic acid
Lysine	Glutamine
Threonine	Glycine
Tryptophan	Proline
Valine	Serine
Methionine	Cysteine[1]
Phenylalanine	Tyrosine[1]

[1] Semi-essential amino acids

The dietary protein requirement of fish larvae is influenced by several factors, including species, feeding rate, and energy content of the diet. A diet, which promotes optimal growth and food efficiency, must have a well balanced protein and energy content. The recommended protein level of trout fry feeds, as a percentage of the diet, is 45-50%, whereas for catfish larvae is 35-40% (Barrows and Hardy, 2001). Also, Metailller et al. (1981) demonstrated that European sea bass (*D. labrax*) showed best growth and best feed conversion when fed a diet with a protein level of 60%.

Lipids

Lipids are indispensable in the early stages of fish life as they constitute the main source of energy. The highly unsaturated fatty acids (HUFA), 20:5n-3 (eicosapentaenoic acid; EPA) and 22:6n-3 (docosahexaenoic acid; DHA) that are predominately in the form of phospholipids in foodstuffs, are required for the normal growth and survival of larval fish (Sorgeloos et al. 1995; Watanabe and Kiron, 1994). Larvae in the wild have an easy access to food rich in phospholipids since the aquatic environment is characterised by a great richness in polyunsaturated fatty acids (PUFA) and their long chain derivatives HUFA (Corraze, 2001). However, HUFA supplementation to larvae reared in hatcheries is considered as a major problem because the live prey, rotifers and *Artemia*, normally given to the larvae contains low levels of EPA and DHA (Dhont and Van Stappen, 2003; Lubzens and Zmora, 2003). This is why, the manipulation of the fatty acid profile of live prey by "bio-encapsulation" of lipid emulsions and whole cell preparations of unicellular algae and protists is considered as a significant progress achieved in larviculture today (Shields, 2001).

In addition to improved growth and survival, optimum fatty acid levels and rations (DHA/EPA) in feeds were found to promote better pigmentation and stress resistance. Gapasin and Durai (2001) showed that survival was significantly better when milkfish (*Chanos chanos*) larvae were fed DHA-

enriched live prey. Feeding of DHA also led to increased resistance to stress in *Coryphaena hippurus* (Watanabe and Kiron, 1994). In addition to the essentiality of EPA and DHA, the requirement for 20:4n-6 (arachidonic acid; ARA) should also be considered. Improvements in dorsal pigmentation in turbot and halibut can be achieved by providing ratios of DHA/EPA of >2:1 but, perhaps more importantly, an EPA/ARA ratio of >5:1 (Bell et al. 2003).

The determination of fatty acid requirements and the biochemical interaction (ratio) between DHA/EPA and EPA/ARA for different larval species is vital for the normal vitality and health of the fish.

Also, the overall dietary lipid level was found to influence the larval development and maturation of digestive tract. Zambonino, Infante and Cahu (1999) conducted an experiment with different lipid levels in compound diets, ranging from 10 to 30%. The results showed that the higher the lipid level, the greater the growth and survival of the European sea bass (*D. labrax*) larvae. The maximal synthesis level of digestive enzymes was reached in the larvae fed with the diets containing more than 20% lipids. In a similar experiment Buchet et al. (2000) demonstrated that a diet with 30% lipids promoted increased development and digestion in red drum (*Sciaenops ocellatus*) when compared with 0% and 15% lipid levels.

Vitamins

Reports on the need of vitamins during the early stages of fish life have been focused on vitamin C and E. The fact that fish eggs contain high concentrations of ascorbic acid is an indication of the importance of this micronutrient during early development. The importance of vitamin C on growth and stress resistance of the larvae of catfish, *Clarias gariepinus*, and sea bass, *D. labrax*, was illustrated by Merchie et al. (1995). Specifically, an enrichment of 20% ascorbyl palmitate (AP) in the live food of catfish larvae increased growth by 30% (compared with 0% AP), and the same enrichment enhanced stress resistance in sea bass larvae. Moreover, Ortu–o et al. (2003) reported that vitamin C and E interfere in tertiary stress responses, such as immunodepression in gilthead seabream (*S. aurata* L.). Vitamin C and E were found to exert a synergistic effect enhancing the respiratory burst activity of seabream phagocytes rendering the innate immune system more resistant against diseases (Ortuno et al. 2001).

Vitamins are incorporated to the larvae through live prey, which is commonly enriched with oil emulsions. In Mediterranean hatcheries seabream larvae fed with rotifers enriched with emulsion containing 5% ascorbyl palmitate (AP; derivative from water-soluble vitamins) showed a reduction in problems that had to do with stress and structural malformations (Merchie et al. 1997).

Minerals

Minerals are considered as essential nutrients for larval fish. They are classified as macro or micro, depending on the amount required in the diet. Table 2 shows the seven macrominerals and nine microminerals required by larval fish (Barrows and Hardy, 2001). Larvae absorb minerals directly from water by their gills and skin or take them up through swallowed water and feed. Mineral elements are involved in the formation of hard structures, such as bones and teeth, production of red blood cells, regulation of body processes and osmoregulation. Deficiencies in some minerals can cause anemia (iron, copper, and cobalt) or bilateral cataracts (zinc) (Barrows and Hardy, 2001).

Table 2. Macro and micro minerals with confirmed nutritional roles in fish larvae.

Macro-minerals	Micro-minerals
Calcium (C)	Iron (Fe)
Phosphorus (P)	Copper (Cu)
Sulfur (S)	Iodine (I)
Sodium (Na)	Manganese (Mn)
Chlorine (Cl)	Cobalt (Co)
Potassium (K)	Zinc (Zn)
Magnesium (Mg)	Molybdenum (Mo)
	Selenium (Se)
	Fluorine (F)

Carotenoid pigments

It was only recently demonstrated that carotenoid pigments present in live prey contribute to the visual function and pigment development in flatfish larvae. The carotenoids, lutein and astaxanthin, present in marine copepods derive vitamin A in the eyes of Atlantic halibut larvae, which facilitates the normal visual function (Shields, 2001). Also, Japanese flounder and turbot showed a higher pigmentation success when vitamin A was included in the diet (Planas and Cunha, 1999).

Feeding

Only in the recent years feeding gained some appreciation from the fish culturists. In the past, food was given to the fish with little attention being paid to feeding rates, food conversion ratio (FCR) and feeding habits. Today, feeding is considered as important as feed composition. In larviculture, the first aspect one should consider is the time of initial exogenous feeding. The most reliable visual guide to determine when initial feeding must start is the absorption of the yolk sac. Therefore, fish larvae with large yolk sac start feeding at an older age than larvae with small yolk sac. For example, brown trout fry start feeding approximately 31 days after hatching (DAH; at 11°C) (Barrows and Hardy, 2001), whereas Senegalese sole larvae start feeding 2 DAH (at 18°C) (Ribeiro et al. 1999a). Another important aspect in feeding fish is the frequency with which fish should be fed. Generally, when fish are in the first stages of life, is better to supply food in small amounts and frequent intervals. This usually gives some advantages, such as lower FCR, better size uniformity, and lower feed waste. Guinea and Fernadez (1997) reported that the increase in feeding frequency also resulted in more stable oxygen consumption (no high peaks) from the fish. Frequent feeding however, is not an option in some hatcheries because of the available feeding methods. When hand feeding is used feeding is usually done a few times per day, whereas when mechanical or demand feeding is available frequent feeding does not constitute a problem.

5.4.2.1.4 Materials and methods

Larval supply

Eggs were obtained from wild broodstock, kept at IPIMAR Aquaculture Research facilities (Olho, Portugal). The larvae were reared until 17 DAH in 200 L tanks using a standard protocol (Dinis and Reis, 1995). The larvae were fed on rotifers (L-type), *Artemia* nauplii, live enriched (Algamac 2000) *Artemia* metanauplii and frozen enriched *Artemia* metanauplii (M24) 3 times/day.

Experimental conditions

Larvae were kept in nine white trays with flat bottom. In each tray 175 larvae were accommodated. Each tray had a bottom area of 0.076 m^2 and held a water volume of 3 l. The trays were supplied with continuous seawater from recirculated system at 0.025 l/min with 50% volume exchange/hr at 22°C. Light intensity on the surface of the water was 350 Lux. Photoperiod was 24L:0D.

The recirculation system (Fig. 2.1) consisted of nine fish tanks (trays), a sedimentation tank with granular filter (made of synthetic fibers), a protein skimmer, a sand filter, three biological filters and a water distribution tank.

The trays were assigned randomly in 3 treatments (3 trays per treatment). The larvae in the first treatment were fed on frozen enriched (Algamac2000) *Artemia*; this treatment served as a control. The larvae in the second treatment (M10) were weaned from frozen *Artemia* to microencapsulated diet containing 10% fish protein hydrolysate (CPSP90, Sopropeche, France), and the larvae in the third one (M20) were weaned to microencapsulated diet containing 20% fish protein hydrolysate (CPSP90) (Table 3.1).

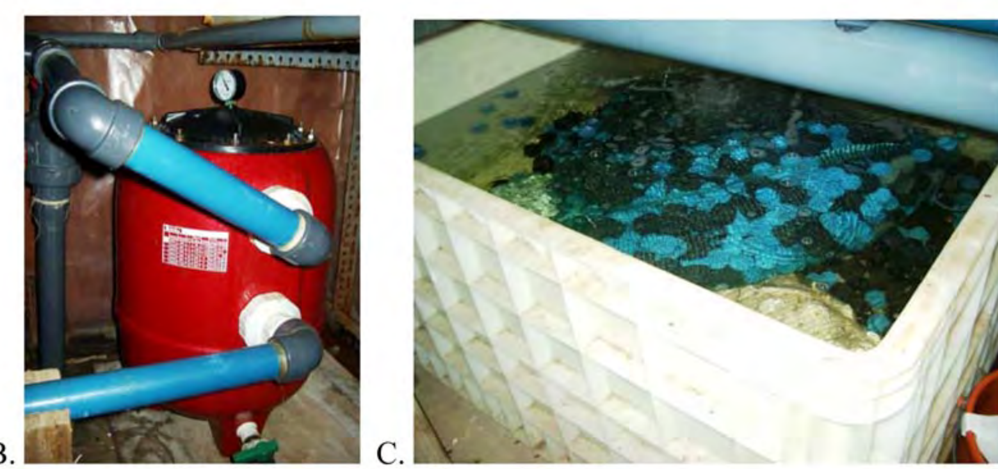

Figure. 2A-C. Some parts of the recirculation system: (A) Fish trays, (B) sand filter, and (C) biological filter.

Table 3. Diet[1] composition (gr/100gr dry matter).

Ingedients	M10 (for 100g)	M20 (for 100g)
Casein	40	40
Squid meal	10	10
Fish meal (Aglonorse)	17	7
Hydrolysate (CPSP90)	10	20
Dextrin(type I)	2	2
Fish oil (A1 DHA Selco)	10	10
Soy lecithin (ICN)	5	5
Baker Yeast	2	2
Vitamin C (ICN)	3	3
Vitamin E (ICN)	1	1

[1] The diet was prepared by Dr Manuel Yufera (Instituto de Ciencias Marinas de Andalucia - CSIC, Puerto Real, Cadiz, Spain).

Weaning trials

Two experiments were performed. For the first experiment, a co-feeding period of 3 days, starting at 19 DAH, was used for treatments M10 and M20. The *Artemia* given to the larvae over the 3-day co-feeding period was reduced to 66, 33, and 15% (of the *Artemia* given to the control) respectively, while the microencapsulated diet was given in a stable amount (Table 3).

During the whole experiment, larvae were fed 5 times a day (10, 12, 14, 16, 18 h). *Artemia* and microencapsulated diet were given alternately, starting with the diet. The amount of daily meals given to the larvae was estimated according to an empirical feeding plan developed by the Centro de Ciencias do Mar (CCMAR) for sole.

All trays were siphoned and cleaned daily. Temperature, salinity, oxygen levels and larval mortality were recorded daily. The experiment lasted until 39 DAH.

For the second experiment, the same experimental equipment and conditions were used. However, this time the co-feeding period lasted for 12 days, starting at 19 DAH. The *Artemia* given to the larvae over the 12-day weaning period was reduced by 25% every four days, while the microencapsulated diet was given in a stable amount (Table 4).

Larvae were fed 5 times a day, following the same feeding times like the first experiment. However, for the 4-day periods that *Artemia* was given to treatments M10 and M20 at 75 and 50% of the amount given to the control, *Artemia* and microencepsulated diet were given in every meal at 5 equal doses. For the last 4-day period (25%), *Artemia* and diet were given alternately, starting with the diet. The second experiment lasted until 38 DAH.

Table 4. Feeding schedule of Experiment 1.

DAH	Treatment	Meals[1] (time)										TOTAL	
		10 h		12 h		14 h		16 h		18 h			
		Artemia (M24/tank)	M10/M20 (mg/tank)	Artemia (M24/tank)	M10/M20 (mg/tank)	Artemia (M24/tank)	M10/M20 (mg/tank)	Artemia (M24/tank)	M10/M20 (mg/tank)	Artemia (M24/tank)	M10/M20 (mg/tank)	Artemia (M24/tank)	M10/M20 (mg/tank)
18	All	50000		50000		50000		50000				200000	
19	Control [2]	30000		30000		30000		30000		30000		150000	
	M10 [2]		60	50000			60	50000			60	100000	180
	M20 [2]		60	50000			60	50000			60	100000	180
20	Control	30000		30000		30000		30000		30000		150000	
	M10		60	25000			60	25000			60	50000	180
	M20		60	25000			60	25000			60	50000	180
21	Control	30000		30000		30000		30000		30000		150000	
	M10		45		45	25000			45		45	25000	180
	M20		45		45	25000			45		45	25000	180
22[3]	Control	30000		30000		30000		30000		30000		150000	
	M10		36		36		36		36		36		180
	M20		36		36		36		36		36		180

[1] The amount of meals was adjusted according to the results of each sampling for WW.

[2] Each feeding regime had three replicates.

[3] After 22 DAH the feeding schedule remained the same. This means that the control was receiving 100% M24, and the M10 and M20 treatments were receiving 100% microencapsulated diet.

Table 5. Feeding schedule of Experiment 2.

DAH	Treatment	Meals[1] (time)										TOTAL	
		10 h		12 h		14 h		16 h		18 h			
		Artemia (M24/tank)	M10/M20 (mg/tank)	Artemia (M24/tank)	M10/M20 (mg/tank)	Artemia (M24/tank)	M10/M20 (mg/tank)	Artemia (M24/tank)	M10/M20 (mg/tank)	Artemia (M24/tank)	M10/M20 (mg/tank)	Artemia (M24/tank)	M10/M20 (mg/tank)
18	All	30000				25000				25000		80000	
19	Control [2]	18200		18200		18200		18200		18200		91000	
	M10 [2]	13700	40	13700	40	13700	40	13700	30	13700	30	68500	180
	M20 [2]	13700	40	13700	40	13700	40	13700	30	13700	30	68500	180
20	Control	21200		21200		21200		21200		21200		106000	
	M10	15900	40	15900	40	15900	40	15900	30	15900	30	79500	180
	M20	15900	40	15900	40	15900	40	15900	30	15900	30	79500	180
21	Control	24600		24600		24600		24600		24600		123000	
	M10	18500	40	18500	40	18500	40	18500	30	18500	30	92500	180
	M20	18500	40	18500	40	18500	40	18500	30	18500	30	92500	180
22	Control	28600		28600		28600		28600		28600		143000	
	M10	21400	40	21400	40	21400	40	21400	30	21400	30	107000	180
	M20	21400	40	21400	40	21400	40	21400	30	21400	30	107000	180
23	Control	33200		33200		33200		33200		33200		166000	
	M10	16600	40	16600	40	16600	40	16600	40	16600	40	83000	200
	M20	16600	40	16600	40	16600	40	16600	40	16600	40	83000	200
24	Control	38600		38600		38600		38600		38600		193000	
	M10	19300	40	19300	40	19300	40	19300	40	19300	40	96500	200
	M20	19300	40	19300	40	19300	40	19300	40	19300	40	96500	200
25	Control	40700		40700		40700		40700		40700		203500	
	M10	20400	40	20400	40	20400	40	20400	40	20400	40	102000	200
	M20	20400	40	20400	40	20400	40	20400	40	20400	40	102000	200

Table 5 cont.

26	Control	40000		40000		40000		40000		40000		200000	
	M10	20000	40	20000	40	20000	40	20000	40	20000	40	100000	200
	M20	20000	40	20000	40	20000	40	20000	40	20000	40	100000	200
27	Control	36700		36700		36700		36700		36700		183500	
	M10		120	23000			120	23000			120	46000	360
	M20		120	23000			120	23000			120	46000	360
28	Control	42100		42100		42100		42100		42100		210500	
	M10		120	26300			120	26300			120	52600	360
	M20		120	26300			120	26300			120	52600	360
29	Control	48200		48200		48200		48200		48200		241000	
	M10		120	30200			120	30200			120	60400	360
	M20		120	30200			120	30200			120	60400	360
30	Control	41600		41600		41600		41600		41600		208000	
	M10		120	26000			120	26000			120	52000	360
	M20		120	26000			120	26000			120	52000	360
31[3]	Control	30000		30000		30000		30000		30000		150000	
	M10		60		60		60		60		60		360
	M20		60		60		60		60		60		360

[1] The amount of meals was adjusted according to the results of each sampling for WW.

[2] Each feeding treatment had three replicates.

[3] After 31 DAH the feeding schedule remained the same. This means that the control was receiving 100% M24, and the M10 and M20 treatments were receiving 100% microencapsulated diet.

Sampling

Sampling was performed in frequent intervals looking at the parameters of total length (TL), wet weight (WW), dry weight (DW), gut content, and enzyme activity (Tables 5, 6). The larvae samples for enzyme analysis were kept at -80°C freezer and the rest of the samples at -20°C.

Table 6. Sampling schedule for first experiment.

DAH	Number of larvae sampled per replicate	Parameter
17	30	TL and WW
21	3	Gut content
26	5	TL, WW and DW
26	15	Enzyme analysis[1]
32	5	TL, WW and DW
39	30	Enzyme analysis
39	30	TL, WW and DW

[1] Only trypsin, amylase, and protein.

Table 7. Sampling schedule for second experiment.

DAH	Number of larvae sampled per replicate	Parameter
17	30	TL, WW and DW
24	5	TL, WW and DW
30	3	Gut content
31	15	Enzyme analysis[1]
31	10	TL, WW and DW
38	30	TL, WW and DW
38	30	Enzyme analysis

[1] Only trypsin, amylase, and protein.

Analytical methods

TL was measured by placing the larvae on a graphic paper. The larvae were weighted for WW and DW.

The gut content observations were performed in order to trace any *Artemia* or microencapsulated particles. Each larva was placed under the microscope, dissected and observed.

The quantification of enzyme activity was determined using the analytical methods described by Ribeiro et al. (1999). The abdominal cavities of the larvae were removed by dissection (Fig. 3) and stored in eppendorfs. Samples were homogenized in 15 volumes (v/w) of ice cold deionised water. Trypsin activity was measured using Na-Benzoyl-DL-arginine-*p*-nitroanilide (Bapna) as a substrate; amylase activity was measured using starch; alkaline phosphatase (AP) activity was measured using *p*-nitrophenylphosphate (pNPP) MgCl2 and pepsin activity was measured using bovine haemoglobin as a substrate. Protein was determined by the Bradford procedure.

Enzyme activities were calculated as micromoles of substrate hydrolysed per minute (e.g. U or mU) at 37°C for AP and pepsin, and 25°C for trypsin. Amylase was expressed as the equivalent enzyme activity, which was required to hydrolyse 1 mg of starch in 30 min at 37°C. Enzyme activities were expressed also as specific (U/mg protein or mU/mg protein), and segmental (total activity per larvae; U/segment or mU/segment) activities.

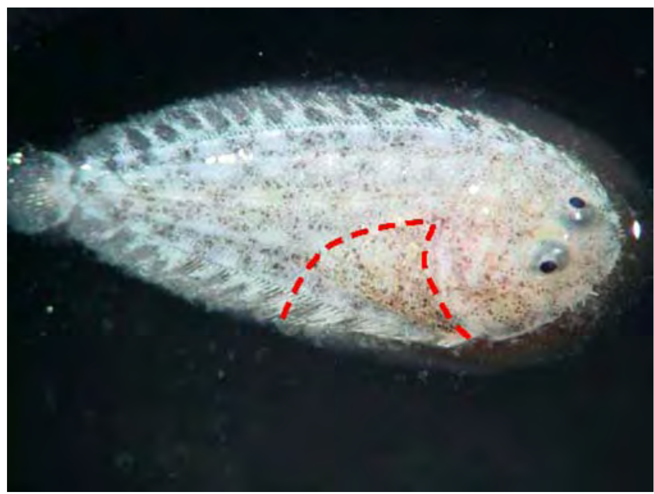

Figure 4. The removal of the abdominal cavity was performed by cutting, with a scissor, along the red dashed line under a binocular microscope.

Data and statistical analysis

Growth is reported as relative growth rate (RGR,% BW/day) and calculated from average body weights as follows:

$$RGR = (e^{g}-1) \times 100 \quad \text{with: } g = [\ln(W_2) - \ln(W_1)]/\text{time}$$

Where, W and W are the initial and final weights, respectively.

The data were analysed by one-way ANOVA and t-test. A probability level of $P < 0.05$ was used to judge whether any effects were significant.

5.4.2.1.5 Results

Water parameters

Water parameters (Table 4.1) were relatively stable during both experiments (tested daily). For the whole experimental period, salinity ranged between 30 and 36‰, temperature between 20.5 and 23.0°C, and dissolved oxygen between 5.9 and 8.6 mg/l.

Table 7. Water parameters during Experiment 1 and 2.

	Salinity		Temperature		Dissolved Oxygen	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Mean	30.4	34.3	21.6	21.9	6.8	6.7
St. Dev.	0.82	1.10	0.72	0.41	0.28	0.47
Max	32.0	36.0	23.0	22.6	7.5	8.6
Min	30.0	33.0	20.5	21.0	6.2	5.9

Growth and survival

Experiment 1

Larvae in treatments M10 and M20 had similar physical development during the experimental period. This is indicated by the measurements of total length (TL), wet weight (WW), dry weight (DW), and relative growth rate (RGR) at the different sampling times. Control group showed a more rapid growth achieving two times the TL and five times the WW achieved by the groups fed on the microencapsulated diet (Table 8).

During the experiment one of the control replicates was affected by an infection the infection first caused corrosion of the caudal fin and later the death of the larvae. The source of the infection was not identified). Formalin treatment was used to combat the infection that caused unexpected mortalities. This replicate was not taken into account in the calculation of mean values.

Table 8. Total length (TL), wet weight (WW), dry weight (DW), and relative growth rate (RGR)[1] measurements during Experiment 1.

Control[2]				
DAH	**TL (mm)**	**Mean WW**	**Mean DW**	**RGR (%BW/day)**
17	5.1 ± 0.7	2.5	0.38 ± 0.2	
26	10.0 ± 2.1	11.6 ± 5.3	2.10 ± 0.9	18.6 ± 1.1
32	13.2 ± 1.6	19.0 ± 6.6	3.66 ± 1.3	8.6 ± 1.4
39	17.3 ± 2.2	36.9 ± 11.3	9.18 ± 2.7	9.9 ± 2.5
M10				
17	5.1 ± 0.7	2.5	0.38 ± 0.2	
26	8.4 ± 1.3	5.5 ± 2.1	0.91 ± 0.4	9.2 ± 0.8
32	9.4 ± 1.2	6.7 ± 2.6	1.15 ± 0.4	3.2 ± 2.0
39	10.0 ± 1.8	7.3 ± 4.1	1.70 ± 0.9	1.3 ± 2.0
M20				
17	5.1 ± 0.7	2.5	0.38 ± 0.2	
26	8.5 ± 1.4	5.1 ± 1.9	0.97 ± 0.3	8.3 ± 1.6
32	9.1 ± 1.8	6.2 ± 3.3	1.13 ± 0.6	3.2 ± 4.7
39	9.8 ± 1.6	6.6 ± 4.0	1.50 ± 0.9	1.0 ± 3.4

[1] Values are means ± S.D.

[2] Only two out of the three replicates were taken into account.

The control group reached a final TL of 17.3 ± 2.2 mm, whereas the M10 and M20 groups reached a final TL of 10.0 ± 1.8 mm and 9.8 ± 1.6 mm respectively. Observing Fig. 4, it can be seen that the TL development of the larvae fed on the two diets was almost identical.

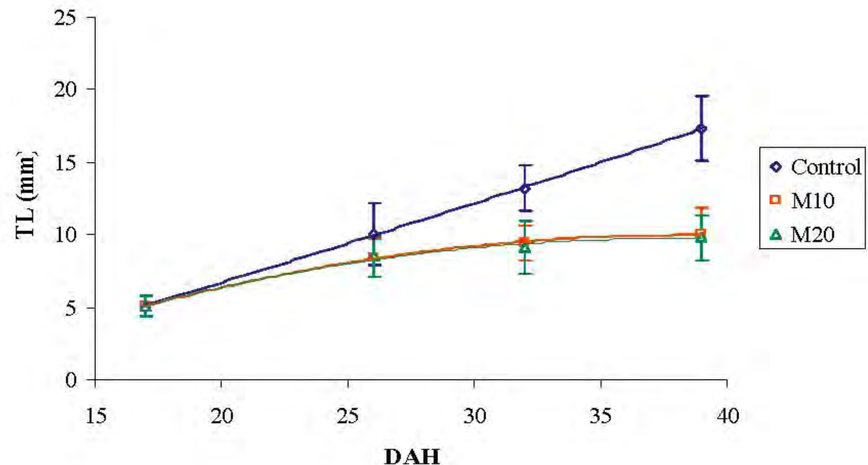

Figure 4. Total length of sole larvae fed *Artemia* (control) and compound diet (M10 and M20) in Experiment 1. Means ± S.D.

Experiment 2

Larvae at the start of Experiment 2 were about double the length and weight of the larvae at the start of Experiment 1. Throughout the Experiment, larvae fed on *Artemia* showed better physical development than larvae fed on the microencapsulated diet (Table 9). RGR for the control group decreased from 14.5 ± 1.7% BW/day to 1.3 ± 0.03% BW/day, whereas for the M10 and M20 groups RGR decreased from 12.9 ± 0.7% BW/day to 0.5 ± 2.2% BW/day and from 13.0 ± 1.1% BW/day to -0.3 ± 0.1% BW/day respectively.

Table 9. Total length (TL), wet weight (WW), dry weight (DW), and relative growth rate (RGR)[1] measurements during Experiment 2.

Control				
DAH	**TL (mm)**	**Mean WW**	**Mean DW**	**RGR (%BW/day)**
17	9.8 ± 1.1	6.2 ± 2.1	1.58 ± 0.4	
24	12.5 ± 0.9	15.9 ± 3.1	3.04 ± 0.6	14.5 ± 1.7
31	15.3 ± 1.4	29.6 ± 7.3	6.12 ± 1.5	9.3 ± 1.7
38	16.2 ± 1.4	32.5 ± 6.7	7.60 ± 1.6	1.3 ± 0.03
M10				
17	9.8 ± 1.1	6.2 ± 2.1	1.58 ± 0.4	
24	12.3 ± 0.8	14.4 ± 2.2	2.80 ± 0.5	12.9 ± 0.7
31	13.1 ± 1.3	18.1 ± 4.5	3.56 ± 0.9	3.4 ± 1.4
38	13.7 ± 1.4	18.8 ± 6.5	4.02 ± 1.4	0.5 ± 2.2
M20				
17	9.8 ± 1.1	6.2 ± 2.1	1.58 ± 0.4	
24	12.1 ± 0.9	14.5 ± 3.1	2.76 ± 0.6	13.0 ± 1.1
31	13.1 ± 1.2	17.9 ± 4.0	3.43 ± 0.8	3.1 ± 0.6
38	13.4 ± 1.5	17.5 ± 6.9	3.75 ± 1.5	0.3 ± 0.1

[1] Values are means ± S.D.

The WW development of the larvae in treatments M10 and M20 was almost identical from 17 to 31 DAH. However, after 31 DAH larvae in treatment M20 started to loose weight (Fig. 6) resulting in a negative RGR (-0.3 ± 0.1% BW/day).

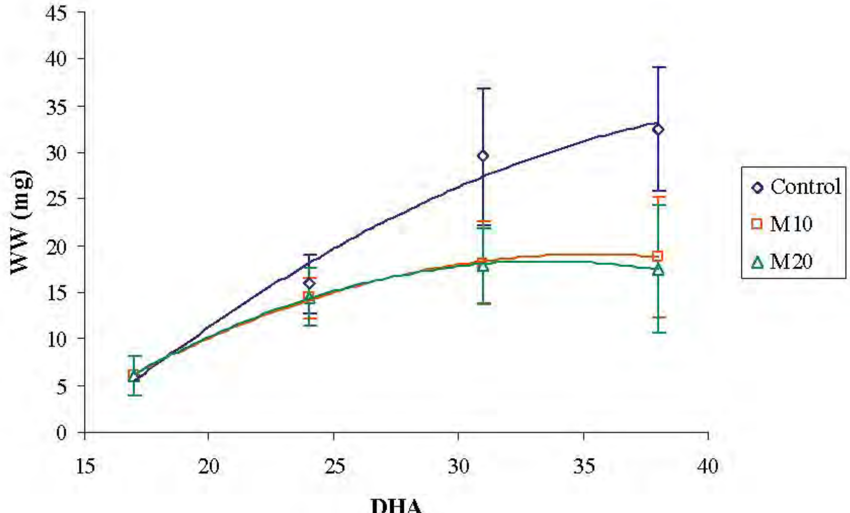

Figure 5. Wet weight of sole larvae fed on *Artemia* (control) and compound diet (M10 and M20) in Experiment 2. Means ± S.D.

Figure 6 shows the frequency distribution of larvae based on their final DW (at 38 DAH). Groups fed on the microencapsulated diet (M10 and M20) show a sharper distribution than the control group. The 60% of the larvae in treatment M10 had a DW between 2.5 and 4.5 mg and the 63% in treatment M20 between 2 and 4 mg. On the other hand, the DW of the 62% of the larvae in the control treatment ranged from 6.5 to 9.5 mg.

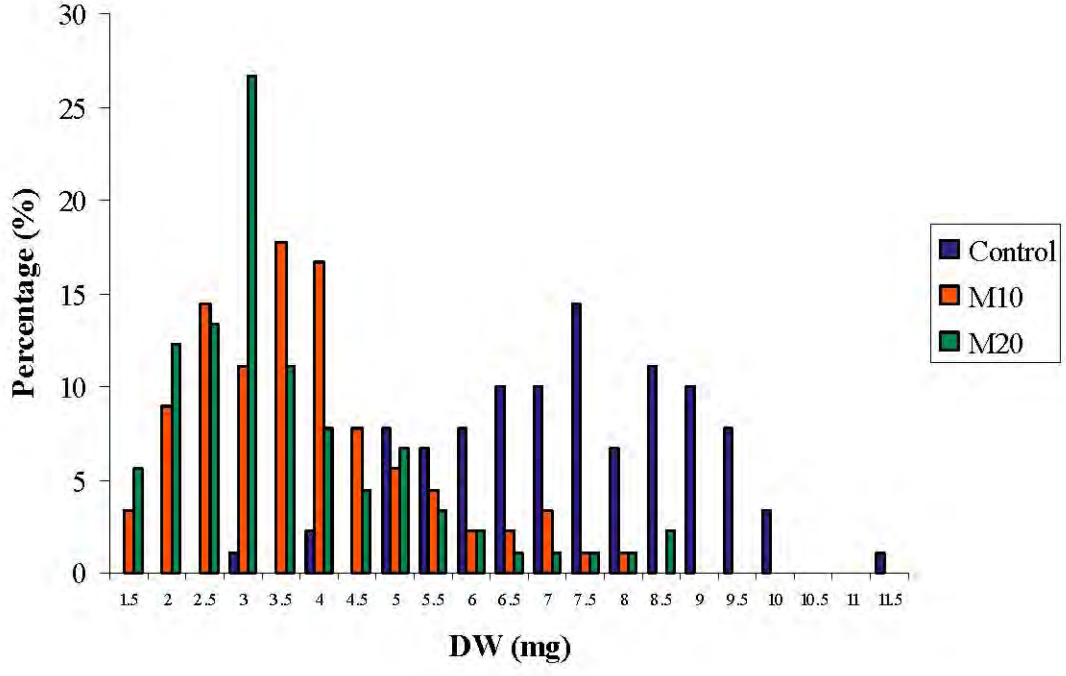

Figure 6. Frequency distribution based on the dry weight of the larvae in Experiment 2.

Survival

In Experiment 1, survival was 85.4 ± 2.4% for the control treatment, 59.6 ± 20.5% for treatment M10, and 58.5 ± 8.9% for treatment M20. Survival was only significantly different between the control and treatment M20. However, in Experiment 2, survival was significantly different between all three treatments. The control had a survival of 99.1 ± 1.1%, the treatment M10 a survival of 58.9 ± 8.0%, and the treatment M20 a survival of 46.9 ± 4.7% (Fig. 7).

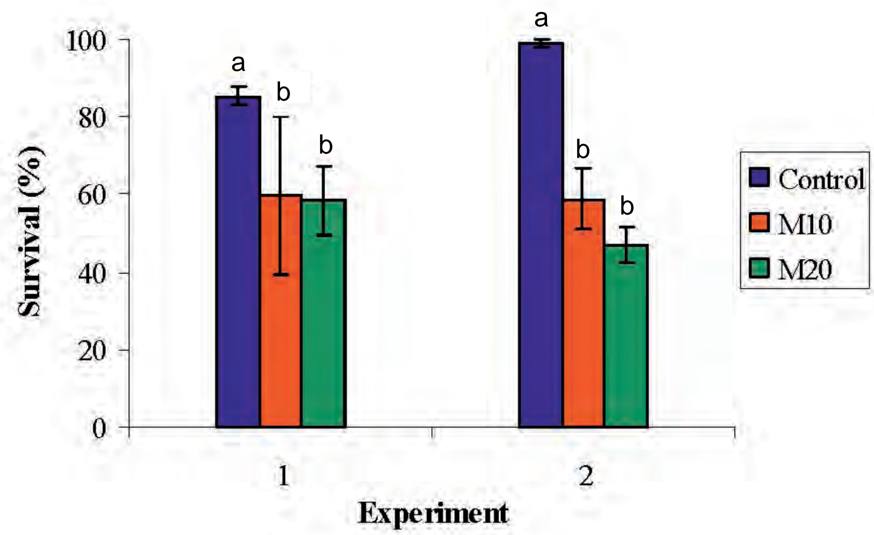

Figure 7. Survival in Experiments 1 and 2. Means ± S.D., with the same superscript letter are not significantly different (P < 0.05).

Gut content

The gut content observation was done at the last day of co-feeding. For Experiment 1, the gut content of the larvae co-fed *Artemia* and the microencapsulated diet had an orange colour (Fig. 8A), which indicates that the main food ingested by those larvae was *Artemia*. There was no sight of any microncapsulated particles in their gut. On the other hand, when larvae were co-fed for 12 days during Experiment 2, their gut content had a white colour (Fig. 8B), which indicates that the larvae had ingested the microencapsulated diet.

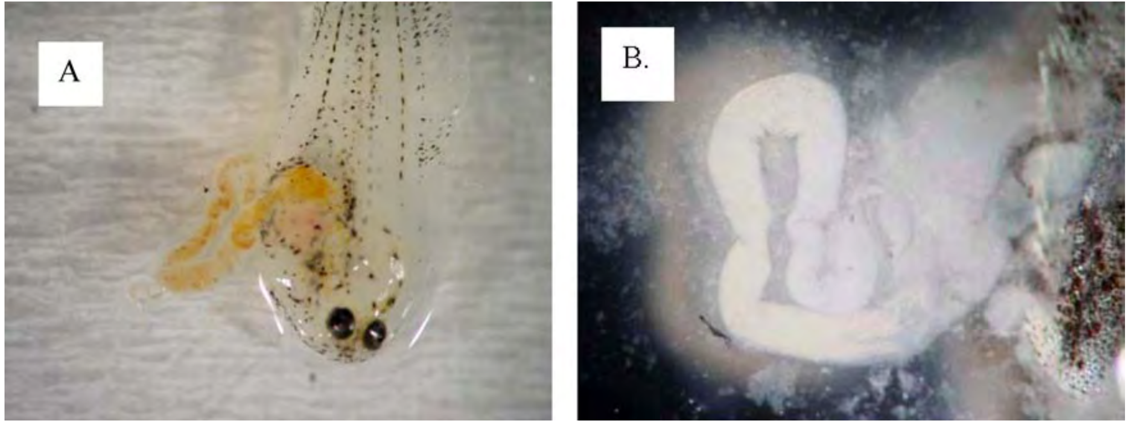

Figure 8. Gut content observations of the larvae co-fed during (A) Experiment 1, and (B) Experiment 2.

Enzyme activity

Experiment 1

Only trypsin and amylase were measured at both sampling times, 26 DAH (after co-feeding) and 39 DAH (end of experiment). Trypsin total, specific, and segmental activities are significantly higher in control than in treatments M10 and M20 for both sampling times (Table 10). Specific activity of all treatments decreases from 26 DAH until 39 DAH, whereas segmental activity increases (Fig. 9A, C).

Table 10. Total, specific and segmental trypsin activities[1] at 26 and 39 DAH in Experiment 1.

Treatment		26 DAH	
	Total Activity	Specific Activity (mU/mg protein)	Segmental Activity
Control	79.28 ± 19.58[a]	65.43 ± 17.05[a]	5.29 ± 1.31[a]
M10	30.16 ± 7.90[b]	34.12 ± 3.91[b]	2.06 ± 0.52[b]
M20	29.30 ± 3.95[b]	35.70 ± 3.78[b]	1.95 ± 0.26[b]
		39 DAH	
Control	214.95 ± 57.34[a]	37.63 ± 3.44[a]	14.33 ± 3.82[a]
M10	40.07 ± 10.70[b]	28.57 ± 6.13[b]	2.67 ± 0.71[b]
M20	39.36 ± 6.69[b]	32.48 ± 6.25[b]	2.67 ± 0.48[b]

[1] Values are means ± S.D. For each sampling day, means within a column with the same superscript letter are not significantly different (P < 0.05).

Amylase specific activity decreases from 26 DAH until 39 DAH (Fig. 11B). There is no significant difference between treatments, with the only exception of treatment M20 at 39 DAH. Amylase segmental activity increases between 26 and 39 DAH only in the case of the control treatment (Fig. 9D). Treatments M10 and M20 have a small decrease (Table 11).

Table 11. Total, specific and segmental amylase activities[1] at 26 and 39 DAH in Experiment 1.

Treatment		26 DAH	
	Total Activity	Specific Activity	Segmental Activity
Control	3.04 ± 1.10[a]	2.51 ± 0.94[a]	0.20 ± 0.07[a]
M10	1.31 ± 0.51[b]	1.51 ± 0.59[a]	0.09 ± 0.04[b]
M20	1.75 ± 0.87[ab]	2.09 ± 0.90[a]	0.12 ± 0.06[ab]
	39 DAH		
Control	3.94 ± 0.82[a]	0.69 ± 0.04[a]	0.26 ± 0.05[a]
M10	1.15 ± 0.29[b]	0.82 ± 0.20[a]	0.08 ± 0.02[b]
M20	1.27 ± 0.34[b]	1.04 ± 0.23[b]	0.09 ± 0.02[b]

[1] Values are means ± S.D. For each sampling day, means within a column with the same superscript letter are not significantly different (P < 0.05).

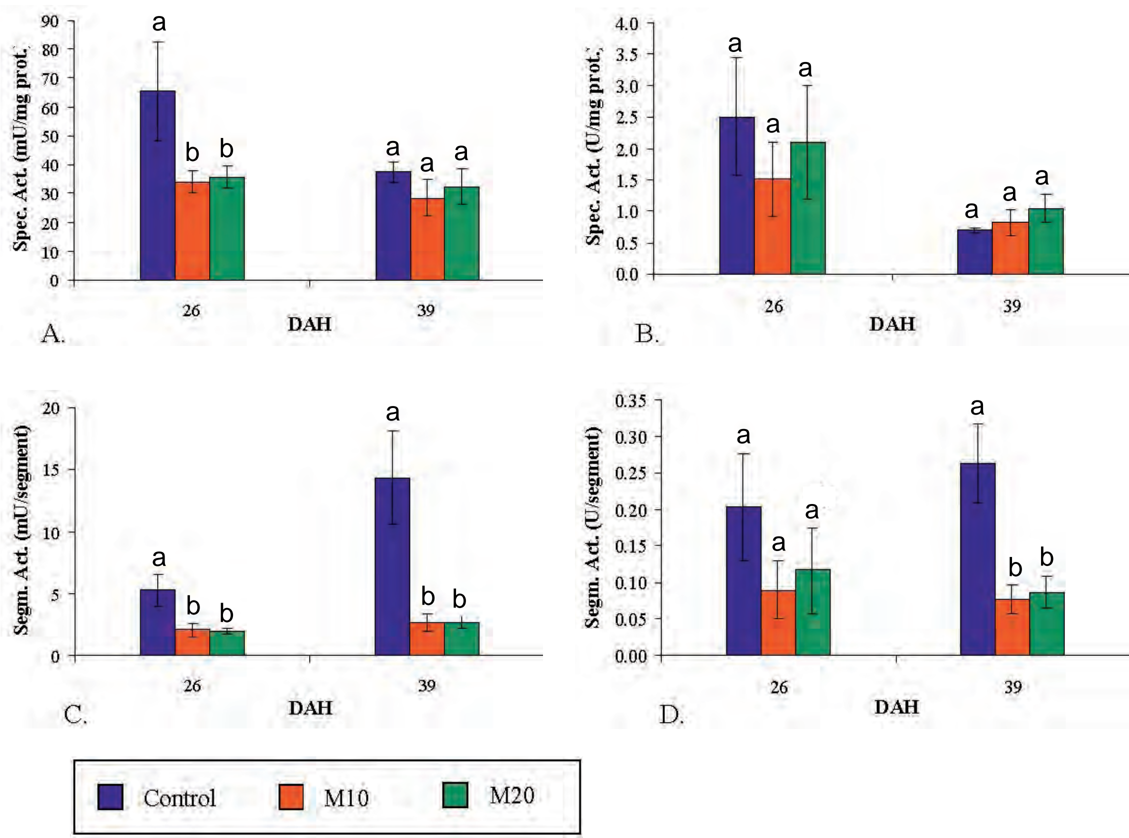

Figure 9. Specific activity of (A) trypsin, and (B) amylase; segmental activity of (C) trypsin, and (D) amylase at 26 and 39 DAH in Experiment 1. Means ± S.D., for each sampling day, columns with the same superscript are not significantly different (P < 0.05).

Protein level is significantly different between control and treatments M10 and M20 at both 26 and 39 DAH (Table 12). The larvae fed on *Artemia* have a higher protein level than the larvae fed on the microencapsulated diet.

Table 12. Protein content of the gut[1] at 26 and 39 DAH in Experiment 1.

Treatment	26 DAH Protein (mg)	39 DAH Protein (mg)
Control	1.22 ± 0.04[a]	5.68 ± 1.16[a]
M10	0.87 ± 0.13[b]	1.40 ± 0.17[b]
M20	0.82 ± 0.06[b]	1.23 ± 0.19[b]

[1] Values are means ± S.D. Means within a column with the same superscript letter are not significantly different (P < 0.05).

Alkaline phosphatase total and segmental activities are higher in control than in treatments M10 and M20. Also, there is a significant difference between treatment M10 and M20 with the latter having lower activities. On the other hand, specific activity is higher in M10 and M20 than in control treatment (Table 13).

Table 13. Total, specific and segmental alkaline phosphatase activities[1] at 39 DAH in Experiment 1.

Treatment	39 DAH		
	Total Activity	**Specific Activity (mU/mg protein)**	**Segmental Activity**
Control	748.48 ± 113.58[a]	133.94 ± 18.23[a]	49.90 ± 7.57[a]
M10	372.05 ± 44.42[b]	267.73 ± 29.28[b]	24.80 ± 2.96[b]
M20	290.77 ± 49.74[c]	239.50 ± 42.77[b]	19.66 ± 3.09[c]

[1] Values are means ± S.D. Means within a column with the same superscript letter are not significantly different (P < 0.05).

Pepsin activity showed a pattern similar to the one of alkaline phosphatase, with the total and segmental activities being higher in the control treatment and the specific activity being higher in treatments M10 and M20 (Table 14).

Table 14. Total, specific and segmental pepsin activities[1] at 39 DAH in Experiment 1.

Treatment	39 DAH		
	Total Activity	**Specific Activity**	**Segmental Activity**
Control	6.72 ± 2.22[a]	1.17 ± 0.26[a]	4.48 ± 1.48[a]
M10	3.06 ± 1.26[b]	2.21 ± 0.95[b]	2.04 ± 0.84[b]
M20	3.25 ± 2.44[b]	2.58 ± 1.56[b]	2.20 ± 1.63[b]

[1] Values are means ± S.D. Means within a column with the same superscript letter are not significantly different (P < 0.05).

Experiment 2

Total, specific, and segmental trypsin activities showed a small increase from 31 DAH until 38 DAH, with the exception of specific activity of treatment M20, which showed a small decrease (Table 15; Fig. 12A, C). Control has higher activities than the other two treatments, which are generally significantly different.

Table 15. Total, specific and segmental trypsin activities[1] at 31 and 38 DAH in Experiment 2.

Treatment	Total Activity	Specific Activity (mU/mg protein)	Segmental Activity
		31 DAH	
Control	74.11 ± 15.80[a]	28.25 ± 4.79[a]	4.94 ± 1.05[a]
M10	42.23 ± 3.95[b]	21.59 ± 2.47[b]	2.88 ± 0.20[b]
M20	40.50 ± 5.38[b]	22.22 ± 2.62[ab]	2.70 ± 0.36[b]
		38 DAH	
Control	113.76 ± 18.64[a]	28.77 ± 3.22[a]	7.58 ± 1.24[a]
M10	43.38 ± 9.65[b]	21.81 ± 3.19[b]	2.98 ± 0.60[b]
M20	35.91 ± 10.69[b]	21.54 ± 3.35[b]	2.97 ± 0.96[b]

[1] Values are means ± S.D. For each sampling day, means within a column with the same superscript letter are not significantly different (P < 0.05).

At 31 DAH, the specific activity of amylase for M20 was higher than control for the M10 treatments, but only significantly different from the latter (Fig.12B). At 38 DAH, both treatments, which were given the microencapsulated diet, have higher amylase specific activity. Amylase segmental activity shows an increase between 31 and 38 DAH, with the control treatment to have a higher activity than the other two (Table 16; Fig. 12D).

Table 16. Amylase total, specific and segmental activities[1] at 31 and 38 DAH in Experiment 2.

Treatment	Total Activity	Specific Activity	Segmental Activity
		31 DAH	
Control	1.75 ± 0.43[a]	0.67 ± 0.15[a]	0.12 ± 0.03[a]
M10	0.85 ± 0.14[b]	0.43 ± 0.07[b]	0.06 ± 0.01[b]
M20	1.46 ± 0.57[ab]	0.79 ± 0.27[a]	0.10 ± 0.04[ab]
		38 DAH	
Control	2.55 ± 0.66[a]	0.65 ± 0.15[a]	0.17 ± 0.04[a]
M10	1.76 ± 0.28[b]	0.91 ± 0.19[b]	0.12 ± 0.02[b]
M20	1.59 ± 0.41[b]	0.98 ± 0.22[b]	0.13 ± 0.03[b]

[1] Values are means ± S.D. For each sampling day, means within a column with the same superscript letter are not significantly different (P < 0.05).

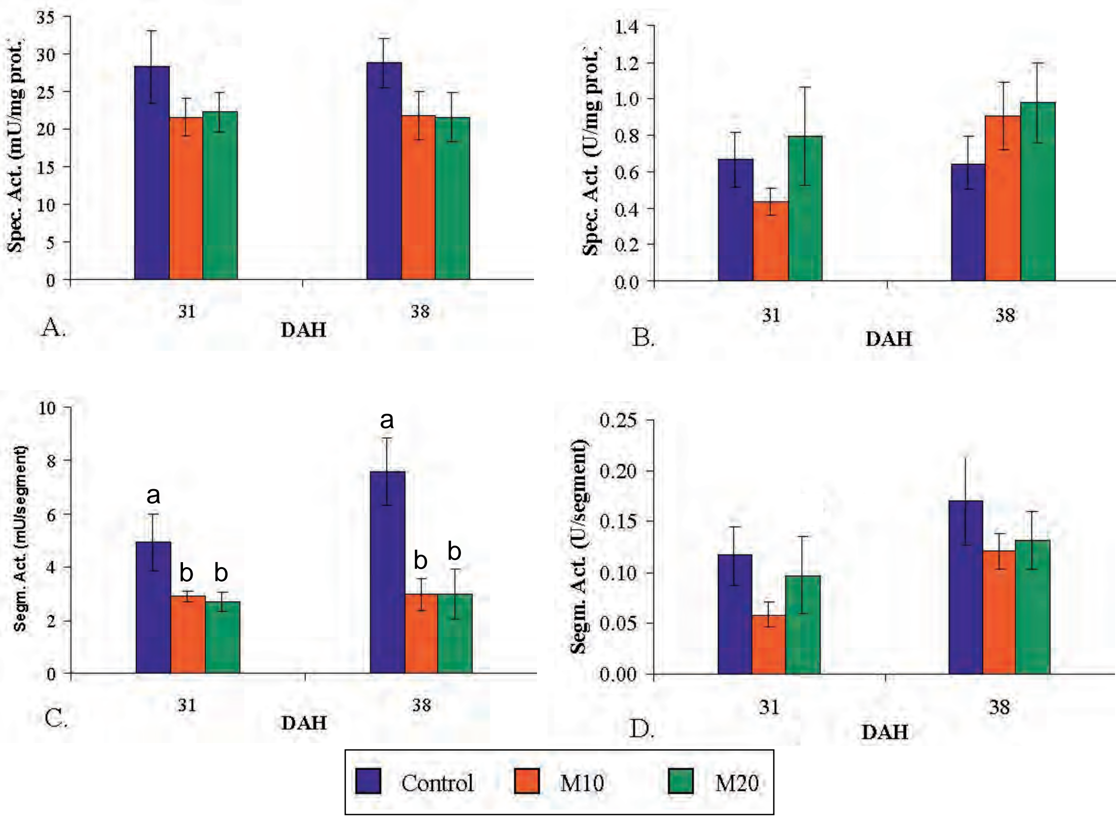

Figure 10. Specific activity of (A) trypsin, and (B) amylase; segmental activity of (C) trypsin, and (D) amylase at 26 and 39 DAH in Experiment 2. Means ± S.D., for each sampling day with the same superscript are not significantly different (P < 0.05).

Protein levels increased between 31 and 38 DAH, and like in Experiment 1, the control had higher levels than treatments M10 and M20 (Table 17).

Table 17. Protein content of the gut[1] at 31 and 38 DAH in Experiment 2.

Treatment	31 DAH Protein	38 DAH Protein
Control	2.61 ± 0.16[a]	3.94 ± 0.28[a]
M10	1.96 ± 0.05[b]	1.99 ± 0.37[b]
M20	1.83 ± 0.17[b]	1.66 ± 0.37[c]

[1] Values are means ± S.D. Means within a column with the same superscript are not significantly different (P < 0.05).

Alkaline phosphatase specific activity has no significant difference between treatments. However, segmental activity is higher in control treatment than in treatments M10 and M20, which have no significant difference between them (Table 18).

Table 18. Total, specific and segmental Alkaline phosphatase activities[1] at 38 DAH in Experiment 2.

Treatment	38 DAH		
	Total Activity	**Specific Activity (mU/mg protein)**	**Segmental Activity**
Control	499.78 ± 43.53[a]	127.56 ± 14.41[a]	33.32 ± 2.90[a]
M10	255.77 ± 55.17[b]	129.36 ± 20.96[a]	17.55 ± 3.22[b]
M20	197.56 ± 51.87[c]	119.52 ± 20.63[a]	16.22 ± 3.30[b]

[1] Values are means ± S.D. Means within a column with the same superscript are not significantly different (P < 0.05).

Pepsin specific activity is higher in the treatments given the microencapsulated diet, whereas segmental activity is higher in control treatment given *Artemia* (Table 19).

Table 19. Total, specific and segmental pepsin activities[1] at 38 DAH in Experiment 2.

Treatment	38 DAH		
	Total Activity	**Specific Activity**	**Segmental Activity**
Control	5.53 ± 1.52[a]	1.39 ± 0.32[a]	3.69 ± 1.01[a]
M10	3.88 ± 0.78[b]	2.00 ± 0.49[b]	2.66 ± 0.43[b]
M20	3.10 ± 1.17[b]	1.88 ± 0.72[b]	2.62 ± 1.18[b]

[1] Values are means ± S.D. Means within a column with the same superscript are not significantly different (P < 0.05).

Comparison between Experiment 1 and Experiment 2

The comparison is done between the enzyme activities obtained for each treatment from the final sampling of each experiment. The difference in larval age is only one day, so age effect does not have a big influence in any enzyme activity difference attained.

The specific activities of trypsin and alkaline phosphatase in the larvae that fed on the microencapsulated diet are found to be higher in Experiment 1 than in Experiment 2 (Fig. 11A, C). Concerning the specific activities of amylase and pepsin, there is a tendency for higher activities in Experiment 1 than in Experiment 2, with the only exception being the amylase specific activity of treatment M10 (Fig. 11B, D).

A different picture is observed, when the segmental enzyme activities are compared. Amylase and pepsin (only treatment M10) segmental activities are higher in the larvae that fed on the microencapsulated diet in Experiment 2 than in Experiment 1 (Fig. 12B, D). The same tendency is also observed for trypsin and pepsin (treatment M20) segmental activities (Fig. 12A, D). However, alkaline phosphatase segmental activity in treatments M10 and M20 is higher in Experiment 1 than in Experiment 2 (Fig. 12C).

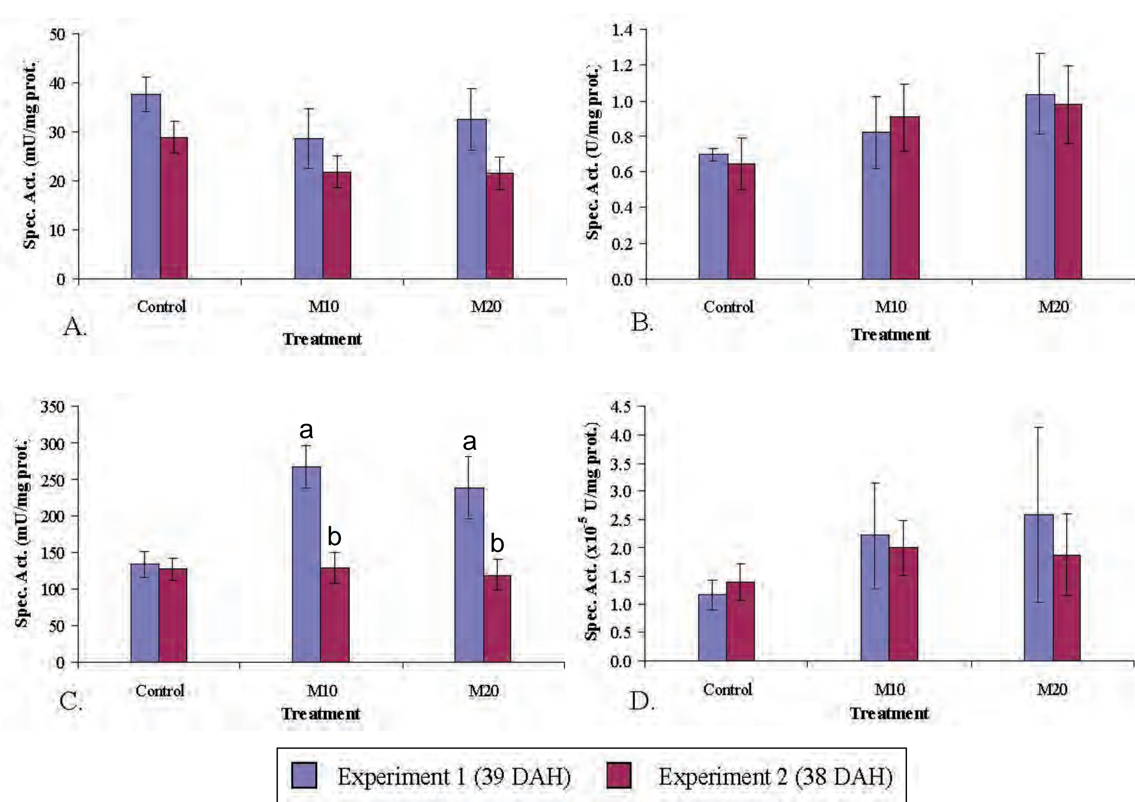

Figure 11. Comparison of the specific activities of (A) trypsin, (B) amylase, (C) alkaline phosphatase, and (D) pepsin, between the two experiments. Means ± S.D., for each treatment with the same superscript letter are not significantly different (P < 0.05).

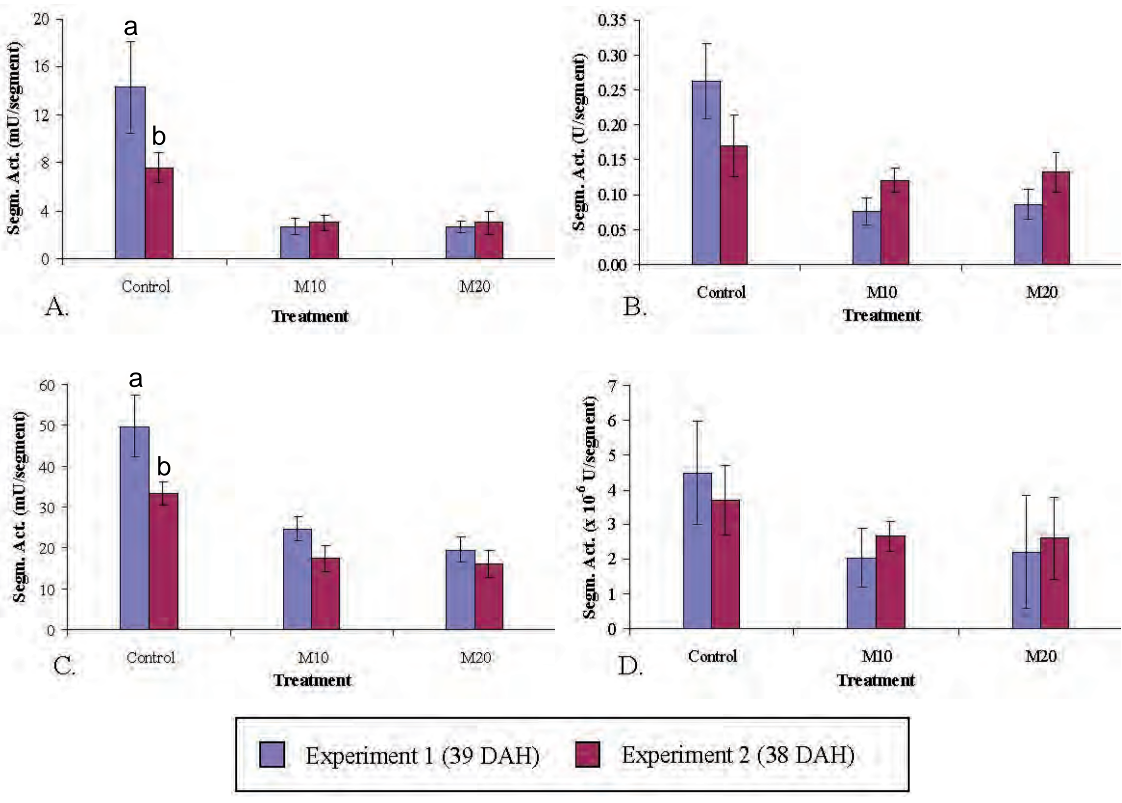

Figure 12. Comparison of the segmental activities of (A) trypsin, (B) amylase, (C) alkaline phosphatase, and (D) pepsin, between the two experiments. Means ± S.D., each treatment with the same superscript letter are not significantly different (P < 0.05).

5.4.2.1.6 Discussion

The growth achieved by the larvae in the control group is similar to the growth achieved in the experiment of Dinis and Reis (1995). The final TL during Experiments 1 and 2 were 17.3 ± 2.2 mm (at 39 DAH) and 16.2 ± 1.4 mm (at 38 DAH) respectively, and Dinis and Reis (1995) reported a TL of 16 ± 0.84 mm at 38 DAH. The corresponding final TL of the larvae that fed on the microencapsulated diet were significantly smaller, ranging from 9.8 ± 1.6 mm (M20 of Experiment 1) to 13.7 ± 1.4 mm (M10 of Experiment 2). This demonstrates that the microencapsulated diet used in this experiment cannot support a growth potential comparable to that of *Artemia*. This conclusion is also supported by the results obtained for WW, DW, and RGR. The minimum final WW and DW achieved from the control treatments were 32.5 ± 6.7 mg and 7.60 ± 1.6 mg (both at Experiment 2) respectively, which were significantly above the maximum final WW (18.8 ± 6.5 mg) and DW (4.02 ± 1.4 mg) achieved on the treatments fed on the microencapsulated diet. The same holds for RGR, where control treatments had significantly higher values than treatments M10 and M20 throughout the two experiments.

Comparing treatment M10 with M20, it can be said that there is not much difference between the two as far as growth is concerned. Figures 7 and 8 show that in both experiments growth during development and final growth were almost identical for both treatments. These findings suggest that larval growth of *S. senegalensis* is the same when 10% or 20% of fish protein hydrolysate is incorporated in the type of larval diet used. In this experiment, it could not be observed if the addition of fish protein hydrolysate promoted growth, as there was not a treatment fed on microencapsulated diet without protein hydrolysate. However, according to the findings of Cahu et al. (1999), and Kolkovski and Tandler (2000) dietary inclusion of protein hydrolysate coming from squid or fish does promote growth in other species. These authors also concluded that there is an optimum range, and not an optimum value of protein hydrolysate that has positive effects on fish growth. Kolkovski and Tandler (2000) suggested that squid protein hydrolysate in microdiets at levels greater than 50% cause suppressed growth in gilthead seabream (*S. aurata*). In addition, Cahu et al. (1999) demonstrated that 19% of fish protein hydrolysate promoted better growth than 38% or 58% in sea bass larvae (*D. labrax*).

The effect of the duration of co-feeding on larval growth was not clear in the present experiments because the larvae at the start of Experiment 2 were almost double the length and three times the weight of the larvae at the start of Experiment 1.

One of the main problems indicated by Dinis and Reis (1995) in the culture of *S. senegalensis* is the high mortality of larvae during weaning. Specifically, weaning the larvae 38 DAH produced a survival around 33%. In this experiment, survival (at 38-39 DAH) of the larvae that were weaned 17 DAH ranged between 46.9 ± 4.7% and 59.6 ± 20.5%. The survival was higher in treatments fed *Artemia* alone than treatments M10 and M20. The low survival of *S. senegalensis* during weaning was also illustrated by Canavate and Fernandez-D'iz (1999); the survival of the control group that fed on *Artemia* was 78.5 ± 5.0% 70 DAH, whereas survival of the groups that were weaned (at 43 DAH) ranged between 0% and 39.0 ± 4.2%. The same problem is also noticed in the larviculture of Dover sole (*S. vulgaris*), where weaning produce high mortality rates (Metailler et al. 1981). However, the larvae of another flatfish, Atlantic halibut (*H. hippoglossus* L.), can survive the weaning period with no apparent mortalities. Naess et al. (2001) reported that survival of weaned halibut ranged between 78% and 96.6%, in comparison with the 96% survival of the control group.

The gut content observations at the end of the co-feeding period suggest that the larvae co-fed for 12 days (Experiment 2) ingested the microencapsulated diet more readily than the larvae co-fed for 3 days (Experiment 1). It appears that 3 days of co-feeding period is not enough for the larvae

to adapt in ingesting the formulated particles. Stoss et al. (2004), and Kolkovski (2001) mentioned that co-feeding does help in the ingestion of inert diets, but the required duration of co-feeding is species specific.

Trypsin specific and segmental activities of the larvae that fed on *Artemia* were higher than the ones of the larvae that fed on the microencapsulated diet. The differences tended to decrease, as the larvae got older. The higher trypsin activity per larva found in the control group may be due to the better growth of this group. Nolting et al. (1998) illustrated that there is a positive correlation between larval length and trypsin segmental activity in sea bass larvae. This correlation was also demonstrated by Oozeki and Bailey (1995) for pollock (*Theragra chalcogramma*) larvae. The values of trypsin segmental activity found by Ribeiro et al. (1999) are lower than the ones obtained in this study. The analysis in Ribeiro et al. (1999) was done from 2 to 18 DAH, therefore the development of the digestive system was at a more precocious stage. However, the values of trypsin specific activity are very similar with the values obtained in this study. In Ribeiro et al. (1999), trypsin specific activity fluctuates between 25 and 30 mU/mg protein (from 12 to 18 DAH), in comparison with 28.25 ± 4.79 to 28.77 ± 3.22 mU/mg protein (from 31 to 38 DAH; Experiment 2) in this study. This observation suggests that the capacity of the *S. senegalensis* larvae to produce trypsin per mg of protein does not change in the later part of their larval development. A similar trypsin specific activity was also found in the larvae of sea bass, *D. labrax* (Zambonino Infante and Cahu, 1994a).

The lower trypsin specific activity in the larvae that fed on the microencapsulated diet can be attributed either to the slow growth or the lack of exogenous enzymes. As already mentioned in paragraph 2.5, it is believed that live prey assists digestion in larvae by contributing digestive enzymes (Kolkovski, 2001). Considering that trypsin levels are related to the protein content, another possible hypothesis is that the protein quantity in the digestive tract was low in the larvae that fed on the microencapsulated diet due to low ingestion rates. Co-feeding period showed to have no effect on trypsin segmental activity, since there was no significant difference between the same treatments. However, the 3-day co-feeding period produced higher trypsin specific activity than the 12-day co-feeding period; this may be due to lower ingestion rates in the larvae co-fed for a shorter time. Between the two fish hydrolysate levels (10% and 20%) there was no significant difference in either specific or segmental activity.

Amylase specific activity at last sampling of each experiment was higher in the larvae that fed on the microencapsulated diet than in the larvae that fed on *Artemia*. A similar result was obtained by Zambonino Infante and Cahu (1994a) in their study on sea bass, *D. labrax*. In their study, larvae from 25 to 40 DAH that fed on compound diet had higher amylase specific activity than larvae that fed on live prey. This phenomenon can be attributed to the ability of larvae to alter their digestive capacity in response to a new diet. Although, adaptation to a new diet seems to be vital, amylase specific activity of *S. senegalensis* larvae was not affected by the duration of co-feeding period.

Looking at the amylase activity per larva, the control group had higher segmental activity than treatments M10 and M20. The values of amylase segmental activity, which were obtained 26 and 39 DAH during Experiment 1, and 31 and 38 DAH during Experiment 2, are similar to the ones obtained by Ribeiro et al. (1999) from 12 to 18 DAH (about 16-21 U/segment). This suggests that after the initial increase of amylase segmental activity during the first stages of their larval life, amylase segmental activity stays rather stable during the later stages. Comparing the two co-feeding periods, it appears that long (12-day) co-feeding produced higher amylase segmental activities than short (3-day) co-feeding, in both treatments M10 and M20. Between the two fish hydrolysate levels (10% and 20%), in most cases, there was no significant difference on either specific or segmental activity of amylase.

In Experiment 1, alkaline phosphatase specific activity was higher in the larvae that fed on the microencapsulated diet than in the larvae that fed on *Artemia*. This was again also observed in sea bass larvae from 25 to 40 DAH (Zambonino Infante and Cahu, 1994a). However, during Experiment 2, this trend was not observed; there was not significant difference in alkaline phosphatase specific activity between treatments. Segmental activity, on the other hand, was higher in the control treatment than in treatments M10 and M20, in both experiments.

Pepsin specific activity was significantly higher in the weaned larvae than in the larvae of the control treatment. This phenomenon was observed also for the sea bass larvae in the study of Zambonino Infante and Cahu (1994b), where pepsin specific activity was 3 times higher in larvae that fed on compound diet than in larvae that fed on live food. This can suggest that pepsin activity is not a determinant factor in the digestion of *S. senegalensis* larvae, and alone cannot ensure high digestion efficiency. Pepsin segmental activity, on the other hand, was significantly higher in the control treatment than in treatments M10 and M20. This can be attributed to the better growth of the larvae that fed on *Artemia*.

5.4.2.1.7 Conclusion

Sole larvae can be adapted to ingest a formulated diet as soon as 17 DAH, if a co-feeding period is employed. The high specific activities of amylase, alkaline phosphatase, and pepsin in the weaned larvae, show that larvae can alter their digestive capacity in response to a new diet. Also, the low growth and survival attained from the weaned larvae cannot be attributed to the lack of digestive enzymes or their specific activity. Incorporation of either 10% or 20% of fish protein hydrolysate in the diet did not produce any significant difference in larvae performances or enzyme activity. However, the possible role of fish protein hydrolysate in sole diets should not be underestimated, when diets that produce better growth are employed. Finally, it can be concluded that *S. senegalensis* larvae seem to have the digestive capacity for an early weaning, but formulation of a species-specific diet, considering nutritional, physical, and attractability aspects, is required.

5.4.2.1.8 Acknowlegments

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5.4.2.1.9 References

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5.4.2.2 Effect of varying dietary lipid and protein levels on reproductive performance of female swordtails *Xiphophorus helleri* (Poeciliidae)

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5.4.2.2.1 Introduction

The guppies (*Poecilia reticulata*), mollies (*P. latipinna, P. sphenops*), swordtails (*Xiphophorus helleri*), and platies (*X. maculatus*) are a very popular group of ornamental fish species due to the existence of variety of body colors and fin patterns. The culture of swordtail is concentrated in Singapore, Malaysia, Indonesia, Thailand, India and China in earthen ponds or floating net cages. Feeding practices however, are of poor standard due to over-reliance on live macro-organisms such as *Tubifex* worms and freshly prepared wet feeds (Fernando et al., 1991). Development of proper formulated diets to avoid potential problems dealing with such diets is therefore needed.

In teleosts, nutrients such as protein, fatty acids, vitamin E, ascorbic acids and carotenoids have been implicated to play influencing roles affecting various reproductive-related processess such as gonadal maturation, gamete quality and spawning performances (Izquierdo et al. 2001; Watanabe and Vassallo-Agius, 2003). However, broodstock nutrition for many species is still poorly understood due to difficulties in conducting studies involving proper feeding and reproduction.

Swordtails are viviparous breeders with females storing transferred sperms within the ovary for internal egg fertilisation, followed by hatching of eggs and a gestation period of approximately 27 days prior to release of free-swimming embryos (Siciliano, 1972). Studies have shown that protein and lipid have important roles in improving reproductive performances of live bearers. (Dzikowski et al., 2001; Kruger et al., 2001a). In teleost reproduction, supply of protein and lipid are critical to developing embryos prior to initiation of exogenous feeding (Brooks et al., 1997 Fernandez-Palacios et al., 1997; Izquierdo et al., 2001; Mazorra et al., 2003)

We have earlier reported on the dietary protein requirements for both growth and reproductive performances in female swordtail breeders (Chong et al. 2004). This present study investigates the interactive role of dietary protein and lipid levels on reproductive performances of female swordtails.

5.4.2.2.2 Materials and methods

Eight practical diets were formulated based on composition outlined in Table 1. Two protein levels (20% and 30%) with 4 lipid levels (8%, 12%, 16%, 20%) within each protein level were formulated. Diets were labeled as 20P8L, 20P12L, 20P16L, 20P20L, 30P8L, 30P12L, 30P16L and 30P20L, respectively. Size of experimental pellets was 0.2-0.3 mm. Proximate compositions of diets are also shown in Table 1.

The processes of raising, conditioning and selection of experimental fish for feeding trial were as in Chong et al. (2004). Virgin females aged 22 weeks old (1.2 -1.3 g) were used for all experiments.

Experimental design and feeding trials

Experimental design also follows that of Chong et al. (2004) with five replicate tanks stocked with 5 females used for evaluation of each diets. Virgin males aged 20 weeks were kept separately in a large tank (4' x 3' x 3') and fed frozen bloodworms twice daily. These males were randomly selected with 2 males introduced to each experimental tank every 30 days. Males were left in

these tanks for 5 days before returning to the common holding tank. During feeding, males were separated from females using plastic sheets. Shelters in the form of bundles of tied-nylon strings were offered to avoid cannibalism of new free-swimming fry by parental fish.

Feeding was carried out to satiation twice daily at 0900 and 1700. Inspection for newborn fry was also carried out at this time. The feeding trial lasted for 26 weeks. At the end of both experiments, the individual weight of females was measured before they were sacrificed for proximate analysis of muscle and ovary tissues. Parameters analyzed were:

Specific growth rate (SGR% / day): $[(\ln W_t - \ln W_i) / T)] \times 100$

where W_t = Mean final weight, W_i = Mean initial weight, T = total experimental days

Feed conversion ratio (FCR): total feed fed (g) / total wet weight gain (g)

Total fry production: Total fry harvested throughout experimental period

Relative fecundity: Total fry production at throughout experimental period / mean weight of female (g): Total fry produced / dietary protein intake: Total fry production at end of experiment / total protein given (g).

HIS hepatosomatic index: (weight x100 / body weight)

GSI gonadostomatic index: (ovary weight x 100 / body weight)

VSI Visceralsomatic Index: (viscera weight x100 / body weight)

In order to investigate to effect of dietary protein level on fry quality, fry were sampled and immediately frozen at -20°C for measurement of total length, dry weight and proximate composition.

Data and Statistical analysis

Comparison of various growth and reproductive parameters from different dietary treatments was carried out using analysis of variance (ANOVA) and where applicable, Tukey's HSD ($P < 0.05$). Percentages data was transformed to arc-sin before being subjected to statistical analysis.

5.4.2.2.3 Results

The final weight of females was relatively higher when fed with diets containing 30% protein (Table 2). For the 20% dietary protein, larger females were obtained with the 12% and 16% dietary lipid diets. As for the 30% dietary protein diets, the 8% dietary lipid diet (30P8L) treatment resulted in heaviest females. Weight gain and SGR values were also generally higher with the 30% protein diets as compared with the 20% level. Among the 20% protein diets, growth rate was poorest with diets 20P8L and 20P20L. Highest growth rate was obtained with the 30P8L and 30P16L diets. Feed consumption was also affected by dietary treatments, with fish receiving diets with highest lipid level (20P20L and 30P20L) showing lowest feed intake. In addition, fish receiving the 20P8L diet also showed low amount of intake. Highest feed intake was recorded with diets 30P8L. Poor FCR values were obtained with diets 20P8L and 20P20L. The 20% dietary protein diets also resulted in significantly shorter females in comparison to the 30% diets. However, there was no difference among the 4 dietary lipid levels for the 30% protein level. As for the 20% protein level diets, the 20% lipid resulted in the lowest total length, followed by 8%, 12% and 16% lipid respectively.

GSI values showed that the highest index was obtained with 30P20L diets while diet 20P8L gave the significantly lowest value. There was no significant difference in GSI among the rest of the diets. HSI was significantly lowest in diets 20P8L, 20P12L and 20P16L while highest HIS values were obtained by the 30P12L and 30P16L respectively. Diet 30P16L, together with diet 20P20L

also caused the highest visceral to body weight ratio while no significant differences were obtained among the rest of the diets.

Proximate analysis showed differences in composition of female muscle among different dietary treatments. Significantly higher muscle protein deposition occurred in the 30% protein diets as compared to the 20% level. As for muscle lipid levels, there was no obvious trend among different lipid levels for the 20% protein diets. Lipid levels were generally lower than the 30% dietary protein diets, who showed a positive correlation when dietary lipids increases. There was no clear trend in ash levels. Analysis of ovary shows that both protein and lipid levels were lowest with the 20P8L diet as compared to other dietary treatments.

Mean overall fry production was significantly higher in 30% dietary protein diets as compared to the 20% level (Figure 1). Among the 20% protein level, diet 20P8L produced the least number of fry. There was however no significant difference between fry production among the different lipid levels in the 30% protein category. Significant correlations were also obtained between fry production and final weight (r=0.73) and final total length (r = 0.64) of females respectively (Fig. 2). When fry production was calculated as relative fecundity, results showed that diet 20P8L resulted in the lowest fecundity while diet 30P8L and 30P20L resulted in the highest fecundity (Fig 3).

Figures 4, 5 and 6 compare fry production among different diets taking into consideration amount of feed and nutrient intake. When reproduction was measured in terms of total fry production per g of feed intake, results showed that diet 30P12L, 30P16L and 30P20L gave the highest significant values. A similar trend was also observed with total fry production per g of protein intake (Fig 5). Evaluation of fry production in terms of amount of lipid intake also showed significantly higher values with 30% dietary protein diets as compared to the 20% protein levels (Fig 6). There were however no significant differences among the 4 lipid levels.

There was no significant difference in both total length and weight measurements of fry produced among the different dietary treatments (Table 3). Proximate analysis of larva showed that while protein content was lowest in diet 20P8L, there was no clear trend in lipid and ash levels.

5.4.2.2.4 Discussion

This present study demonstrates the combinatory roles of dietary protein and lipid levels on growth and reproductive performances of swordtail females. Our results demonstrate that better growth is obtained with diets containing 30% dietary protein as compared to 20%. This is in agreement with our earlier study which showed that growth rate of female swordtail brood was significantly higher with the 30% dietary protein treatment as compared to the 20% level (Chong et al., 2004). This reinforces the fact that 20% dietary protein is below the optimal requirement needed for growth in swordtail. The present study shows that increasing the dietary lipid level from 8% to 12% and 16% for the 20% dietary protein level caused significant increase in final weight, SGR and body length of female swordtails brood. Improved growth parameters as a result of feeding with higher dietary lipid levels have been reported numerous teleost species (Hemre and Sandnes, 1999; Vergara et al., 1999; Chaiyapechara et al., 2003). This could be attributed to the mechanism of protein sparing action where lipid spares the utilisation of protein as energy. As for the 30% dietary protein level, there were no significant differences in growth parameters among the four lipid levels. We hypothesize that at this protein level, the supply of protein was enough to fulfill requirements of the female brood. Several studies have pointed out the lack of protein sparing effect by lipid when adequate or excessive proteins are supplied (Gaylord et al., 2003; Hebb et al., 2003). The slightly inferior SGR obtained for diets 20P20L and 30P20L as compared to the 12% and 16% lipid levels diets could be attributed to the lower feed intake due to satiation by high lipid content. Reduced appetite and hence lower feed intake was reported in salmon with relatively higher body fat levels due to utilisation of high fat diets (Shearer et al., 1997).

The present study showed that diet 20P8L also caused the lowest GSI index and levels of protein and lipid in the ovary. Increasing the lipid level, however, significantly improved these parameters. Besides the possible role in sparing protein from being used for energy, lipid is also crucial for the formation of membrane structures in developing embryos. In comparison with marine species, relatively fewer studies have been reported on freshwater broodstock lipid requirements. Although freshwater species possess the ability to convert 18:3n-3 to HUFAs, several studies evidently showed that dietary requirements for some fatty acids such as arachidonic acid exists to properly modulate reproductive activities (Mercure and Van Der Kraak, 1996; Tamaru et al., 1997). Therefore, the detailed role of dietary lipids in general and fatty acids in particular in freshwater teleost reproduction awaits further studies. Our results showed that female swordtail hepatosomatic index was lower with the 20% protein level as compared to the 30% protein level. Studies have shown a general increase in HSI value during vitellogenesis due to high protein synthetic activities (van Bohemen et al., 1981; Koya et al., 2000). This higher rate of vitellogenesis probably explains the resulting higher fecundity performance in the 30% protein level diets. Our study does not seem to show any specific trend in the role of dietary treatments on visceral weight. Elsewhere, Chaiyapechara et al. (2003) also did not obtain any significant increase in VSI when high lipid levels were fed to rainbow trout.

The general increase in muscle lipid deposition when fed with increasing dietary lipid levels in swordtail females also agrees with observations reported in many species (Chou and Shiau, 1996; Chaiyapechara et al., 2003; Gaylord et al., 2003; Ai et al., 2004; Boujard et al., 2004). The relatively lower lipid deposition in muscle among the 20% protein diets could also be attributed to the use of lipid as a source of energy as protein is being spared for either growth or reproduction. Studies have indicated the possibility of fatty acid transfer from body reserve to eggs in situations where requirement for such fatty acids increases during spawning season (Furuita et al., 2000). Oocyte maturation involves transportation and accumulation of large amounts of protein and lipid in oocytes. Therefore, the lower protein obtained in both muscle and ovary of female swordtail fed with the 20% protein probably indicates limited or insufficient protein for maintenance and oocyte development and hence, utilisation of body reserves. Other studies elsewhere have reported inferior protein content in fish muscle and oocytes when dietary levels are insufficient especially during spawning seasons (Gunasekera et al., 1997; Al Hafedh et al., 1999).

In tandem with the female growth parameters, this study also shows that the 30% dietary protein level resulted in a significantly higher fry production as compared to the 20% level. The positive correlation obtained between fry production and female final weight and length reinforces the fact that female size is an important function for reproductive performance (Milton and Arthington, 1983; Gunasekera et al., 1996; El-Sayed et al., 2003). More precisely, positive relationships between fecundity and size (length, weight) in female swordtail have been reported earlier (Kruger et al., 2001; Tamaru et al. 2001; Chong et al., 2004). Optimised growth rate has been implicated in allowing fish to undergo ovary maturation earlier (Gunasekera et al., 1995). Our study also showed that raising the lipid level from 8% to 12%-20% for both the 20% and 30% dietary protein levels caused significantly improved relative fecundity. This implies that there is a dietary lipid requirement for reproduction in female swordtails. Our results also demonstrated that when an amount of feed, protein or lipid is taken into consideration, best fecundity performances were obtained with the 30% protein diets with lipids ranging from 12-20%. Therefore, taking into consideration the number of fry produced per amount of diet/nutrient intake, the optimised dietary protein and lipid requirements for female swordtails is at 30% and 12%, respectively.

This present study also further demonstrates the potential of utilising formulated feed for optimized growth, maintenance and reproduction of female swordtails. The average spawn per female in this study for the 30% protein level diets ranged from 25-45 fry per female, which is well within the range reported for this species (Tamaru et al., 2001). Encouraging results have been reported in attempting to replace traditional moist diets based on trash fish with formulated pellets as broodstock feed (Mazorra et al., 2003; Emata and Borlongan, 2003). Most Poeciliids including swordtails readily accept a wide range of feed and could therefore be easily weaned onto formulated feeds, further demonstrating the possibility of utilizing cheaper sources of protein and lipid.

Studies, especially in marine species, have shown the importance of balancing composition of unsaturated fatty acids such as arachidonic acid, docosahexaenoic acid and eicosapentanoic acid dietary levels to ensure optimized broodstock reproductive performances and enhance larval quality (Sargent, 1995; Mazorra et al., 2003). In order to further obtain a more effective formulation for swordtail broodstock diet, future studies will investigate the role of these essential unsaturated fatty acids in regulating the reproduction activities of this species.

5.4.2.2.5 Tables and figures

Table 1. Composition and proximate analysis of test diets.

	Diets							
	20P8L	20P12L	20P16L	20P20L	30P8L	30P12L	30P16L	30P20L
Fish meal	13.6	13.6	13.6	13.6	27.9	27.9	27.9	27.9
Krill meal	10	10	10	10	10	10	10	10
Fish hydrolysate	5	5	5	5	5	5	5	5
Algrow1	5	5	5	5	5	5	5	5
Fish oil	4.6	8.6	12.7	16.7	3.3	7.3	11.3	15.4
Pregelled starch	10	10	10	10	10	10	10	10
Cellulose	50.8	46.8	42.7	38.7	37.8	33.8	29.8	25.7
Vitamin mix[2]	1	1	1	1	1	1	1	1
Proximate composition (% dry matter)								
Moisture	4.6	4.8	5.2	4.2	4.7	4.7	4.9	4.5
Crude protein	20.5	20.3	19.8	19.3	30.4	29.6	29.3	30.3
Crude lipid	7.6	11.5	16.1	20.0	8.1	11.6	16.3	20.3
Ash	8.1	7.6	7.7	7.5	9.9	10.0	9.5	9.5
GE* (kJ/g)	19.9	20.4	21.4	22.3	19.9	20.9	22.1	22.8

*Gross energy, calculated based on 0.17, 0.40 and 0.24 kJ/g for carbohydrate, lipid and protein respectively.
1. Algrow, Dunaliella sulina powder, Cognis Pty Ltd.
2. Vitamin mix, standard fish mix, Curios Pty Ltd.

Table 2. Mean values (± S.D.) of various growth parameters and proximate composition (% dry matter) of female swordtail fed different levels of dietary protein and lipids. Mean values in similar rows with different letters are significantly different (Tukey's HSD, $P < 0.05$).

	20P8L	20P12L	20P16L	20P20L	30P8L	30P12L	30P16L	30P20L
Initial weight (g)	1.26 ± 0.02	1.24 ± 0.02	1.29 ± 0.04	1.29 ± 0.03	1.30 ± 0.03	1.31 ± 0.01	1.22 ± 0.03	1.26 ± 0.02
Final weight (g)	2.15 ± 0.47a	2.98 ± 0.66ab	3.50 ± 0.72ab	2.20 ± 0.78a	4.11 ± 0.34b	3.64 ± 1.07ab	4.22 ± 0.40b	3.97 ± 1.44ab
Weight gain (g)	0.88 ± 0.47a	1.74 ± 0.65ab	2.21 ± 0.75ab	0.92 ± 0.78a	2.81 ± 0.34b	2.34 ± 1.06ab	2.89 ± 0.40b	2.71 ± 1.45ab
SGR (%)	0.28 ± 0.12a	0.47 ± 0.11ab	0.54 ± 0.13ab	0.27 ± 0.17a	0.63 ± 0.05b	0.54 ± 0.17ab	0.63 ± 0.04b	0.60 ± 0.19ab
Total feed intake (g)	28.2 ± 1.8a	30.5 ± 1.5ab	30.4 ± 2.5a	21.3 ± 5.5a	39.1 ± 1.6b	33.8 ± 4.3ab	32.8 ± 5.3ab	26.3 ± 9.1a
FCR	30.8 ± 12.1a	17.0 ± 4.6b	15.3 ± 5.9b	30.1 ± 13.7a	14.1 ± 2.0b	16.8 ± 6.6b	11.4 ± 2.0b	11.7 ± 3.6b
GSI (%)	3.1 ± 0.78a	8.5 ± 1.02b	8.2 ± 0.96b	8.3 ± 1.17b	11.3 ± 1.1bc	12.3 ± 5.4c	11.2 ± 2.6bc	13.3 ± 3.5c
VSI	2.5 ± 0.19a	2.9 ± 0.77ab	2.8 ± 0.40a	3.1 ± 0.87ab	2.4 ± 0.40a	2.5 ± 0.13a	2.60 ± 0.38b	2.1 ± 0.34a
HSI	0.39 ± 0.19a	0.30 ± 0.06a	0.52 ± 0.07a	0.79 ± 0.05b	0.68 ± 0.05b	1.48 ± 0.25c	1.56 ± 0.17c	1.06 ± 0.23bc
Final length female (cm)	4.49 ± 0.08ab	4.87 ± 0.18abc	4.95 ± 0.15bc	4.29 ± 0.14a	5.29 ± 0.08c	5.35 ± 0.13c	5.34 ± 0.13c	5.26 ± 0.18c
Female muscle protein (%)	61.5 ± 0.12a	64.4 ± 0.09b	61.8 ± 0.4a	63.4 ± 0.14ab	68.7 ± 0.08c	69.3 ± 1.1c	67.5 ± 0.2c	67.4 ± 1.2c
Female muscle lipid (%)	10.2 ± 0.1a	13.6 ± 0.2b	13.7 ± 0.4b	11.5 ± 0.6a	12.2 ± 0.3ab	14.4 ± 0.2bc	19.3 ± 0.1c	19.8 ± 0.3c
Female muscle ash (%)	16.7 ± 0.3b	16.6 ± 0.4b	14.1 ± 0.3a	14.7 ± 1.0a	15.2 ± 0.4ab	14.3 ± 0.1ab	12.6 ± 0.9a	15.3 ± 0.2ab
Female ovary protein (%)	51.3 ± 0.4a	52.7 ± 0.5ab	55.4 ± 1.3b	54.5 ± 2.0b	55.5 ± 0.6b	54.6 ± 1.6b	53.4 ± 1.1b	53.1 ± 0.8ab
Female ovary lipid (%)	23.5 ± 0.3a	25.8 ± 0.8ab	24.8 ± 1.1ab	27.5 ± 1.2b	27.7 ± 0.1b	26.6 ± 0.1b	27.3 ± 0.2b	25.4 ± 1.2ab

Table 3. Mean values (n=50) of total length (mm), dry weight (mg) and carcass proximately analysis of fry produced by female swordtail fed different levels of dietary protein and lipid. Mean values in similar row with different letter are significantly different (Tukey's HSD, $P < 0.05$).

	20P8L	20P12L	20P16L	20P20L	30P8L	30P12L	30P16L	30P20L
Total length (mm)	5.27 ± 0.51	5.29 ± 0.22	5.29 ± 0.32	5.30 ± 0.61	5.31 ± 0.40	5.26 ± 0.32	5.33 ± 0.01	5.27 ± 0.01
Dry weight (mg)	1.81 ± 0.48	1.93 ± 0.55	1.87 ± 0.62	1.95 ± 0.50	1.88 ± 0.35	2.01 ± 0.91	2.16 ± 0.42	2.14 ± 0.73
Crude protein (%)	55.4 ± 0.4a	56.3 ± 0.1ab	57.7 ± 0.7ab	57.6 ±0.2ab	58.7 ± 0.4b	56.7 ± 0.2ab	58.7 ± 0.3b	56.8 ± 0.3ab
Crude lipid (%)	24.4 ± 0.4	24.4 ± 0.1	25.3 ± 0.3	25.3 ± 0.2	23.7 ± 0.7	23.7 ± 0.6	23.9 ± 0.1	24.8 ± 0.2
Ash (%)	8.7 ± 0.2	9.6 ± 1.1	9.1 ± 0.5	9.5 ± 0.4	9.1 ± 0.4	8.8 ± 0.3	9.9 ± 0.3	8.7 ± 0.4

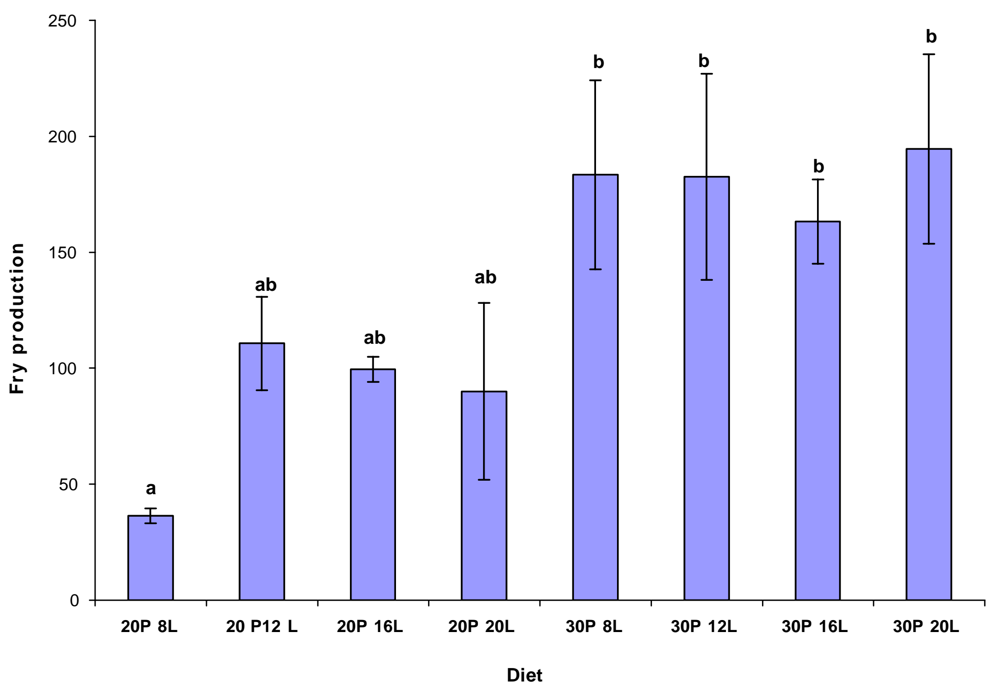

Figure. 1. Mean fry production (± S.E) of female swordtail fed different levels of dietary protein and lipid. Mean values with different letters are significantly different (Tukey's HSD, P < 0.05).

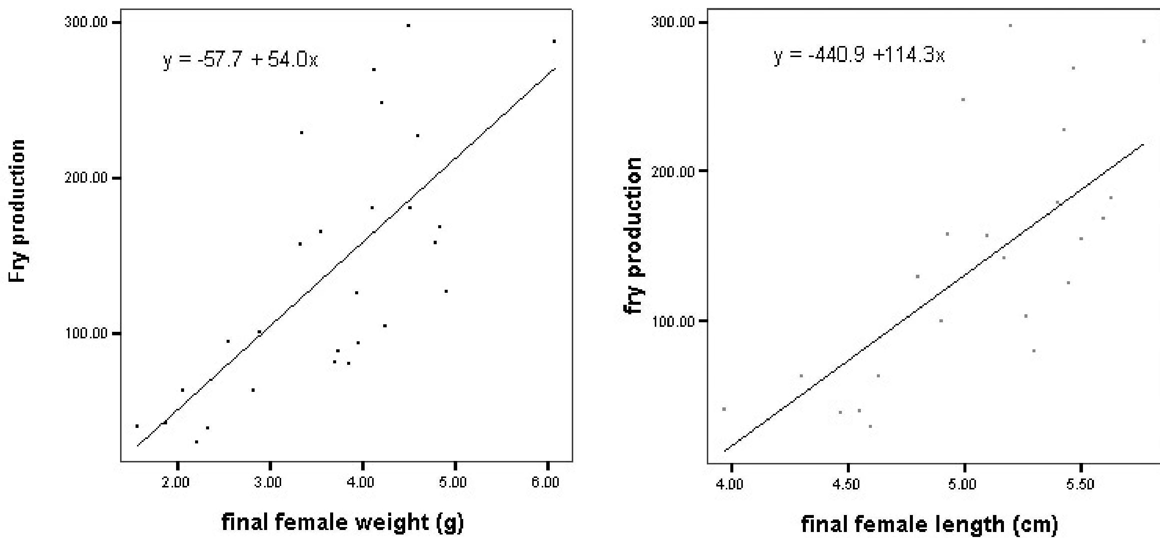

Figure. 2. Relationship between mean fry production and final weight and length of female swordtails (P = 0.05).

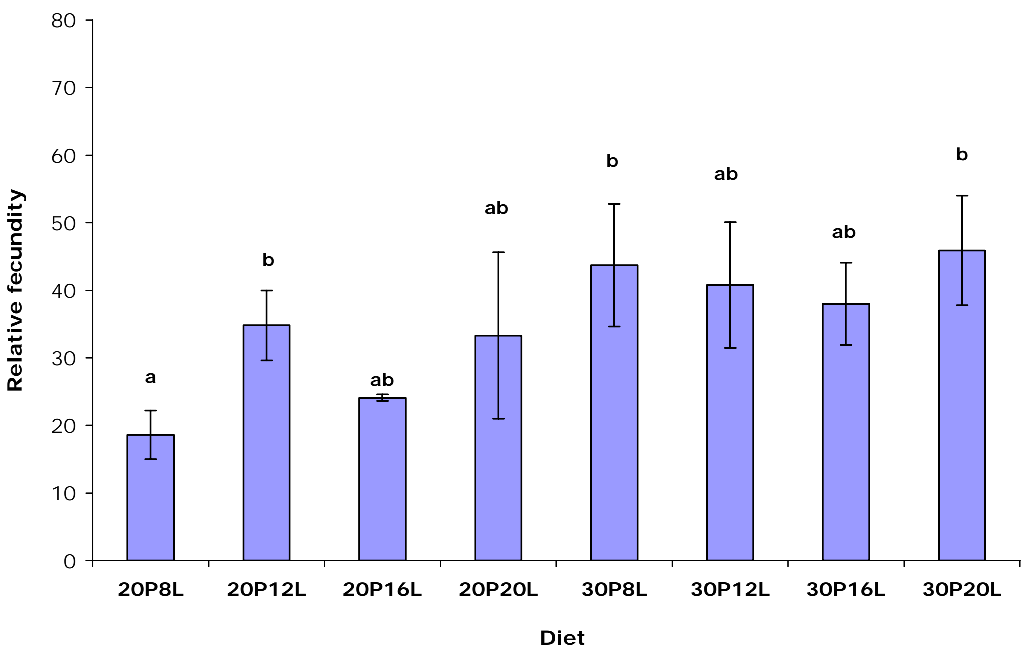

Figure 3. Relative fecundity (± S.E) of female swordtail fed different levels of dietary protein and lipid. Mean values with different letters are significantly different (Tukey's HSD, P < 0.05).

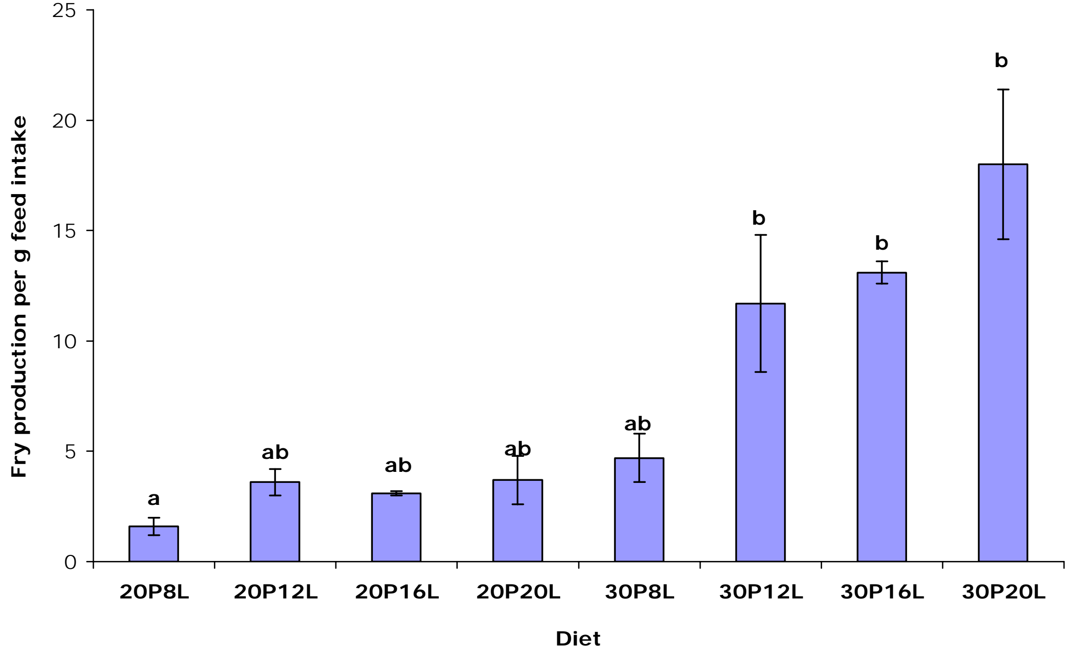

Figure 4. Mean fry production per feed intake (± S.E) of female swordtail fed different levels of dietary protein and lipid. Mean values with different letters are significantly different (Tukey's HSD, P < 0.05).

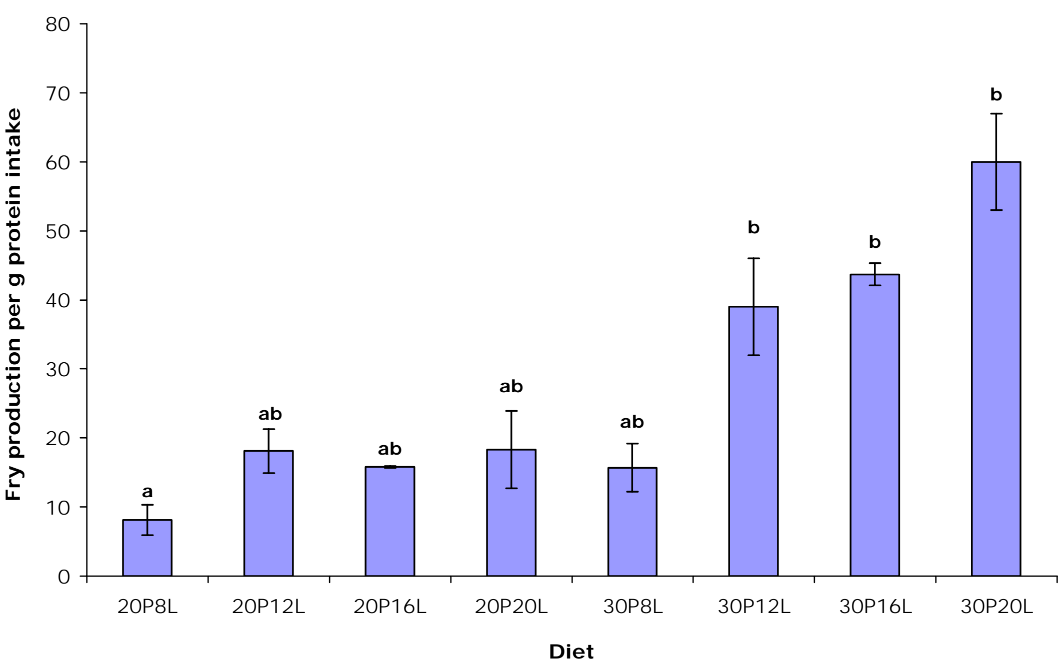

Figure 5. Mean fry production per g protein intake (± S.E) of female swordtail fed different levels of dietary protein and lipid. Mean values with different letters are significantly different (Tukey's HSD, P < 0.05).

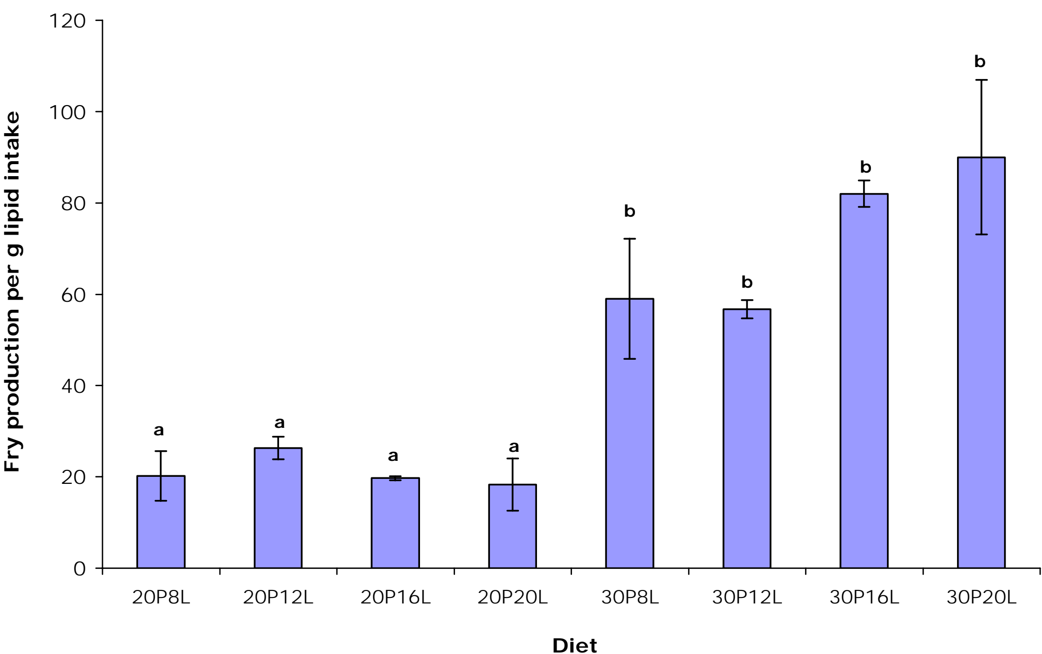

Figure 6. Mean fry production per g lipid intake (± S.E) of female swordtail fed different levels of dietary protein and lipid. Mean values with different letters are significantly different (Tukey's HSD, P < 0.05).

5.4.2.2.6 References

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6.0 INDUSTRY COLLABORATION

Throughout the project, the PI established close links with many industry partners, stakeholders and companies within WA as well as around Australia. A 'service centre' was established through the project where the Department of Fisheries, Western Australia (DoFWA) supplied, free-of-charge, special live food enrichments and broodstock additives to private and government hatcheries. These 'tailor-made' nutritional products are not available commercially and are specifically designed to meet specific nutritional and physiological requirements of fish and other marine organisms. Other industry links includes collaboration on specific issues such as larvae rearing, systems development, and nutrition.

Industry links

- Marine Farms Pty Ltd. The company was established 3 years ago and started a mahi mahi pilot scale hatchery at Rous Head, Fremantle. DoFWA staff collaborate closely with the company regarding system design, feeds and enrichments and broodstock additives specifically designed for mahi mahi. The company established its commercial scale hatchery and grow out facility in Exmouth, WA.
- M.G. Kailis, Exmouth Hatchery. The hatchery is primarily producing pearl oyster spat. In recent years, the company has been involved in rock lobster propagation focusing on the early life stages. Strong collaboration links were established between the hatchery and DoFWA, where different enrichments and larval feeds have been developed for the phyllosoma. DoFWA staff trained and advised Kailis technicians on *Artemia* hatching, enriching and grow out procedures to reduce bacterial loads in the *Artemia* and phyllosoma tanks.
- Clean Seas Hatchery (Steehr group). The company owns one of the two large Yellowtail kingfish hatcheries in South Australia. During 2002-03, the PI supplied free-of-charge broodstock nutritional additives for the yellowtail kingfish. These additives were aimed at stress resistance, immune stimulation, and improved egg quality and viability. The additives were supplied in gel capsules and these were inserted into the fresh sardines given to the fish. According to the hatchery staff, the supplementation of the broodstock nutritional additives resulted in reduced deformities and higher egg viability (although never tested scientifically). Changes in the hatchery management terminated these collaboration links.
- SouthWest Seahorses, Kalbarri, WA. The company is culturing seahorses for the aquarium trade. Specific enrichments and microdiets are supplied to the company.
- AQWA (Aquarium of Western Australia). The company is one of the biggest public aquariums in Australia. The PI and DoFWA staff are working closely with AQWA on different issues and projects including, system design (aquaria, flow-through and closed systems, filtration, and use of UV and ozone), live food production and enrichments as well as specific issues such as leafy sea dragon (*Phycodurus eques*) culture. The PI together with AQWA is currently testing the use of *Dunaliella salina* paste and powder (one of Cognis' products) as a replacement for traditional green algae such as *Nannochloropsis* sp., *Tetraselmis* sp., etc. used in 'green water' systems.
- Sunshine Yabbies. A company established in WA as a commercial hatchery for yabbies. DoFWA staff constantly advise and help the company with the design of systems, live food production and supply of *Artemia* (enriched, frozen and 'pickled').
- Skretting Australia. The biggest fish feed manufacturer in Australia. Collaborative links were established between DoFWA and Skretting to test and develop co-feeding and weaning protocols for their Gemma microdiet range. These links also involved the Darwin Aquaculture Centre, NT (see research collaboration).

7.0 RESEARCH COLLABORATION

The PI established research collaborations with several national and international centres. These collaboration links involved several research projects and resulted in the development and adaptation of new methods, scientific publications and staff exchange.

Research links

- Aquaculture Development Unit (ADU), Challanger TAFE. The ADU and DoFWA are partners in the current project, as part of a broader collaborative relationship between the two agencies in WA. The PI leads the Mariculture Research & Advisory Group, DoFWA which is currently situated at the ADU facilities. The close proximity and relationship benefits both agencies.
- Tasmanian Aquaculture and Fisheries Institute (TAFI), Tasmania. During the project, TAFI and DoFWA collaborated on several issues, such as striped trumpeter larvae nutrition, *Artemia* enrichments and bacteria associated with both larvae and enrichments. These collaborative links also involved CSIRO Marine Research (Hobart, Tasmania) in analysing fatty acids and vitamins. Recently, TAFI purchased the microdiet feeding system developed at DoFWA as part of the current project.
- The Darwin Aquaculture Centre (DAC), NT. The DAC is the biggest barramundi hatchery in Australia producing all the fingerlings to the barramundi industry in NT (estimated at 1500 metric tonnes). During the last two years, the DAC supplied the barramundi larvae to DoFWA free-of-charge for the co-feeding protocols trials. These trials were designed in close collaboration with DAC, taking into consideration current protocol and improvments developed at DAC. These links resulted in two major publications (submitted) where barramundi larvae were succesfully reared with minimum (or no) *Artemia* supplementation. These rearing protocols are used as standard protocols at DAC.
- CSIRO Marine Research, Cleveland, QLD. The possibility of including amino acids in microdiet beads for prawn larvae was tested by Dr David Smith. The PI together with Dr Manuel Yufera produced the microencupsulated particles for this research project.
- National Institute for Water and Atmosphere (NIWA), NZ. Due to the fact that both centres, DoFWA and NIWA, are working with yellowtail kingfish, strong collaborative links were established. A large scale experiment was conducted at the Aquaculture Park, Bream Bay, NZ. 'Tailor-made' enrichments containing 'mega' doses of vitamins E and C were tested in both rotifers and *Artemia* in an attempt to reduce deformities in the larvae. The initial results show significant reduction in deformities coupled with increased stress resistance. Several collaborative projects are currently in different stages of design and development.
- Instituto de Ciencias Marinas de Andalucía (CSIC), Cadiza, Spain. Strong research collaboration links exist between the PI and Dr Manuel Yufera (also co-investigator in the current Project). These links led to several publications related to novel methods in microdiet preparation i.e. encapsulation through protein cross-linking, and recently a new method of internal gelation. During the project, Dr Yufera visited WA and conducted an experiment with the PI. A reciprocal visit of the PI to Spain and Portugal resulted in several publications.
- Centre for Marine Sciences, FCMA, Universidade do Algarve, Portugal. Collaborative links were established as a result of the PI winning an international exchange grant from the Australian Academy of Science. The PI spent a month in Portugal and conducted experiments involving Senegal sole, microdiets and hydrolysates as part of the protein component of the microdiets.

This project also involved Spain (the microencapsulated diets produced by Dr Yufera) and Universitiet of Ghent, Belgium (Professor Sorgeloos).

- National University of Malaysia. Initial links were established after a workshop (recent developments in hatchery technology) where the PI was invited as a keynote speaker. A research project looking at the development of microdiets through nutritional requirements of fresh water ornamental fish was developed. DoFWA supplied the diets for the experiments (without revealing the specific ingredients and/or method of manufacture).
- Tropical Marine Science Institute (TMSI), National University of Singapore. The PI has had a long term relationship with the institute. In 1999, the PI designed the life support systems and all the tanks systems for TMSI. In the current project, *Artemia* products from Cognis Pty. Ltd. were (and still currently are) evaluated as a food source for seahorses (the main organism under investigation at TMSI).
- Nagasaki University. The PI and Dr Hagiwara from the Nagasaki University have been collaborating for many years. In 2004, the PI won a Japanese Society for the Promotion of Science grant for scientific exchange and spent 3 weeks in Japan. As part of the visit, several projects were designed and are currently under evaluation in Japan. Another outcome from the visit was a scientific paper (yellowtail kingfish, from larvae to adult - problems and opportunities) presented at the Nutrition Symposium, Hermosillo, Mexico (November, 2004) with Dr Sakakura from Nagasaki University as co-author.

8.0 EXTENSION

As part of the project and as stated in the project proposal, the PI organised a national workshop titled 'The Second Hatchery Feeds and Technology Workshop'. The workshop focused on recent developments in both industry and research as related to new species, feeds and technologies. Specifically the workshop objectives were:

1. To assess the current status and advances of hatchery feeds, technologies and larvae rearing techniques in commercial hatcheries.
2. To assess the current R&D activity, advances and research capabilities made since 2000 in the major R&D centres.
3. To assess priorities for research and development needs in the area of marine hatcheries.
4. To identify constraints to the continued development of Australian aquaculture in the area of marine hatcheries.
5. To identify opportunities to enhance collaboration and information exchange amongst researchers and industry.
6. To develop a national R&D plan for marine hatcheries for 2005-2010.

The workshop was held in Sydney on 30 September and 1 October, 2004, in conjunction with the Australasian Aquaculture Symposium. The first day of the workshop was devoted to status reviews from both industry representatives and research providers. The second day was devoted to a round-table discussion to identify knowledge gaps and priorities for the different areas of marine hatchery production.

There were fifty two participants from around Australia, as well as five overseas speakers. The workshop budget allowed for the participation of five keynote speakers: from Japan (rotifers), Spain (nutrition), Greece (systems), France (microdiets) and Norway (health), which contributed to a successful workshop. As part of the workshop, a dinner was organised between the two days which facilitated networking between the participants.

The workshop was organised by the Department of Fisheries, Western Australia, in collaboration with the Tasmanian Aquaculture and Fisheries Institute and the Queensland Department of Primary Industries and Fisheries, and was kindly sponsored by the following organisations: Frontiers of Science &Technology, Mission and Workshop component of the Innovation Access Program, part of the Australian Government's Innovation Statement, Backing Australia's Ability, Fisheries Research and Development Corporation, Challenger TAFE, Western Australia, CRC – Aquafin, Cognis Australia Pty Ltd, Skretting and M.G. Kailis. The organising committee consisted of: Dr Sagiv Kolkovski, Dr Mike Rimmer, Dr Stephen Battaglene and Dr Robert van Barneveld.

The proceedings of the workshop are available through the PI, the Department of Fisheries, Western Australia and included as Appendix 5 (on CD as a PDF). It is planned to have a one day seminar in South Australia to update the yellowtail kingfish hatcheries personnel on the outcomes of the workshop (these hatcheries were not able to attend the workshop due to the spawning season) and to get their input regarding the gaps and priorities for the 2005-2010 R&D plan. Following that, the PI together with the organising committee will finalise the draft R&D plan for submission to the FRDC.

9.0 PUBLICATIONS

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White, M. 2003. Investigating free amino acid leaching rates from fish larvae microdiets. Honours. Department of Chemical Engineering. Curtin University of Technology, Perth, Western Australia.

Visits / exchanges

2000. Perth, WA. Dr Manuel Yufera (Spain). Funded by the current FRDC project.

2003. Faro, Portugal and Cadiz, Spain. Dr Sagiv Kolkovski. Funded by the Australian Academy of Science.

2004. Kagoshima and Nagasaki, Japan. Dr Sagiv Kolkovski. Funded by the Japanese Society for Promotion of Science (JSPS).

10.0 BENEFITS AND ADOPTION

The project benefits, outputs and related outcomes can be divided into four sections (as per objectives):

i) System development

ii) Microdiet development

iii) Feeding protocols

iv) *Artemia* production and enhancement

In general, outcomes and benefits will (and in fact already) flow to marine hatcheries (finfish and other marine organisms), industry and R&D centres as well as other industry sectors.

System development

The development of the larvae rearing system, and the adjoining live food enrichment system, involved innovative solutions in terms of automated systems, dosing and feed delivery systems, and filtration. These systems allow better control and save time and money. Some parts of the systems have already been adopted by industry. For example, the tank design is currently being tested at the M.G. Kailis Exmouth hatchery. The innovative microdiet feeding system is currently being installed at TAFI in their larvae tanks. The larvae rearing system and/or other specific systems can benefit any R&D centre involved in marine larvae rearing as well as commercial hatcheries. It is envisaged that these systems will be progressed as a commercial product, depending on demand.

The outcome of this activity is a better controlled environment for marine larvae, reduced maintenance time, and reduced manpower, resulting in more efficient hatcheries.

Microdiet development

Several different microdiet preparation methods were tested during the project as well as different nutritional parameters. Chemical and physical parameters were assessed for both commercial and experimental diet particles. Some of these diets match or even outperform commercially available diets and therefore, present a commercial opportunity for microdiets that can be tailor-made to specific species and/or conditions. Moreover, characterising the chemical and physical parameters such as leaching and sinking rates enables recommendations to be made to commercial hatcheries for specific feeding methods and rates, e.g. small amounts in short intervals for fast sinking diets.

Comparing commercial diets also resulted in specific recommendations to hatcheries such as mixing two different diets, one with higher attractability and the other with a better nutritional profile.

These recommendations resulted in optimising food and feeding practices in commercial hatcheries, leading to better larvae growth and reducing tank fouling by inert diets.

Feeding protocols

The development of co-feeding and weaning protocols was done in close collaboration with industry (WA). Strong links were developed with the Darwin Aquaculture Centre (DAC), NT (the largest barramundi hatchery in Australia) and Skretting Australia, TAS (the largest fish feed manufacture in Australia). Feeding protocols were developed, validated and adopted with strong input from DAC. Based on this collaborative work, two papers were published describing co-feeding protocols with a reduction of *Artemia* use by 90% through to its complete elimination from the protocol.

The outcome of this activity is optimising the utilisation of microdiets by the larvae and reducing the reliance on live food with minimal or no effect on larval performances.

Artemia production and enhancement

Strong links were established with Cognis Australia Pty Ltd in relation to *Artemia* production. Pilot scale grow out trials proved to be quite successful. Based on these results Cognis, together with FRDC and Department of Fisheries, Western Australia, are now investing in a new project aimed at commercialisation of *Artemia* production. Several products were developed, some of which are new. These products are already commercially available in Australia.

The outcome of this activity is local *Artemia* (cysts and biomass) becoming available in Australia so that imported *Artemia* can be replaced thereby reducing the risk of supply shortage and introduction of unknown pathogens.

Commercially available live food enrichments were surveyed in terms of nutritional value as well as bacterial loads. Tailor-made enrichments as well as broodstock additives were manufactured for specific sectors and found to be effective.

The outcome of this activity is the availability of specific products designed for specific fish species and/or addressing specific nutritional and/or physiological issues, rather than use of off-the-shelf general products. This can lead to a better, stress resistant, and faster grown larvae with fewer deformities or other problems.

11.0 FURTHER DEVELOPMENTS

The current project initiated R&D activities in two major areas: microdiets and *Artemia* cysts and biomass. The outcomes from the project led the way to the future commercialisation in these two areas. A follow up FRDC project is currently aiming at 1: Finalising the research on the prototype microdiet as commercial diet and, 2: Developing and initiating a commercial-scale system for the production of *Artemia* cysts and biomass together with Cognis Australia Pty Ltd.

As part of the new project, a monitoring program will be initiated together with Cognis to predict future natural *Artemia* outbreaks.

New *Artemia* products that were initiated in the current project will be further developed as commercial products, depending on market demand.

In consultation with FRDC, the concept of automatic hatchery and other specific systems, developed in the current project, will be progressed as commercial products / systems. Some of these products have already been implemented in other centres i.e. feeding systems.

At the end of the current project, the PI organised a national hatchery feeds and technology workshop (Sydney 30 September – 1 October, 2004). One of the outcomes was an R&D plan for 2005-2010. It is envisaged that during early 2005, the PI together with Drs Robert Van Barneveld and Stephen Battaglene will consult with the SA hatcheries and complete and submit the R&D plan to FRDC.

12.0 CONCLUSION

The current project focused on marine fish larvae feeds and rearing systems. The project achieved all the objectives set in the project proposal. A rearing system and adjunct live food system was developed. The system contained several innovative sub-systems such as a live food distributing system and microdiet automated feeders.

Weaning and co-feeding protocols for snapper, yellowtail kingfish and barramundi were developed using commercially available diets as well as experimental ones. These protocols are now being used in commercial hatcheries.

Several live food enrichments (commercial and experimental) were tested with the species mentioned above for larvae performance and bacterial levels. Results were given to industry and to the manufacturers. In one case, these results contributed to a change in the production line procedures to reduce bacteria loads.

Several chemical and physical aspects of microdiets were investigated, including those related to different preparation methods. Different formulations were tested to determine the optimal ingredients in terms of ingestion, digestion and assimilation. An experimental diet was compared to a commercially available one and has proven to give the same and, in some cases, better performance.

Artemia cysts and biomass production was initiated and some products are already commercially available in Australia. The project set the foundation to a commercial scale production of both cysts and biomass in Western Australia. New products derived from *Artemia* biomass will be further developed in the subsequent FRDC project.

During the project life span, strong links to industry were developed. Collaboration with several R&D institutes in Australia and overseas was initiated and will carry on in the new FRDC project. These links and collaborations were demonstrated during the national hatchery feeds and technology workshop organised as part of the current project.

13.0 APPENDICES

APPENDIX 1 – INTELLECTUAL PROPERTIES

During the current project, several systems and methods were developed. Some of the details on these systems were published in appropriate scientific journals. However, others products and activities remain unpublished due to IP and commercial confidentiality.

Microdiet feeding systems

Department of Fisheries, Western Australia developed a unique feeding system to enable the distribution of microdiets for marine larvae (with particle size down to 75 µm). Currently, this system is unique. The majority of the feeding systems available commercially are too big and suit only larger particles or are too small for domestic use (e.g. only suit aquaria). The product should have potential as a commercial product worldwide in hatcheries and R&D centres as well as public aquariums.

An investigation into the market potential of the system and the cost to patent it should be undertaken. If found to be commercially viable, negotiations with an aquaculture product distributor (worldwide) should be initiated.

Microdiet formulation

The microdiet for marine fish larvae developed collaboratively with Department of Fisheries, Western Australia has potential as a commercial product. One of the objectives of the next FRDC project is to develop it to a commercial prototype that may be taken up by a feed company. Therefore, it is recommended that the formulation and ingredients of the microdiet will remain confidential and will not be published until the development of the microdiet is completed.

Artemia production

The production and processing of *Artemia* cysts and biomass in the current project as well as in the new FRDC project was carried out in collaboration with Cognis Australia Pty Ltd. An IP agreement for the new project is currently under development. However, the current project did not have a specific IP agreement with the company. It is recommended that the entire *Artemia* section in the current report will be under commercial confidentiality and will not be published and/or distributed. This section contains results and recommendations from trials conducted at the Cognis site (Hutt Lagoon) as well as information on new products and processing of *Artemia* cysts and biomass. These results are highly relevant to the commercial production of *Artemia* and should not be released without agreement.

APPENDIX 2 – STAFF

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Dr Sagiv Kolkovski, Lead Scientist (Principal Investigator)

Mr John Curnow (Senior Technician)

Mr Justin King (Technician)

Dr Greg Maguire (Research Supervisor)

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Ms. Helen Clifton (Research Assistant)

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Mr. David Poppi (Honours Student)

Mr. Michael Horne (Research Assistant)

Mr. Kevin Kane (Research Assistant)

APPENDIX 3 – THE R&D PRIORITIES (FRDC PLAN) FOR *ARTEMIA* AND ARTIFICIAL DIETS

Key to Symbols

👍 High priority	➢ Links to	💰 High return	Longer term

Group B: Rotifers and brine shimp

👍 💰	***B1. Benchmarks for rotifer/brine shrimp production*** Many different techniques are used to feed rotifers and brine shrimp in Australian hatcheries. We need to identify the best practices being used overseas for live food production and to translocate this technology to Australian hatcheries. In order to provide a benchmark for Australian production, we need information from individual hatcheries, such as the quantity of *Artemia* used and the level of fish production. Methods to improve feeding efficiency and reduce the use of *Artemia*, such as on-growing of nauplii, need to be introduced to hatcheries.
💰 ➢B8	***B2. Technology transfer*** There is a great deal of information on the production of live feeds throughout the world. The degree of technology used in Australian hatcheries is extremely variable. In order to raise the standard of live feed production across-the-board in Australian hatcheries, we must increase the level of technology transfer. This may be achieved by introducing regular workshops, producing manuals (printed and electronic) and reports, and initiating a website or mailing list through the internet.
👍	***B3. Assessment and production of Australian strains and alternative species*** There is a need to establish a research program to identify new strains of endemic rotifer species that may be suitable for culture in Australian hatcheries. The program will require that we (a) initially isolate rotifers and (b) then develop techniques to mass culture them. If this is possible, the suitability of the rotifer as a live feed for marine fish will need to be evaluated in research and commercial hatcheries. New rotifers could be selected for size (very small for first feeding fish larvae; very large as a potential *Artemia* replacement) or productivity. Improvements in rotifer size and productivity may also be sought by initiating a selective breeding program for rotifer strains already cultured in Australian hatcheries.
👍	***B4. Australian production of* Artemia *in ponds*** Australia has a large resource of saline ponds situated on the coast (usually for salt manufacture) and in inland Australia (natural ephemeral saline lakes; man-made saline evaporation basins as part of rising saline groundwater interception schemes). These lakes may be suitable for culture of either endemic brine shrimp such as *Parartemia* spp. or exotic *Artemia* spp. There is a need to evaluate the potential for commercial production of brine shrimp in Australian salt lakes.
👍	***B5. Weaning and co-feeds*** There is a need to reduce the use of *Artemia* as a live food for marine fish. This may be achieved by developing new feeding strategies for fish larvae, such as an extension of the rotifer feeding phase and early weaning of larvae with artificial diets.

B6. Enrichment

Rotifers need to be enriched, particularly with (n-3)HUFA's prior to feeding to marine fish larvae. Several commercial enrichment diets are readily available to Australian hatcheries; however, the efficacy of the enrichment protocols used in Australian hatcheries is in doubt. Procedures need to be developed to regularly analyse HUFA content of enriched rotifers to ensure that target concentrations are being reached. Best practice methods currently in use in overseas hatcheries need to be determined and translocated to Australia. Flow-on effects of the enrichment of rotifers with vitamins etc. on the production of Australian marine fish larvae need to be quantified.

B7. Evaluation of alternative species from overseas

Possible alternative species to rotifers as live feeds may be cultured overseas. A program to identify new genera (e.g. the cladoceran *Moina*) that may be suitable for marine hatcheries would be useful. Once overseas genera are identified, similar species endemic to Australia may be isolated and evaluated.

➢ B2 ### *B8. Evaluation of new rotifer systems*

Large-scale batch culture of microalgae is generally expensive and can account for 30–40% of total hatchery costs. Continuous microalgal production systems may reduce the cost of producing algae to feed rotifers. This technique should be investigated. Development and commercialisation of microalgae concentration in Australia may also provide an off-the-shelf feed for rotifer production. This may reduce the cost and increase the reliability of rotifer production. Significant mass-culture technology of rotifers has been developed overseas. For example, ultra-high-density production systems have been developed in Japan, which are based on feeding concentrated freshwater *Chlorella*. Final harvest densities in these systems can be 100 times greater, and production costs can be 65% less, than those of traditional culture methods. This technology needs to be transferred or adapted to Australian hatcheries.

B9. Evaluating ongoing technology

Large-scale production of juvenile rock lobsters requires large quantities of *Artemia*. To reduce costs and increase production we need to develop techniques for reliable production of advanced (on-grown) *Artemia*.

B10. Culture systems

The development of extensive, fertilised-pond larval rearing techniques may overcome the need to conduct large-scale live-feed production in Australian hatcheries. Extensive larval rearing has been used successfully for a number of marine fish species. The suitability of this technique for larval rearing may be highly species specific, and this needs to be evaluated. There may be advantages in having an initial 10–14 day rearing phase in an intensive hatchery, followed by on-growing in extensive ponds. This would reduce the dependence on *Artemia*, and could significantly reduce the cost of fish production.

Group D: Artificial diets

D1. Test currently available diets

➢ B5

A survey of the larvae and weaning diets commercially available in Australia is needed. The survey should focus on the main commercial species currently reared (i.e. barramundi and snapper).

The survey should use standard protocols and include the following topics:
- cost versus profit
- growth versus cost
- labour efficiency

D2. Develop standard testing systems

A standard system for testing microdiets needs to be developed. The performances of a given microdiet are greatly affected by the shape, size and volume of the larval tanks. The inert movements of the diet particles depend on hydrodynamics in the rearing tank. A standard testing system for both tropical and temperate areas will have the advantage of testing different diets with different fish species in the same conditions. Two standard systems, one for tropical fish species such as barramundi (at James Cook University, Townsville) and the other for temperate species such as snapper (at Fisheries Western Australia and/or Fremantle Maritime Centre, Perth) could be developed for this purpose.

The testing system should be on a commercial scale to allow immediate transfer of results to industry, without the need for up-scaling. A standardised system would also improve hatchery skills in general.

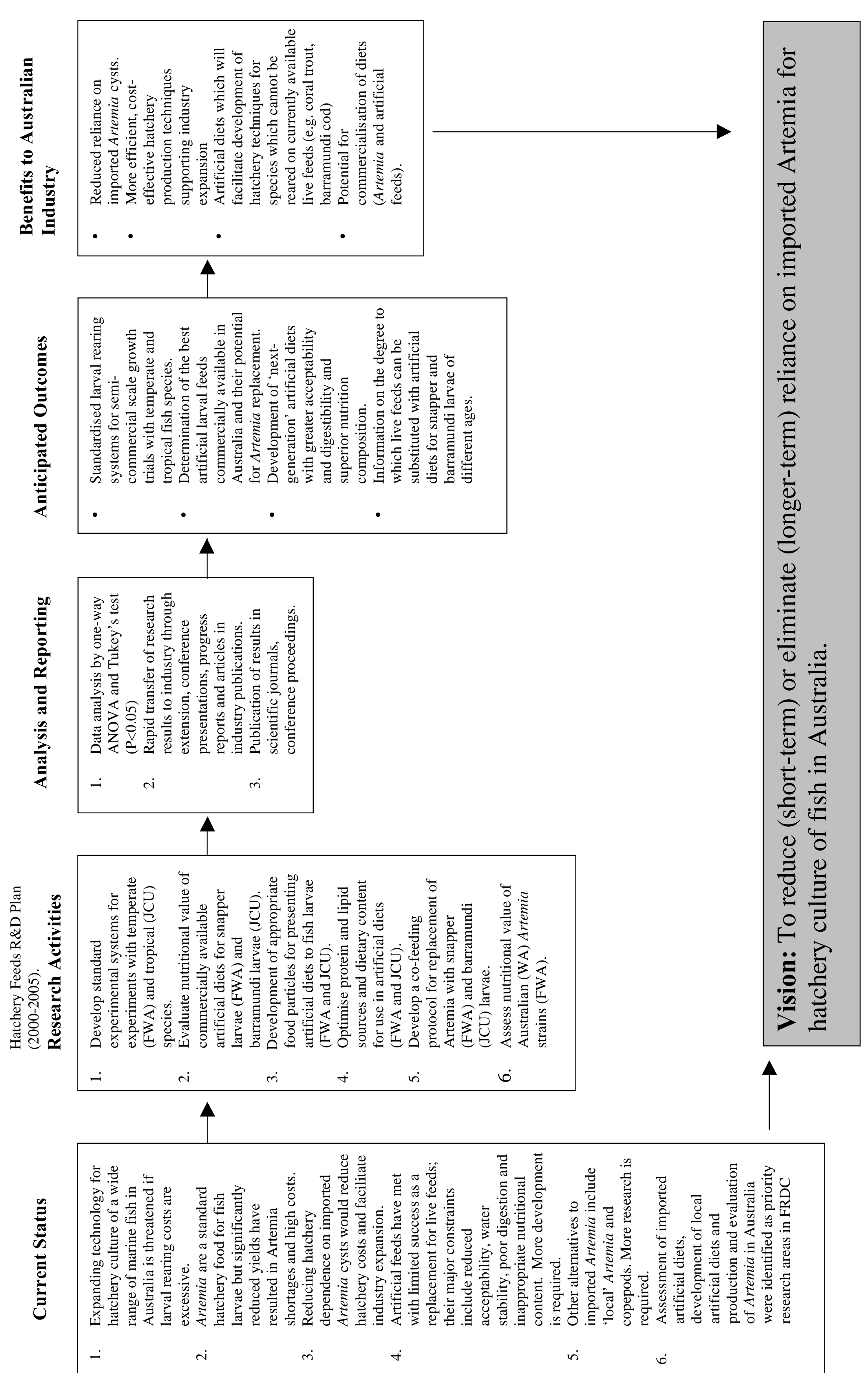
D3. Develop local diets

In the short term the development of local diets should focus on co-feeding, using both dry and live feeds. Research in this area should aim to shorten the weaning period and decrease the amount of *Artemia* being used. In the longer term research should aim at the complete replacement of *Artemia* with microdiets. Local microdiets will need to compete with overseas diets in terms of cost and performance.

The R&D of local microdiets will need to focus on improving:
- ingestion by using feed attractants;
- digestion by using easy-to-digest proteins, binders and dietary enzymes etc.

Communication between hatcheries, research institutions and feed providers needs to be improved to aid development of local diets and to get feedback from the hatcheries that are using a particular diet.

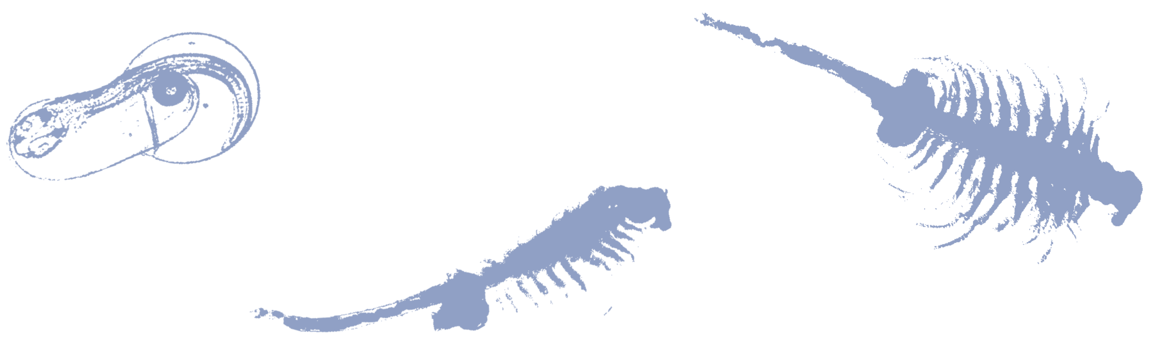
APPENDIX 4 – FLOW DIAGRAM SUMMARISING THE PROJECT ACTIVITIES AND OUTCOMES AS SUBMITTED IN THE PROPOSAL



APPENDIX 5 – THE SECOND HATCHERY FEEDS AND TECHNOLOGY WORKSHOP, 2004

The Second Hatchery Feeds and Technology Workshop

NOVATEL CENTURY SYDNEY



September 30 – October 1

2004

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Introduction

The Second Hatchery Feeds and Technology Workshop

Novotel Century Sydney

September 30 – October 1, 2004

Aquaculture in Australia has developed rapidly over the last decade in both production and value. During recent years, research into the culture of new species has received a lot of attention and a range of species are already commercially cultured. In common with many countries around the world, production of marine fish in Australia will mainly depend on the production of seedstock from hatcheries. Because of this dependence, the continued development of Australian aquaculture will rely heavily on the development of cost-effective hatchery production technologies, with low or negligible environmental impacts. Production technologies for most cultured species are still heavily dependent on live feeds, particularly during the early life stages. A wide range of variables affect the suitability of live prey organisms for hatchery use, including ease of culture, prey size, behaviour and nutritional composition. There has been increasing interest in developing alternative feeds for hatcheries, including alternative zooplankton species and formulated diets. With the expansion of aquaculture in Australia there is a need to improve coordination among the research organisations studying feeds for larval fish, and a need to identify opportunities and priorities for future research that more closely match the needs of industry.

In 2000, the first National Hatchery Feeds Workshop was convened in Cairns. The workshop brought together a wide range of industry and research participants from around Australia together with keynote speakers from overseas. The outcomes of that workshop were Hatchery Feeds Research and Development Plan 2000-2005 and the workshop proceedings, which included reviews of major topics and state and species summaries. These outputs provided a review of the current world status of hatchery feeds and where Australian industry was placed. The R&D plan identified the gaps and research priorities for future development of hatchery feeds.

Five years later, the time has come to re-visit these priorities, review the R&D outcomes in related projects, states and species and to look at current industry status and needs for the next five years.

The Second Hatchery Feeds & Technology Workshop will be held over two days in Sydney, 30 September – 1 October 2004, immediately following the 'Australasian Aquaculture 2004' conference and trade show.

The workshop will focus on recent developments in both industry and research as related to new species, feeds and technologies. The workshop objectives are :

1. To assess the current status and advances of hatchery feeds, technologies and larvae rearing techniques in commercial hatcheries.
2. To assess the current R&D activity, advances and research capabilities made since 2000 in the major R&D centres.
3. To assess priorities for research and development needs in the area of marine hatcheries.
4. To identify constraints to the continued development of Australian aquaculture in the area of marine hatcheries.
5. To identify opportunities to enhance collaboration and information exchange amongst researchers and industry.
6. To develop a national R&D plan for marine hatcheries for 2005-2010.

The first day of the workshop will be devoted to status reviews from both industry representatives and research providers. The second day will be devoted to a round-table discussion to identify knowledge gaps and priorities for the different areas of marine hatchery production.

The workshop is organised by the Department of Fisheries, Western Australia, in collaboration with the Tasmanian Aquaculture and Fisheries Institute and the Queensland Department of Primary Industries and Fisheries and is kindly sponsored by the following organisations: Frontiers of Science & Technology Mission and Workshop component of the Innovation Access Program, part of the Australian Government's Innovation Statement, Backing Australia's Ability, Fisheries Research and Development Corporation therough its nutrition sub-program, Challenger TAFE, Western Australia, CRC – Aquafin, Cognis Australia Pty Ltd, Skretting, M.G. Kailis and Embassy of France and FEAST – France.

Organising Committee

Dr Sagiv Kolkovski

Dr Mike Rimmer

Dr Stephen Battaglene

Dr Robert van Barneveld

Keynote Speakers

Nutritional requirements for finfish larvae

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Abstract

From on-start feeding, fish larvae nutritional reserves are very limited and their survival dramatically depends on exogenous feed. Hence, complete and balanced nutrition is critical of rearing success during early life stage. But most fish larvae, particularly marine ones, despite on the wild fed on a wide range of live preys, under culture conditions are forced to fed on a very limited number of preys (two or three) which frequently are not part of their natural food and hence their nutritional composition is not always the most suitable for maximum growth, development and survival of the larvae. Moreover, along larval development the fish will undertake several morphological and physiological changes which in nature are simultaneous with changes in behaviour and even habitat and type of prey fed. All these changes will affect to nutrient availability and feed utilization by the larvae in order to match their nutritional requirements. In practice, most of these problems will be simplified by the proper development of inert diets which are able to cover nutritional requirements at different moments of larval development. In order to achieve those diets we need, among many other important things, to have a complete knowledge of nutrient requirements for the different fish species.

Whereas protein composition of live preys is genetically determined, lipid qualitative and quantitative composition is greatly affected by their diet and significantly varies among batches of the same type of prey, as well as among different species. Early studies on the 80´s had determined that lipids are the most important factor affecting the nutritional quality of live preys and since then a vast amount of the research conducted on larval nutrition have focussed on these nutrients. Essential long chain polyunsaturated fatty acids, as key components of bio-membranes play many important roles in their functioning and are particularly indispensable for larval development. Their presence and quantity in the diet are determining to the efficiency of digestion, absorption, and transport of some nutrients, and to the capacity of dietary energy deposition and utilization. They markedly affect eye and brain development as well as larval behaviour. Finally, as sources of eicosanoids they regulate several physiological functions including some related with larval development, immune function and stress resistance, globally affecting larval growth and survival and rearing success. Recently, molecular studies have denote the presence and activation by the fatty acid composition of the diet of a delta-6 desaturase like gene, involved in long chain polyunsaturated fatty acids synthesis. Besides, the different essential fatty acids compete among them at many different points of fish physiology, dietary unbalances among them leading to detrimental consequences for the larvae. To complicate the picture a bit more, the molecular form in which they are administered is determinant of the utilization efficiency of the dietary essential fatty acids. Other lipids such as phospholipids are considered indispensable for fish larvae, since they distinctly promote the limited ability of larvae to absorb, to re-acylate and to transport triglycerides and provide additional sources of nutrients. Fat-soluble vitamins and pigments have also prove to play important roles along larval development and their inadequate dietary levels either by shortage or excess are negative for the larvae. In a similar manner to what is found in juveniles, dietary protein utilization has been found to be affected by the dietary source, particularly during early larval stages and playing a central role in the development and maturation of the larval gut. Besides, certain free amino acids also constitute a very important source of energy, act as attractants and play a significant role in gut function and development. Finally, despite their importance, only a very limited number of studies have focused other nutritional aspects of larval development such as water-soluble vitamin and mineral requirements and energy utilization by fish larvae.

Key words

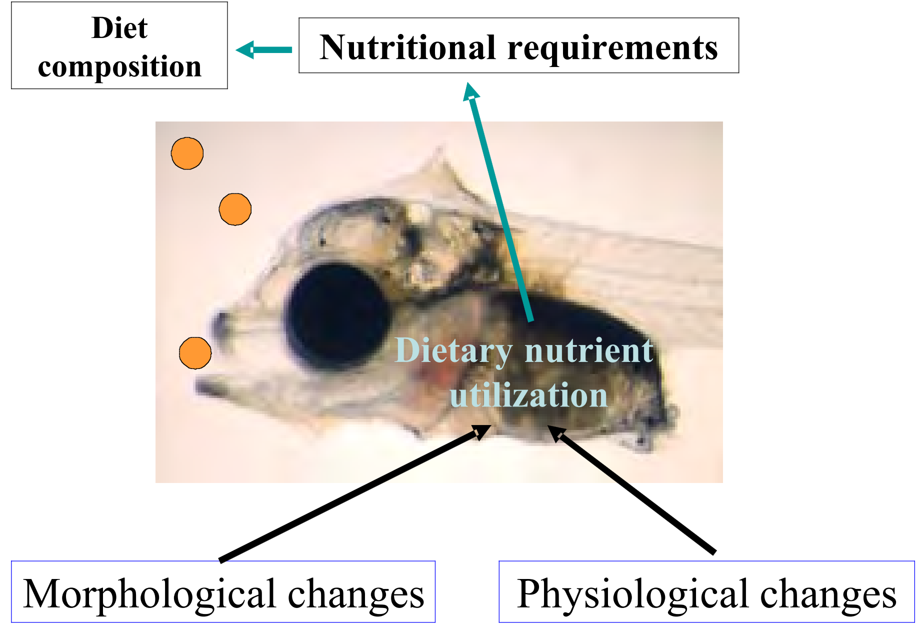
Arachidonic acid, broodstock nutrition, docosahexaenoic acid, eicosapentaenoic acid, fish nutrition, larval nutrition, essential fatty acids.

Abbreviations

EFA: essential fatty acids; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ARA: arachidonic acid; PUFA: polyunsaturated fatty acids with 18 or more carbon atoms and 2 or more double bounds; HUFA: highly unsaturated fatty acids with 20 or more carbon atoms and 2 or more double bounds; PI: phosphatidyl inositol; AA: amino acids; EAA: essential amino acids.

Introduction

From on-start feeding, fish larvae nutritional reserves are very limited and their survival dramatically depends on exogenous feed. Hence, complete and balanced nutrition is critical of rearing success during early life stage. But most fish larvae, particularly marine ones, despite on the wild fed on a wide range of live preys, under culture conditions are forced to fed on a very limited number of preys (two or three) which frequently are not part of their natural food and hence their nutritional composition is not always the most suitable for maximum growth, development and survival of the larvae. Moreover, along larval development the fish will undertake several morphological and physiological changes which in nature are simultaneous with changes in behaviour and even habitat and type of prey fed. All these changes will affect to nutrient availability and feed utilization by the larvae in order to match their nutritional requirements. In practice, most of these problems will be simplified by the proper development of inert diets which are able to cover nutritional requirements at different moments of larval development. In order to achieve those diets we need, among many other important things, to have a complete knowledge of nutrient requirements for the different fish species.



Requirements for essential fatty acids

Three very long chain polyunsaturated fatty acids, namely docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) have a variety of very important functions in fish species, particularly in larvae. Despite freshwater fish seem to have sufficient Δ5 and Δ6 desaturases and elongases activities to produce ARA, EPA and DHA if their precursors linoleic (18:2n-6) and linolenic (18:3n-3) acids are present in the diet, such enzymatic activity is very restricted in marine fish larvae and as a consequence, DHA, EPA and ARA have to be included in the diet and are considered essential. A Δ6 desaturase-like gene has been isolated in larval gilthead sebream (Seilez et al., 2003). More recently,

experiments in our laboratory have found that its expression is affected by the diet, denoting a higher activity of this enzyme when low EFA and high 18 carbon atoms polyunsaturated fatty acids are provided in the rotifers. Inadequate contents of those EFA in the diet give rise to several behavioural and morphological alterations such as poor feeding and swimming activities, poor growth and dropping mortality, fatty livers, hydrops, deficient swim bladder inflation, abnormal pigmentation, disgregation of gill epithelia, immune-deficiency and raised cortisol levels (Izquierdo, 1996; 2004).

Since environmental factors such as temperature, salinity and light affect lipid composition of fish tissue (Izquierdo, 2004), EFA requirements could be also affected by environmental conditions. For instance, larvae of the euryhaline species *Galaxias maculatus* have been found to be higher in EPA, DHA and ARA acids when they were obtained from marine environments in comparison with those from freshwater (Dantagnan et al., submitted), denoting the important role of some of these fatty acids in osmotic regulation. Moreover, before first feeding, synthesis of those EFA was activated in larvae from freshwater environment but not in those obtained in the estuary, suggesting the influence of environment salinity on activation of elongation and desaturation enzymes.

In the wild, types and contents of EFA differ among the different steps of the trophic chain, and EFA requirements would then rely on the trophic behaviour of each fish species. Being fish larvae visual feeders, larval trophic behaviour is closely related to the development of the visual capacity. In sparids, such as gilthead seabream and red porgy (Roo et al., 1999) the most important changes in the eye structure occur along the lecitotrophic stage as a preparation for prey capture, rod photoreceptors necessary for accurate vision at low light intensity appearing in gilthead seabream about 18th day after hatch. N-3 PUFA, and particularly DHA play a critical role in neural and retinal tissue functions. Bell and Dick (1993) found that both rod and cone photoreceptors in herring eye, accumulate and selectively retain DHA, and thus, feeding herring a DHA poor Artemia during the period of rod development resulted in impaired vision at low light intensities. Moreover, elevation of dietary DHA and eicosapentaenoic acid (EPA) increase eye diameter in gilthead seabream (Izquierdo et al., 2000; Roo et al., submitted) and this fact, together with a high density of cone photoreceptors in these larvae, implied a total higher number of cones and a potentially improved visual accuracy (Roo et al., submitted). Thus, restriction in light intensity applied in some commercial hatcheries, particularly during the first two weeks of larval development when only cone type receptors of maximum light capture effectiveness at high light intensity are sufficiently developed, may impose higher DHA requirements in broodstock and larvae than in fish cultured at higher light intensities. Besides, inadequate lighting regimes may constitute an stress factor in larval culture conditions, which in turn increase the EFA demand in this fish.

Despite the retention of EFA, particularly DHA, in seabream brain, appearance of larval swimming reaction to a visual stimulus is delayed in fish fed low EFA rotifers, suggesting the delay in functional development of brain and visual system (Benítez et al., submitted). Moreover, larvae fed low EFA rotifers showed lower cruising and escaping swimming speed than those fed high EFA.

Along larval development several authors have shown a requirement of EFA for gilthead seabream very close to 1.5 % n-3 HUFA in dry matter when larvae were fed either live preys (Rodríguez et al., 1998) or microdiets (Salhi et al., 1999), regardless dietary lipid level (Salhi et al., 1994). Much higher requirements are estimated in the literature when EPA contents are 2 or 3 times higher than those of DHA (Rodríguez et al., 1994, 1997), due to the very high incorporation of EPA into the larval polar lipids and the displacement of DHA from certain polar lipids (Izquierdo et al., 2000). However, as it happens in the other life stages, provided other nutrients such as antioxidants are also balanced, elevation of dietary n-3 HUFA up to 8 % keeping a DHA/EPA ratio of 1.7 further improves larval growth and survival (Liu et al., 2002). High n-3 HUFA requirements have been also estimated for red porgy (3.39 % at 1.35 DHA/EPA, Hernández-Cruz et al., 1999) and Dentex dentex (Mourente et al., 1999) despite in the latter the high EPA content in Artemia may have caused an overestimation of the requirements as we have seen in gilthead seabream (Rodríguez et al., 1997). On the contrary, carp larvae seemed to require as low as 0.05% n-3 fatty acids from cod liver oil (Radunz Neto et al., 1993) to cover the essential fatty acid requirements along this period of life.

The particular structure of DHA provides this fatty acid with many important functions in fish metabolism. Its incorporation into cell membranes regulates membrane integrity and function, this fatty acid being an important component of phosphoglycerides, particularly phosphatidyl ethanolamine and phosphatidyl choline,

in larvae. It is specifically retained in starved or low-EFA fed fish, possibly due to the lower cell oxidation rates than other fatty acids (Madsen et al., 1999). It is necessary for growth, survival, flat fish metamorphosis and disease prevention. It may be a substrate for some lypoxigenases and several studies have shown that it has a greater potential as an essential fatty acid for marine fish larvae than EPA (Watanabe et al., 1989; Watanabe, 1993), its requirement being more limiting for growth and survival than those for n-3 HUFA (Izquierdo, 1996). Minimum dietary levels in diets for larval gilthead seabream seem to be 0.8 (Izquierdo, 2004). In larvae, high levels (5 % in dry basis) of dietary DHA in microdiets for gilthead seabream did not caused any excess problem, but further promoted growth and larval survival (Liu et al., 2002). Regarding other sparids, requirements along larval development seem to be about 1.5 % for red porgy larvae when DHA/EPA ratios are about 1.4 (Hernández-Cruz et al., 1999) and close to 2.3 % for *Dentex dentex* fed a very low DHA/EPA ratio (0.32) (Mourente et al., 1999).

Eicosapentaenoic acid is also particularly important for larval growth (Watanabe et al., 1989) playing general and particular roles in fish metabolism. Its presence in rotifers enhances non-specific lipase activity in larval seabream (Izquierdo et al., 2000), neutral lipids esterified with EPA being a preferred substrate for this enzyme. In marine fish it is a main component of polar lipids and it regulates membrane integrity and function, indeed its incorporation into phosphoacylglycerides enhances fluidity of cell membrane (Sipka et al., 1996) in a higher degree than ARA (Hagve et al., 1998) but lower than DHA (Hashimoto et al., 1999). Moderate dietary levels of this fatty acid also enhance DHA incorporation into larval PL (Izquierdo et al., 2000, 2001), causing a sparing effect on such an important fatty acid. It is a good substrate for some cycloxygenases, being precursor of some prostanoids in marine fish and also a main substrate for some lypoxigenases, being the main precursor for leukotriene synthesis in some species. Its competition with ARA for these two types of enzymes enables it to be an important regulator of eicosanoid synthesis. Best growth, survival, resistance to stress and spawning quality have been obtained in larval gilthead seabream with EPA dietary levels of 0.7-0.8 (Rodríguez et al., 1998; Salhi et al., 1999) in dry basis. In larvae, increase of EPA up to 2.9 % in dry basis when DHA/EPA levels where high (1.72) and ARA contents were only 0.05 significantly improved growth, survival and resistance to a shock temperature stress of gilthead seabream (Liu et al., 2002), denoting its high value as EFA. However, increase of dietary EPA up to 1.8 reduced growth when ARA levels are as high as 1.8 % and DHA/EPA about 1.3, denoting how the EFA value of EPA is dependant on the dietary levels of DHA and ARA.

Arachidonic acid is a main component of a minor but very important polar lipid class, phosphatidyl inositol (PI). In vitro, ARA is a preferred substrate for most cycloxigenases, being the main precursor for prostaglandin synthesis, whereas in some marine fish in vivo, EPA is the main substrate due to its high presence in the diet. ARA also constitutes a good substrate for several lypoxigenases, its derivative hydroxi-fatty acids having important physiological functions in marine fish. Its content in the PI of cell membranes possibly regulates eicosanoid synthesis. In gilthead seabream larvae, increase of ARA up to 1 % enhances survival and growth when DHA and EPA dietary contents are 1.3 and 0.7, respectively (Izquierdo, 1996; Bessonart et al., 1999). Increase in ARA contents in the rotifers also prevent post-stress mortality (Koven et al., 2001). ARA seems to play also important roles in turbot juveniles (Castell et al., 1994) and in flatfish pigmentation (Estévez et al., 1997).

Evidences of competition among two or more of these essential fatty acids have been suggested for digestive enzymes, fatty acid binding proteins, phosphoacylglycerides synthetases, lypoxigenases and cyclooxigenases, and probably in beta-oxidation as it happens in rats (Izquierdo, 2004). Not only absolute dietary values for each of these essential fatty acids but also optimum dietary ratios among them must be define since both factors will affect at least to their incorporation into the tissue lipids and hence membrane fluidity and function, the energy values obtain from their beta-oxidation and the production of metabolically active compounds. Thus, optimum DHA/EPA ratios have been defined for turbot larvae around 2 (Reitan et al., 1994) and for seabream around 1.2 at least (Rodríguez et al. 1997). Considering both the sum of the three EFAs and the ratios among them, if we plot the dietary value of the ratio (DHA+EPA+ARA)*DHA/EPA/ARA against growth in some of our recent studies (Figure 6), we found a significant correlation. If we apply the same equation to dietary fatty acids in other gilthead seabream studies (Rodríguez et al., 1994, 1995, Salhi et al., 1998, Liu et al., 2002, Koven et al., 2001, Fernández et al., 1995 and others), we found that for ARA values higher than 0.5% the closer the value of the equation (DHA+EPA+ARA)*DHA/EPA/ARA to 50 the better the growth performance.

Phospholipids

Feeding larvae low dietary contents of PL reduces growth and lipid transport from larval enterocytes to hepatocytes (Kanazawa 1993; Izquierdo et al., 2000). For instance, feeding larval gilthead seabream diets without lecithin supplementation produces accumulation of lipidic vacuoles in the basal zone of the enterocyte and esteatosis in the hepatic tissue, both of them being markedly reduced by a 2% addition of soybean lecithin, denoting an enhancement in the lipid transport activity in gut and liver (Izquierdo et al., 2000). This reduction in lipid transport could be related with a limited capacity for "de novo" synthesis of phospholipids in the larvae. Reacilation of phospholipids in the enterocyte is known to occur through the glycerol-3-phosphate pathway in both the rough and the smooth endoplasmic reticulum (Izquierdo *et al.*, 2000). But since marine fish larvae fed microdiets show enterocytes with a poor development of endoplasmic reticulum and Golgi system, reacilation capacity may be limited in these larvae. Moreover, inappropriate dietary lipids have been found to markedly affect re-esterification pathways in seabream gut (Caballero et al., submitted), modifying the type of lipoprotein formed. For instance, addition of soybean oil promotes PC synthesis by both gycerol-3-phosphate acyltransferase and monoacylglycerol pathways, thus providing material for VLDL formation, whereas addition of rapeseed oil inhibits lipid re-esterification, particularly into TG (Caballero et al., submitted).

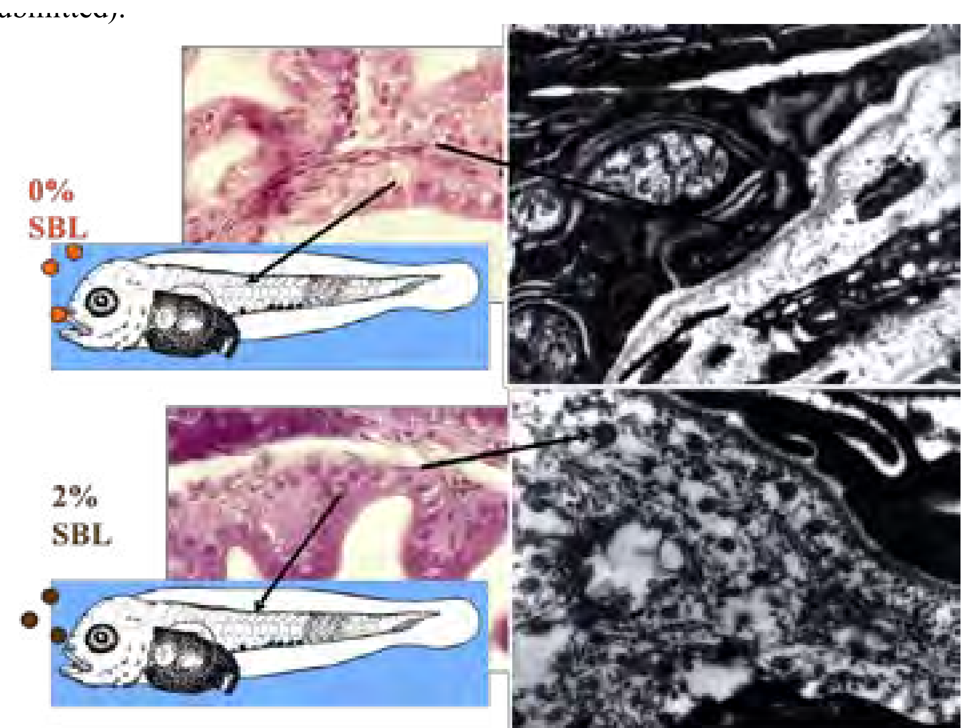


Figure 2 Inclusion of different types of phospholipids in larval microdiets markedly enhance reacilation, lipoprotein synthesis and lipid transport.

On the contrary, when gilthead seabream larvae are fed TG of marine origin, rich in n-3 HUFA it was observed an accumulation of lipid vacuoles in the basal zone of the enterocyte and hepatic steatosis, denoting the good absorption of dietary TG but also a reduced lipid transport to peripheral tissues, whereas feeding with marine PL markedly reduced lipid accumulation in both type of tissues. A higher lipid content due to accumulation of TG and cholesterol esters was found in larvae fed marine TG, whereas in larvae fed marine PL relative proportions of PC and phosphatidyl-ethanolamine (PE) were higher and richer in n-3 HUFA (Salhi et al., 1999). These results agree well with the higher incorporation into larval polar lipids of fatty acids from dietary polar lipids than from dietary triglycerides. In studies with labelled fatty acids dietary n-3 HUFA PL, significantly improved the incorporation of free eicosapentaenoic acid, but not of free oleic acid, into larval polar lipids in comparison to n-3 HUFA rich TG. This specific incorporation of eicosapentaenoic acid when dietary polar lipids are rich in n-3 HUFA could be related to the enhancement of lipid transport, mobilization and deposition in the peripheral tissues by n-3 HUFA rich dietary phospholipids. As a consequence, growth of larval gilthead seabream was improved when they were fed microdiets containing marine PL instead of marine TG despite the slightly lower dietary n-3 HUFA levels of the former (1.5% versus 1.8%, respectively) (Salhi et al., 1999).

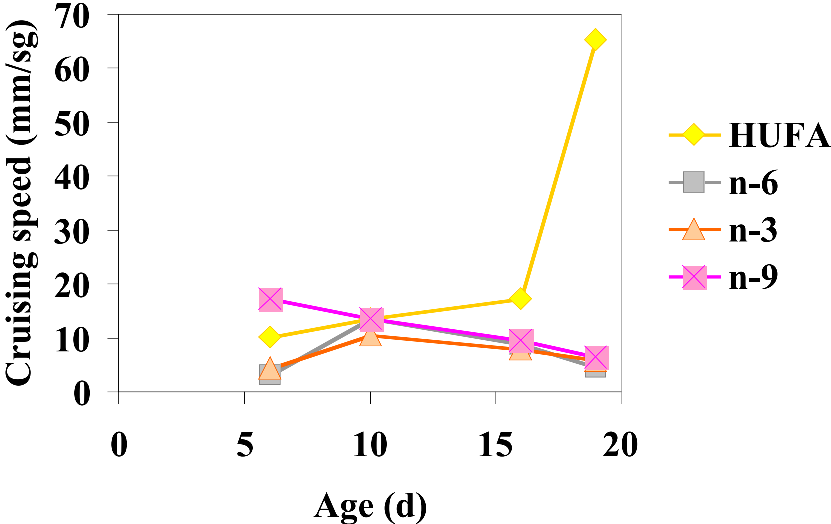


Figure 3. Effect of feeding larval seabream rotifers with different fatty acid composition.

But incorporation of dietary free fatty acids seems to be even lower than that of triglycerides. Thus, labelled oleic acid was better incorporated into both polar or neutral lipids of seabream larvae when it was provided in the diet esterified in a triglyceride than as a free fatty acid, suggesting again a limited capacity of reacilation or transport for dietary long chain free fatty acids or its preferential utilization as energy source in the enterocyte.

Enzymatic, histological and biochemical evidences suggest that marine fish larvae are able to digest and absorb n-3 HUFA rich TG more efficiently than free fatty acids, but feeding with PL, particularly if they are rich in n-3 HUFA, will enhance PL digestion and specially lipid transport allowing a better n-3 HUFA incorporation into larval membrane lipids and promoting fish growth. This confirms former studies which suggest that in addition to the dietary level of essential fatty acids, the molecular form in which they are present in the diet is also important for good growth and survival of marine fish larvae (Izquierdo, 1988; 1996; Izquierdo *et al.*, 1989).

Accumulation of lipidic vacuoles in the basal zone of the enterocyte caused by feeding diets without lecithin supplementation in gilthead seabream disappeared when 0.1% PC was added regardless of its (squid or soybean) origin (Izquierdo et al., 2000). However, squid PC was more efficient in reducing hepatic steatosis than soybean PC, suggesting a combined effect of dietary PC and n-3 HUFA to further enhance hepatic lipid utilization. Indeed both types of molecules have been found to promote lipoprotein synthesis.

Vitamins

The improvement in production of microdiets for larval feeding has greatly facilitated the determination of the vitamin requirements in fish larvae, allowing to experimentally isolate vitamin deficiencies and describing several types of abnormalities. Most described water-soluble vitamin requirements are much higher for larvae than for juveniles of the same species, not only due to the higher metabolic demand in the former, but also for the high ratio surface/volume in larval diets making the diets more prone to oxidation and leaching. Thus, whereas in juveniles vitamin premix accounts for about 2-3% of the diet, in larval microdiets they may reach up to 6-8% of the diet.

Most water soluble vitamin contents of hatchery microalgae and live prey seem to be able to match the requirements of fish larvae, except for the low levels of pyridoxine described in certain studies (González, 1997). However, fat soluble vitamin contents of microalgae and live prey greatly varied among sample batches and with culture conditions, frequently originating hypo and hypervitaminosis.

Vitamin E and vitamin A decreased in seabream from fertilization to the onset of exogenous feeding and a continuous uptake of both nutrients from live preys is observed from day 10th after hatching. However a decrease in the larvae vitamin A content is found when rotifers are substituted by *Artemia* nauplii. Enrichment

of *Artemia* nauplii with fat-soluble vitamins improves amber-jack growth (*Seriola dumerilii*) and seabream microdiet supplementation with 1756 IU of a retinol and beta-carotene mixture significantly improves larval growth. However, bioavailability of beta-carotene seems to be very poor in gilthead seabream in comparison with retinol and astaxanthin which seems to have a provitamin A function in larvae of this species. Regarding vitamin E requirements, progressive elevation of dietary alpha-tocopherol acetate levels from up to 1500 mg/kg in larval seabream diets containing free ascorbic acid significantly reduced larval survival, whereas the same increase in alpha-tocopherol when vitamin C was supplemented as ascorbic acid polyphosphate caused a significant improvement in larval growth without affecting survival, suggesting a pro-oxidative effect of alpha-tocopherol over vitamin C in the former.

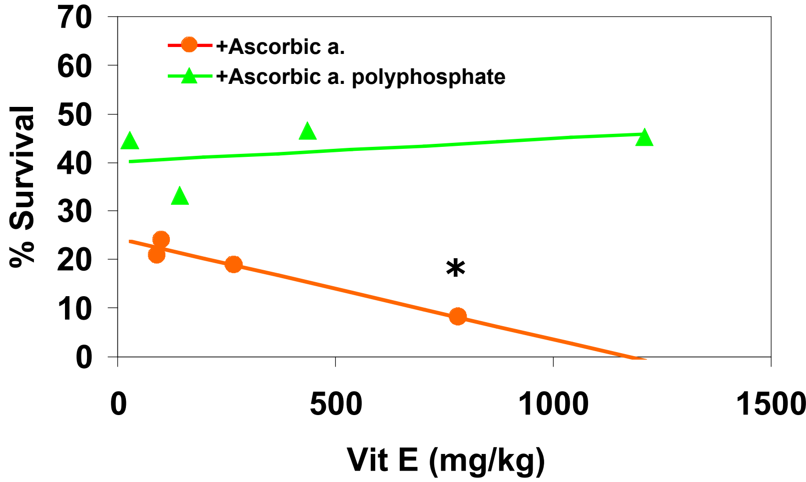


Figure 4. Effect of dietary Vit E in seabream performance in diets containing ascorbic acid in a free or polyphospate form.

Protein and amino acid requirements

Fast growing fish larvae have a high demand for protein requiring more elevated dietary contents than juveniles and adults, microdiets designed for larval rearing containing between 50 and 70 % protein. From the 20 most common amino acids 10 have been found to be essential or indispensable for all studied fish and are required for optimum growth despite fish are not able to synthesize them: Leu, Ile, Val, Thr, Phe, Met, Trp, Arg, His, and Lys. Another two amino acids, Tyr and Cys are only non essential if Phe and Met are present in the diet. At least all those amino acids should be also required by fish larvae. Moreover, the importance of other minor amino acids such as taurine, recently pointed out as essential for best growth and survival of several species of sparids should not be neglected. Methods to determine quantitative requirements of each of those aa in fish larvae include feeding microdiets with graded levels of one amino acid at a time in a test diet containing either all crystalline amino acids, a mixture of casein, gelatin and crystalline amino acids, or a semipurified diet using an imbalanced protein (zein, corn gluten) formulated so that the amino acid profile is identical to the test protein except for the amino acid being tested. As studied by Kanazawa and co-workers for fish larvae of several species, diets are designed to contain protein levels at or slightly below the optimum protein requirement for that species to assure a maximum utilization of the limiting amino acid. Hence, quantitative requirements of several aa have been determined for red sea bream and Japanese flounder larvae (López-Alvarado, 1995). Relations among aa, such as competition or common synthesis pathways, need also be considered. Moreover, aa leaching in the relatively long water staying microdiets, cause difficulties to accurately determine physiological requirements. Hence other methods previously utilized in juveniles have been applied to fish larvae. For instance, from the early 80´s it has been shown that there is not difference between the relative proportions of individual essential aa required in diet and the relative proportions of the same 10 aa present in fish carcass. Since the essential aa profile of fish muscle protein does not differ greatly between individual fish species the pattern of requirement for individual species will also be similar. Thus, analysis of the larval aa composition has been frequently used to predict its essential aa requirements (Watanabe and Kiron, 1994).

Comparison of live prey and fish larvae aa profile would also allow us to predict if such feed would cover the larval aa requirements. For instance, when turbot larvae and live food eaa profiles are compared, the profile of

the latter seems to be deficient in some eaa such as leucine, arginine, threonine or methionine (Conceiçao et al., 1997), depending on the larval age and type of prey, whereas rotifers seem to be deficient in threonine and leucine for larval seabream.

Other methods utilized in juveniles consider that when an essential amino acid is deficient in a diet the major proportion will be used for protein synthesis and only a little fraction will be oxidized to carbon dioxide to obtain energy, whereas if that amino acid is supplied in the diet in excess plasma levels will increase and it will be more available for oxidation. A force feeding method including labelled eaa has been recently developed for fish larvae (Conceiçao et al., 2003), denoting a high retention of labelled doses of eaa in the body (>60%), and low catabolism as measured by liberated 14CO2 (< 25%). In contrast, non essential aa were faster catabolized (>40%).

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Aspects of host-microflora interactions in marine aquaculture: From disease problems to microflora management?

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In most countries the efforts of the aquaculture industry to provide high quality fish and prevent diseases have been remarkable, considering the complexity of the biological systems and the relative novelty of aquatic husbandry. The dramatic decrease in use of antimicrobial agents is mainly due to health promoting measures, including vaccines. However, the production of larvae and fry is still unpredictable for some species, owing to a lack of control of the microbiota in the rearing systems. So far, conventional approaches such as the use of disinfectants and antimicrobial drugs, have had limited success in the prevention or cure of aquatic disease. It is now generally accepted that the use of antibiotics does not constitute a sustainable solution, and may result in microflora imbalance for the larvae [1], and with possible long-term effects. Better means for disease prevention or control should be pursued for intensive aquaculture production systems. Several alternative strategies to the use of antimicrobials in disease control have been proposed and have already been applied successfully in aquaculture. Further progress will depend upon an efficient microbial control, particularly in the production of larvae and during ongrowth, in order to prevent the proliferation and spread of (opportunistic) pathogens, as reviewed in [2, 3].

In marine ecosystems the intimate relationship between bacteria and their hosts, and the relatively open production systems, adds to the complexity of this problem. Intensive aquaculture provides an excellent medium for proliferation and growth of opportunistic bacteria. In the aquatic environment bacteria may travel easily between habitats and hosts, and during intensive larval production, eggs and larvae are in intimate contact with bacteria. Thus the risk of transfection and epizootics is high. Early host-microbe interactions may result in the formation of a protective microflora, or be the first step in infection. Molecular mechanisms of early host-microbe interactions have so far been poorly described. A better understanding of such factors is imperative for successful mass-production of larvae.

In intensive egg production the numbers of bacteria in incubators may be controlled by disinfection, antibiotics or other methods which may disturb the balance of microbial communities, favour proliferation of opportunistic bacteria or result in unpredictable developments. The egg chorion is a well-suited substrate for adhesion of bacteria, and fish eggs become heavily overgrown with bacteria towards hatching. In aquaculture eggs are kept at high densities in incubators with a microflora that differ in numbers and characteristics from that in the sea, and are rapidly overgrown with bacteria after fertilization [4]. The microflora that develops on the egg surface reflects the bacterial composition of the water, but species-specific adhesion to surface receptors may also affect the composition of the epiflora. Members of the epiflora may damage developing eggs, but we do not yet know whether a natural, diverse epiflora may prevent microcolony formation or domination by harmful bacteria. Factors that may protect eggs from bacterial invasion or infection are still poorly understood. Marine fish larvae ingest bacteria by drinking and are thus primed with antigens before active feeding commences [5]. This may result in the formation of an indigenous larval microflora, but at present we know few of the details of host colonisation by commensal bacteria or pathogens in the aquatic environment. Sequestering of intact bacterial antigens by newly hatched larvae may affect their development, but the mechanisms of early host-microbe interactions in aquaculture are still poorly described. The microflora of marine invertebrates resident in fish farms or used as feed may harbour bacteria that are pathogenic to the farmed fish. Thus there is also a need for better understanding of invertebrate–microflora interactions.

Successful aquaculture will rely on better insight into the complex interactions between the cultured organisms and the complex bacterial communities that develop in the rearing systems. The use of probiotics or microflora manipulation may also have a potential in aquaculture, for reviews see [3, 6].

All animals have developed the means to support complex and dynamic consortia of microorganisms during their life cycle. They maintain these societies of nonpathogenic microbes on their mucosal surfaces, and in healthy individuals microbial cells may outnumber somatic and germ cells by a ratio of 10:1. Although the stability of the microflora is important for animal health, very little is known about how its constituents communicate with us to make up stable and mutually advantageous relationships. While considerable attention has been devoted to studying the molecular mechanisms of pathogenic host–microbial relationships, relatively little is known about the molecular foundations of commensal or indigenous host–microbe relationships, and their contributions to normal animal development and physiology. The 'indigenous' microflora comprise microorganisms that inhabit body sites in which surfaces and cavities are open to the environment. However, the term 'indigenous flora' is not clearly defined, and describe the indigenous intestinal microflora composition of a given animal species as a combination of the 'autochthonous flora' present during the evolution of the animal, the 'normal flora' consisting of microorganisms that become established in practically all its members, and some 'pathogens', which are acquired accidentally and are capable of persisting in tissues**.** Today the concepts ”indigenous flora” and ”normal flora” are usually used interchangeably to describe the collection of microorganisms that normally inhabit the GI tract. For a discussion see [7].

The vast majority of these indigenous microbes reside in the intestine, where studies of gnotobiotic mice have disclosed that the microbiota affects a wide range of biological processes, including nutrient processing and absorption, development of the mucosal immune system, and epithelia. The intestinal tract is densely populated with microbes. The microflora of an individual varies along the gut, and as a function of development and environmental factors. The interplay between the microflora, the epithelium, and the lymphoid tissue is dynamic and reciprocal. Thus the indigenous gastrointestinal (Gl) tract microflora may exert extensive effects on the anatomical, physiological and immunological development of the host. The indigenous microflora stimulates the host immune system to respond more quickly to pathogen challenge and, through bacterial antagonism, inhibits colonization of the GI tract by overt exogenous pathogens. Indigenous GI bacteria may also include opportunistic pathogens that can translocate across the mucosal barrier to cause systemic infection in weakened hosts.

Comparisons of conventionally raised, germ-free animals, and animals that were colonized with components of the microbiota during or after completion of postnatal development (conventionalized) have revealed that a range of host functions are affected by the indigenous microbiota, such as educating the immune system and the gut-associated lymphoid tissue, affecting the integrity of the intestinal mucosal barrier, modulating proliferation and differentiation of epithelial cells and processing nutrients consumed in the diet, reviewed in [8]. Understanding how we establish and sustain mutualistic relationships with the components of our gut microflora is important in understanding the basis of health and the origins of a variety of diseases. Comparisons of germ-free and conventionally raised transgenic rats or knockout mice have established that the 'normal' microflora is an important contributor to the development of inflammatory diseases. Aggressive decontamination of the gut may help to prevent a number of reactions, such as graft-versus-host diseases or rejection. Other recent work supports the contention that structural similarities between microbial epitopes and epitopes expressed on host cells may lead to self-directed immunity, which may explain why *Helicobacter pylori* colonization of the stomach may lead to parietal cell loss, chronic gastritis, and an increased risk of gastric cancer [9].

It is generally accepted that fish contain a specific intestinal microflora consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria. The composition may change with age, nutritional status, and environmental conditions, but apparently a primary transient microflora become established at the larval stage, developing into a persistent flora in juveniles or after metamorphosis. Opportunistic vibrios are common members of the indigenous microflora of healthy fish. Pathogens such as *V. salmonicida* and *V. anguillarum* have been shown *in vivo* to adhere to the intestinal epithelium of fish larvae [2]. Adhesion to mucus is usually considered a first step in the infection process. The *in situ* association of pathogenic and non-pathogenic bacteria isolated from fish to different mucosal surfaces of Atlantic salmon (*Salmo salar* L.) were tested by immunohistochemistry [10]. The majority of tested bacteria, including *V. anguillarum* serotype O1*, V. salmonicida*, *V. viscosus, Flexibacter maritimus*, and apathogenic "gut group vibrios", all adhered to mucus on salmon epithelial surfaces. In contrast, *V. anguillarum* serotype O2, did not associate with mucus, but to other tissue components. No strict surface tropism could be demonstrated as mucous-associating bacteria could be observed in all the salmon

mucosal tissues tested as well as with mucus in piglet ileum sections. Thus adhesion to mucus appeared to be a widespread trait of marine bacteria, and not restricted to pathogens or virulent strains.

It is difficult to determine whether or not a particular microorganism is truly indigenous to a particular host. Colonization begins at birth and is followed by progressive assembly of a complex and dynamic microbial society. Assembly is presumably regulated by elaborate and combinatorial microbial–microbial and host–microbial interactions relying on principles refined through animal evolution. These consortia of indigenous microorganisms may also change during the life-cycle, and in fish particularly during development from larval to adult stages. A complete view of vertebrate biology therefore requires an understanding of the contributions of these indigenous microbial communities to host development. These microorganisms also colonize an amazingly wide variety of habitats, including ecosystems where nutrients are extraordinarily scarce and where environmental stresses are extreme, and as in most natural habitats, it appears that the vast majority of our microbial partners can not be cultured with available techniques. Moreover, what we do know about the molecular foundations for commensalism comes from a very limited number of model organisms.

Fucose is well represented in mammalian cellular glycoconjugates, where it is almost always a terminal, α-linked sugar [11]. A difference between the small intestinal epithelial surface of germ-free and conventionally raised mice involves fucosylated glycans. It has been known for some time that a shift in fucosylation of epithelial cell glycoconjugates may engineer a shift in the microflora of mammals through weaning. The molecular basis for this difference revealed that a member of the normal weaning microflora uses a signaling mechanism that instructs the host to present a source of fucose that the microbe in turn can use to colonize and proliferate in this competitive ecosystem. Studies of a gut commensal of the mouse, *Bacteroides thetaiotaomicron*, has revealed a signaling pathway that allows the microbe and host to actively collaborate to produce such a foundation that can be used by the bacterium, and be mutually beneficial for the bacterium and the host. This type of dynamic molecular interaction may help to define and understand such commensal relationships [9]. Before weaning, Fuc α1,2Gal-containing glycans are not detectable in epithelial enterocytes. This was found in conventionally raised mice that have acquired a microflora or germ-free mice [11]. In conventionally raised mice, production of Fuc α1,2Gal-glycans is expressed during weaning in all enterocytes covering ileal villi. In contrast, fucosylated glycan expression is completely absent by the time germ-free animals are weaned. However, inoculation of a conventional mouse's microflora into a germ-free recipient during adulthood re-initiates Fuc α1,2Gal-glycan production, and the induction and of host α1,2-fucosyltransferase and production of Fuc α1,2Gal-glycans is sustained in ex-germ-free mice for the remainder of their lives. These results indicate that microbial signaling is required for sustaining synthesis of fucosylated glycans. Thes glycans represent a mutual benefit for both the bacterium and the host. By controlling the production of its nutrient source in the intestinal epithelium *B. thetaiotaomicron* may colonize the intestine at weaning, when this ecosystem is already densely populated with a pre-weaning microflora. Once a critical density of organisms is attained, the population can signal the host to provide a sustained supply of this carbon and energy source. It seems reasonable that this is a general strategy that may be used by other members of the commensal microflora, and may involve other glycoconjugates [9]. The host benefits by gaining some control over the composition of its microflora. The nutrient foundation may serve to help organize initial colonization by a cohort of microbes. These microbes further transform nutrient availability to allow the proliferation of other microbial partners. Consequently the resulting microbial consortium are defined by microbial–microbial and microbial–host cross-talk.

In a similar manner, the glycosylation of mucus glycoproteins changes during metamorphosis in Atlantic halibut *Hippoglossus hippoglossus* (L.). These qualitative changes included a shift in the mucus composition from predominantly neutral to a mixture of neutral and sulphated glycoproteins that occurred during the development from a pelagic larva to bottom-dwelling flatfish [12]. Numerous saccular cells were also observed in the epidermis of the yolk-sac larvae that disappeared simultaneously as the mucous cells increased in number in the epidermis of the metamorphosed halibut. These findings may help to understand the protective role of the mucus layer of Atlantic halibut during development as compared to other fish species in aquaculture, and may propose a molecular reason for a shift in the indigenous microbiota during this change in habitat.

In adult fish local mucosal and secretory immunity is important in protection against bacterial infections. Teleosts possess intraepithelial lymphoid tissue, although less organized than in mammals. Macrophages, lymphoid cells and secretory immunoglobulin-forming cells are infiltrated within the intestinal epithelium,

and intestinal epithelial cells of adult fish ingest intact antigens. However, fish apparently lack specialized intraepithelial cells for such uptake, and enterocytes may thus serve in antigen sampling [5]. The "immune capacity" of fish larvae is apparently not fully developed until relatively late post hatching. Until then larvae probably rely on non-specific defenses, and may be influenced by their indigenous microbial consortia.

The study of how mutualistic relationships (symbiotic or commensal) are established between a microbe and its mammalian host represents an emerging field. One obstacle to defining the molecular foundations of mutualistic relationships has been the complexities of the ecosystems where such relationships are negotiated. This is particularly true in the intestine, where identifying a 'language' involving one or more of its resident commensals is difficult, because a multitude of signals are occurring at the same time and because we do not yet know what to look for. These models permit experimental analysis of how both host and microbe actively collaborate in shaping and manipulating the gut's nutrient foundation, and the approaches used, and the results obtained, are likely to be applicable to other animal ecosystems [8].

Marine models that have contributed significantly to the understranding of host-pathogen interactions are scarce. However, symbiosis is considered a higly evolved and sophisticated form of host-microbe interaction, and marine habitats have abundant examples of symbiotic relationships. One particularly elegant and ground-breaking model is the study of the interaction between a species of marine squid (*Euprymna scolopes*) and its bacterial symbiont (*Vibrio fischeri*). This study was among the first to reveal that bacteria can induce morphological phenotypes in their animal partners, and demonstrated that bacteria can play a crucial inductive role in the normal development of animal organs by effecting fundamental developmental processes, such as cell death and differentiation [13]. The developmental consequences of the squid–Vibrio interaction share some similarities with the microbial impact on mammalian gut development. The squid–Vibrio partnership is founded on mutualistic benefit. The bacteria inhabit the light organ of the squid and produce the light that illuminates the lower surface of the animal, providing camouflage. Thus, both partners benefit from the association: the bacteria are offered a protected nutrient-rich niche, whereas the host is somewhat protected from predators [13]. Squid that are not exposed to *V. fischeri* remain in an arrested state of morphogenesis, and thus the microorganism actively promotes its association with its host.

Comparison of germfree versurs conventional zebrafish (*Danio rerio)* provided an opportunity to investigate the molecular mechanisms underlying such interactions through genetic and chemical means during larval and juvenile stages germ-free and conventional zebrafish [14]. Using a DNA-microarray technology, this work demonstrated that 212 genes were differentially expressed in germ-free fish in respect to conventionalised fish. In addition it was demonstrated that some genes were specifically expressed in response to certain micro-organisms, indicating that the early colonisation of the GI tract by a particular microorganism can be responsible for changed metabolism in the fish larvae. The results with zebrafish demonstrated that microorganisms may stimulate the intestinal epithelial development and affect the enterocyte morphology also in fish. The findings also provided an argument for using gnotobiotic wild-type and or genetically manipulated zebrafish as a model organism for deciphering the molecular foundations of symbiotic commensal host–bacterial relationships in the vertebrate digestive tract.

In aquatic ecosystems the intimate contact between microorganisms and other biota, and the constant flow of water through the digestive tract of fish and invertebrates affect host-microflora relationships. A diverse and natural indigenous microflora, but it is not yet known to what extent the natural microflora of fish may protect against pathogen colonization, for discussion see [2, 3]. There is increasing evidence that microflora manipulation, or addition of microorganisms that are antagonistic or probiotic, may improve health conditions and survival of larvae in intensive rearing. The term probiotic is mostly used for "living cells that exerts beneficial health effects on the host by improving the microbial balance or properties of the indigenous microflora" [15]. The use of probiotic microorganisms has proven advantageous in domestic animal production, and is one of the most significant technologies that have evolved in response to disease control problems. Such "bacterial management" approaches are applied at production level in poultry farming. Considering the recent successes of these alternative approaches, the Food and Agriculture Organization of the United Nations defined the development of affordable and efficient vaccines, the use of immunostimulants and nonspecific immune enhancers, and the use of probiotics and bioaugmentation for the improvement of aquatic environmental quality as major areas for further research in disease control in aquaculture [16]. The characteristics may

include antagonism or colonization prevention towards pathogens, stimulation of immunity or innate defenses or health benefits from released factors. The use of probiotics in aquaculture has been reviewed [6, 17].

Table 1. Some effects of probiotic bacteria, modes of action, aquaculture systems where they have been used, and selection criteria.

Probiotic effect	**Modes of action**
Growth promotion	Produce inhibitory compounds
Effects on other infections	Compete for chemicals or energy
Alleviation of lactose intolerance	Compete for iron
Anticarcinogenic effect	Compete for adhesion sites
Enhancement of immune response	Interaction with phytoplankton
Increase production in aquaculture	
Improvement of water quality	
Aquaculture system used	**Selection criteria for probiotics**
Fish eggs and larvae	Acquisition of putative probiotics
Fish juveniles and adults	Screening and preselection
Crustaceans and Penaid shrimps	In vitro antagonism tests.
Crabs	Colonization and adhesion
Bivalve molluscs	Small-scale tests
Live food	Evaluation of pathogenicity of strains
- Unicellular algae	*In vivo* evaluation of probiotic effects
- Rotifers	Mode of application of putative probiotic
- Artemia	Experimental infections
Microbially matured water	*In vivo* antagonism tests.
Interaction with nutritional effects	

In aquaculture severe microbial problems may start at the egg stage, and consequently manipulation of the egg epiflora would appear reasonable. We tried to manipulate the egg epiflora by incubating aseptically dissected, bacteria-free eggs with defined cultures of antibiotic producing bacteria [4]. However, the antibiotic-producing isolates failed to prevent colonization by a natural seawater microflora. This may suggest that egg-surface receptors that accounts for a colonization of a diverse natural microflora may furnish protection against domination by particular strains [4].

It has been observed that *Artemia* and rotifer cultures may transfect bacteria that are harmful to the fish larvae during start feeding, and various attempts at microbial control of the startfeeding cultures the live food organisms (*Artemia* and rotifers) used for larvae. Microbial control of *Artemia* juveniles has been achieved by pre-emptive colonization by selected bacterial strains [18], and the resulting changes in the *Artemia* microflora appeared stable. In contrast, preincubation in bacterial suspensions may be aimed at suppressing opportunistic bacteria in *Brachionus* or *Artemia* cultures [19] - an ecosystems approach to suppress opportunistic bacteria. Successful attempts at controlling the microflora by manipulating of the ecosystem has been reported, using microbially matured water in a system that competitively selects against opportunistic and potentially pathogenic bacteria [20]. It thus appears that improved microbial control of food organisms used for larvae is feasible through microflora or ecosystem manipulation. However, stable transfection of microorganisms between hosts, or from food organisms to hosts, is difficult and still has to be tested for long-term effects.

In intensive invertebrate culture diseases often prevail as a result of the build-up of organic pollution. Promising results have been obtained in shrimp pond aquaculture in China, by adding immobilized bacteria that effectively reduce sediment organic pollution, an example of probiotic bioremediation [21].

Lactic acid bacteria have been isolated from the intestinal mucosa of a variety of fish species, and may produce growth-inhibiting factors that could inhibit various *Vibrio* spp., especially *V. anguillarum*. Lactic acid bacteria are widely used as probiotics, and the use of a *Carnobacterium* sp. as a probiotic for Atlantic salmon and rainbow trout has been reported [22], and the potential of lactic acid bacteria as probiotics in aquaculture has been reviewed [23].

Vibrios are natural members of the indigenous microflora of healthy fish, and also dominate in the intestinal tract of marine fish, reviewed in [3]. Fish pathogenic vibrios may be present on wild fish or healthy fish in aquaculture, and commensal vibrios with inhibitory activity against pathogens may be isolated from mucosal surfaces of healthy fish. It has been assumed that commensal, apathogenic strains that may help to confer protection against related pathogenic strains, reviewed in [3]. We observed, in agreement with this concept, that survival of halibut (*H. hippoglossus*) larvae could be affected by incubation with indigenous bacteria isolated from fish [24]. Thus, introducing a strain that may compete with the pathogen may increase survival. However, introduction of apathogenic strains may not be unequivocally beneficial. We also demonstrated increase in the number of epidermal saccular mucous cells of halibut larvae following incubation of the larvae in seawater with increased numbers of bacteria and following addition of apathogenic bacteria to the incubators [12], suggesting that changes in the microflora may induce non-specific defenses of the larvae.

Many bacteria that cause diseases of humans and animals are highly motile. The role of motility and chemotaxis in the host-parasite relationship of pathogenic bacteria has been reviewed [25], arguing that for many pathogens, motility is essential in some phases of their life cycle and that virulence and motility are often intimately linked by complex regulatory networks. Possibilities to exploit bacterial motility as a specific therapeutic antibacterial target to cure or prevent disease are discussed. Also for some fish pathogens motility appears to play a dominant role during the infection process. Using a range of motility-mutants, we demonstrated that motility per se was required for the association of *V. anguillarum* O1 with mucus, and that no adhesin-receptor interactions could be demonstrated to play a role in this association (G.K. Knudsen and J. A. Olafsen, unpublished). The motile mutants included one with full motility but no chemotaxis, and three with reduced motility due to a truncated flagellum. The non-motile mutants included four with no flagellum and one with a paralysed flagellum.

Perspectives

The basic mechanisms of microbial interactions during production of fish larvae in aquaculture are not well understood. To achieve improved microbial control in larval rearing systems we still need information about bacterial colonization factors, host regulation of the adherent microflora as well as interaction with egg surfaces and food organisms. Also, we still lack information about development of tolerance to the commensal microflora.

Most marine bacteria adhere well to fish mucus, but we also lack information about microbial adhesion factors as well as non-specific defence systems in mucus. Despite the fact that many fish pathogenic strains are relatively host specific, little is yet known about receptors for bacterial adhesion. Moreover, for marine bacteria there is a conspicuous lack of information about invasion strategies like antigen shift or phase variation, mechanisms that are known to be key factors in microbial pathogenicity.

The use of probiotics, or microbial manipulation, in intensive rearing of marine organisms may have a profound potential in health management. However, it is not likely that improved microbial control may be achieved by finding the “ideal probiotic solution”. Various stages and situations may call for different approaches, such as antagonism, competition, bacteriocin production, immune stimulation, health promotion and bioremediation. Thus it is likely that the use of a selected mixture of beneficial strains, or different approaches at different stages may prove more effective in different situations - and more stable over time.

A feasible approach in aquaculture will be the use of controlled bacterial communities at various critical stages of larval rearing. Such “multifunctional bacterial communities” will have several advantages over single-strain probiotics. Introduction of such measures will require a better basic understanding of various basic aspects of host-microbe interactions in the marine environment and a set of protocols for practice of bacterial management in fish hatcheries for the industry to take advantage of collected experience.

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Current status of live food culture in Japan

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Abstract

Euryhaline rotifer *Brachionus plicatilis* complex has been used as a first live food for marine fish larvae. Even under similar environmental conditions, genetically close rotifer strains have variable size, thus, selection of appropriate size of rotifer strains is useful for feeding fish larvae whose mouth sizes differ among species and growth stages. Rotifer life history parameters such as reproductive characteristics and lorica size can be regulated artificially using chemicals such as neurotransmitters and hormones. In vivo enzyme activity test is useful for detecting instability of rotifer cultures.

Culturists generally feed rotifers several times a day. In this condition, however, rotifers suffer periodical starvation, which results in low quality rotifers that live long and spawn less. Recent development of continuous culture system for rotifer mass production has enabled the aquaculturists in Japanese public hatcheries to carry out stable rotifer production of higher quality live food. This system utilizes large tanks (e.g. 25 m^3) used in conventional rotifer cultures. With continuous culture water inflow, feeding and harvest, L-type *B. plicatilis* can be produced at 0.2-1x10^8 rotifers/day/m^3 for 40-220 days. Rotifer population growth rates of this system are higher than those of conventional batch culture or semi-continuous culture methods, while the production costs are the same. Live rotifers can be stored under low temperature such as 4-12°C. Resting eggs are appropriate for long-term preservation of rotifers.

Keywords: live food; rotifer; practical mass culture; basic research

Introduction

Before 1960, euryhaline rotifer *Brachionus plicatilis* was considered a noxious animal, which caused huge damages in traditional eel cultures conducted in outdoor pond in Japan. Because of their rapid reproductive rate, rotifers completely consume phytoplanktons and completely dominate ponds resulting to a change in water color from green phytoplankton to brown rotifers. This phenomenon is called "Mizukawari" (change of water color). From 1950's, Takashi Ito conducted a series of studies on this phenomenon (e.g. Ito, 1958, 1960, 1971), and revealed the difficulty of eliminating rotifers using chemical treatments, while maintaining good water quality necessary for keeping eels alive. Although ponds are dried off during off season, rotifers still survive since they are capable of producing resting eggs, which can thrive in dried pond sediments. Resting eggs are highly tolerant to toxicants and environmental stressors, thereby posing greater difficulty in removing them from pond sediments.

In the same period, research has been focused to develop techniques for artificial breeding as well as mass rearing of producing juveniles of marine fishes. Culturists however, have encountered difficulty in obtaining sufficient amount of live food for fish larvae (Hirano and Ooshima, 1963). The success of acclimating brackish *B. plicatilis* to seawater (Ito, 1960) enabled its use as diet for rearing larvae of marine fishes. Studies were conducted to establish *Nannochloropsis* and baker's yeast as rotifer food (reviewed by Hirata, 1980), as well as to detect nutritional problems of mass cultured rotifers as live feed and improvement of nutritional value by the fortification of n-3 HUFA (reviewed by Watanabe et al., 1983). The development of marine fish larval rearing technique as well as of rotifer mass culture technology have been reviewed by several authors (Fujita, 1973; Fukusho, 1983). During late 1980's, phytoplankton industry has developed products such as paste of condensed microalgae that enabled them to cultivate high density rotifer of more than 1x10^3 ind./ml (Yoshimura et al., 1996).

Recently, Hagiwara et al. (2001b) reviewed the development of technique on rotifer culture, as well as the progress of laboratory studies, which can be used as a basis for rotifer culture practices. In this paper, we attempt to summarize some progress obtained thereafter.

B. plicatilis strains are quite variable in size and shape. Based on the results of morphometric and genetic (allozyme and karyotype analysis) studies with *B. plicatilis* (reviewed by Hagiwara et al., 1995, 2001b), Segers (1995) reclassified large morphotype into *B. plicatilis* and small morphotype into *Brachionus rotundiformis*. Recent works on rotifer gene analysis provide the possibility of several species boundaries among geographically isolated *Brachionus* strains. Ciros-Pérez et al. (2001) suggested the use of *B. ibericus* as the S-type strain found in Torreblanca marsh in Spain. Based on these facts, we use the term *B. plicatilis* complex with this group, and when further description is necessary, we describe them as one of three morphotypes L, S and SS, according to Hagiwara et al. (2001b).

Diet for Rotifers

The use of baker's yeast as rotifer diet is still beneficial to aquaculturists since it allows rotifer production at a cheaper price. But the culture is less stable due to the decline of water quality. Moreover, the produced rotifers need further nutritional enrichment before feeding to fish larvae. Phytoplanktons are still the ideal food for rotifer cultures, because it results to higher stability of culture, but its low productivity poses a problem in ensuring enough amount of food for rotifer production.

Chlorella Industry Co Ltd. pioneered the production of commercial microalgal products. They started on producing freshwater *Chlorella. Chlorella* has genetically high productivity (e.g. doubling time) compared to other marine microalgae, and they can be mass cultured in organic medium at the same growth rate without light (Maruyama and Hirayama, 1993). Hirayama et al. (1989) succeeded in incorporating Vitamin B_{12}, an essential vitamin for rotifers inside the *Chlorella* cells. In 1997, Hayashi & Maruyama (in prep) developed a technique to introduce n-3 HUFA inside Chlorella cells and such product is already commercially available. Other *Chlorella* products also contain large amount of βcarotein, that make fish larvae to have higher tolerance against viral disease (Tachibana et al., 1997). Frozen paste of marine alga *Nannochloropsis oculata* is also a popular product mainly because of its higher EPA content.

Recent laboratory studies in this area provided basic genetic information that are important for future technology development. A *Chlorella* strain acclimatized at 3°C can survive even after they are frozen, and this mechanism involves the appearance of genes for n-3 fatty acid desaturase (Suga et al., 2002). Through treatments of mutagenesis, Chaturvedi et al. (2004) obtained a *N. oculata* strain that contain higher amount of eicosapentaenoic acid with higher temperature optima for growth.

Rotifer mass culture

Before the introduction of phytoplankton products as major food for rotifer culture system, baker's yeast was widely used for rotifers mass cultured by batch or thinning-out (semi-continuous) methods. Despite its cheaper cost, the overfeeding of yeast tend to bring a decline in water quality and result to instability of rotifer culture. With this system, the amount of food is insufficient to the physiological demand of rotifers, thus, rotifers are likely starved. Yoshinaga et al. (2000) reported however, that periodical starvation of maternal *B. plicatilis* has a positive effect because it can induce higher starvation tolerance in offspring, although it reduces fecundity on the exposed animals.

Due to the commercial availability of microalgal diets, it became feasible to provide ample amount of diet for rotifer population without causing water quality to decline. Thus, continuous feeding of phytoplankton diet would result in higher productivity of rotifers compared to traditional culture system. Based on this concept, high density batch-culture technique of rotifers have been developed (reviewed by Yoshimura et al., 1996, 1997), and recent modification of this technique achieved the culture density exceeding $1x10^5$ ind./ml (Yoshimura et al., 2003). The development of this technique allows small hatcheries to produce sufficient amount of rotifers even in small tanks. It is generally recognized, however, that the physiological condition of rotifers at higher culture density may deteriorate either due to ammonia accumulation, bacteria/protozoa influence, food shortage or oxygen decline. One arising question is, whether rotifers produced at ultra high density would have equal quality as diet for fish larvae than those produced using traditional method.

Recent study on red seabream *Pagrus major* larvae (Tomoda et al., 2004) revealed that nutritional quality of rotifers varies depending on the population growth stage. Furthermore, fish larvae fed on those rotifers have different body chemical composition. In this experiment, batch cultured rotifers were harvested at four different growth stages: lag, exponential growth, late-exponential and stationary phases. Table 1 summarizes these results.

Table 1. Rotifer density, mortality, and egg ratio enriched at different culture days and red seabream *Pagrus major* growth and chemical composition fed enriched rotifers (after Tomoda et al., 2004).

	A	B	C	D
Rotifer				
Density at harvest (ind/ml)	$0.25 \pm 0.1 \times 10^3$	$0.65 \pm 0.1 \times 10^3$	$1.2 \pm 0.2 \times 10^3$	$1.8 \pm 0.4 \times 10^3$
Mortality 6 hrs after enrichment[a]	1.7 ± 0.7[a]	1.8 ± 1.2[ab]	2.9 ± 1.1[ab]	6.2 ± 3.2[c]
Egg ratio 6 hrs after enrichment[a]	46.4 ± 8.3[a]	31.5 ± 7.1[b]	38.1 ± 8.6[ab]	35.7 ± 4.8[b]
Fish Larvae				
Total length (mm)[b]	6.35 ± 0.5[a]	6.30 ± 0.5[ab]	6.25 ± 0.4[ab]	6.0 ± 0.5[c]
EPA content (g/100g)	0.60 ± 0.02	0.58 ± 0.03	0.5 ± 0.05	0.4 ± 0.01
DHA/EPA	5.02 ± 0.33	5.45 ± 0.59	6.24 ± 0.53	7.31 ± 0.80

[a] Values are means and standard deviation of 10 replicates per treatment. Values with common letter are not significantly different (Scheffe's test) at 95% confidence level.

[b] Values are means and standard deviation of 150 replicates per treatment. Values with common letter are not significantly different (Tukey-Kramer test) at 95% confidence level.

A,B,C and D are days 2,4,6, and 8 of *Chlorella* enrichment, respectively.

Although the rotifers received the same nutritional enrichment, rotifers in the late-exponential and stationary phases showed poor quality, such as lower egg ratio (number of carried parthenogenetic eggs per female) and lower survival. Larvae fed rotifers at the stationary phase also showed poor performance in growth and nutritional condition; total length was smaller on day 20 posthatch, and had less quantity of eicosapentaenoic acid (EPA) in larval body and higher content of DHA/EPA.

As discussed in Hagiwara et al. (2001b), the continuous culture system is more advantageous to maintain good water quality for rotifer culture (James and Razeq, 1986, 1989; Walz, 1993), and a possible solution to avoid production of rotifers with low quality. It appears that such system has greater potential to aquaculturists. A high density rotifer culture was developed using continuous culture system in 1-2 m^3 small culture tank. With this system, the culture density of SS type was maintained at 3 to 6×10^3 ind./ml (Fu et al., 1997). This technique, however, can be effectively applied to S and SS-type *B. plicatilis*, while culture practices with L-type have been less common. But high-density continuous culture of rotifers showed less productivity in L-type *B. plicatilis* cultures and the entire equipment is costly for private hatcheries (Fu et al., 1997).

To date, Kuwada's group in Fisheries Research Agency (former Japan Sea Farming Association) developed an extensive continuous culture system for rotifers utilizing large rotifer culture tanks (20-50 m^3). This system has been common in public hatcheries in Japan (reviewed by Anon. 2000), because it does not require extra cost. The system is composed of one tank each for rotifer culture and harvest, which receives continuous inflow of filtered seawater and freshwater to adjust the salinity to 20 ppt, as well as continuous feeding of refrigerated condensed *Chlorella*. To further reduce the cost, a part of *Chlorella* can be replaced by cheaper diet such as baker's yeast (Snell and Hoff, 1985). The rotifer culture was continuously harvested by siphoning (Figure 1).

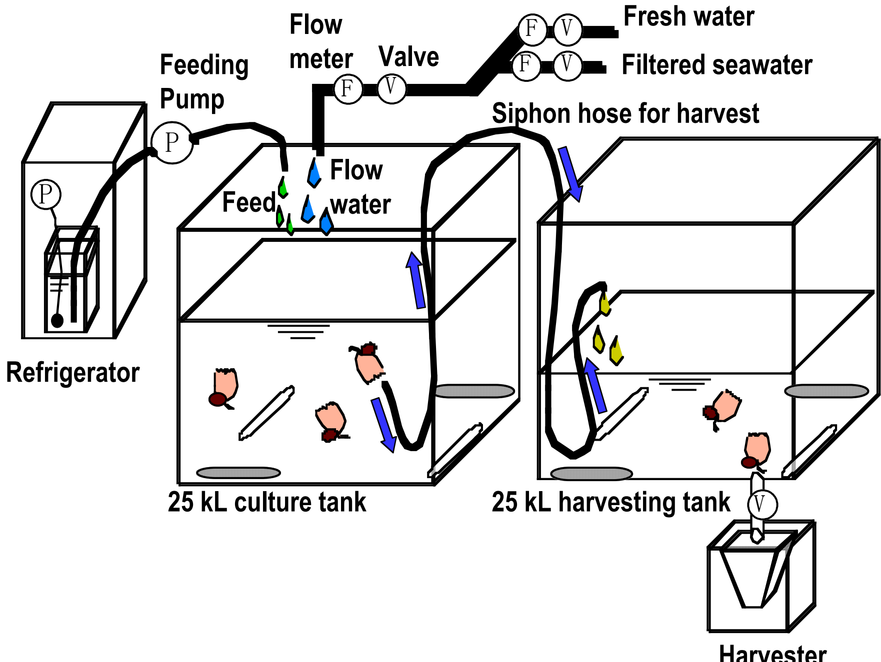

Figure 1. Schematic overview of extensive continuous culture.

This system is advantageous to provide rotifers a stable environment that enables them to maintain stable population growth. This system also utilizes a chemostat system, which maintains the amount of food at food limitation level, and maintains the dilution rate constant. In the steady state condition, therefore, dilution rate equals to rotifer specific growth rate, thus allows the culturists to harvest rotifers automatically, which are decided by spontaneous performance of rotifers based on given amount of diet and environment. With this system, culturists do not need to regulate the amount of feed to attain maximum growth of rotifer population. For example, about 1 x 10^{10} L-type *B. plicatilis* can be harvested daily in a 25 m^3 tank for 40 continuous days at 25°C with daily feeding amount of approximately 2.6 kg dry weight diet composed of *Chlorella* and baker's yeast. In our records of different culture trials, rotifer density was generally maintained between 100-500 ind./ml with population growth ranging between 70 and 100% per day. Culture period can reach to more than 200 days using the same culture unit. This system can also be applied to traditional thinning-out cultures, by regulating the water inflow and constant and continuous feeding, even though rotifer harvest is conducted once a day only. Another advantage of this system is that, culturists can conduct rotifer mass cultures at desired temperatures. In Figure 2, rotifer population growth rates were compared among different methods, and extensive continuous culture system showed a wide spectrum of temperature range, and gave high rotifer production and harvest. Especially at lower temperature between 10 and 20°C, this method produced more rotifers, compared to other methods.

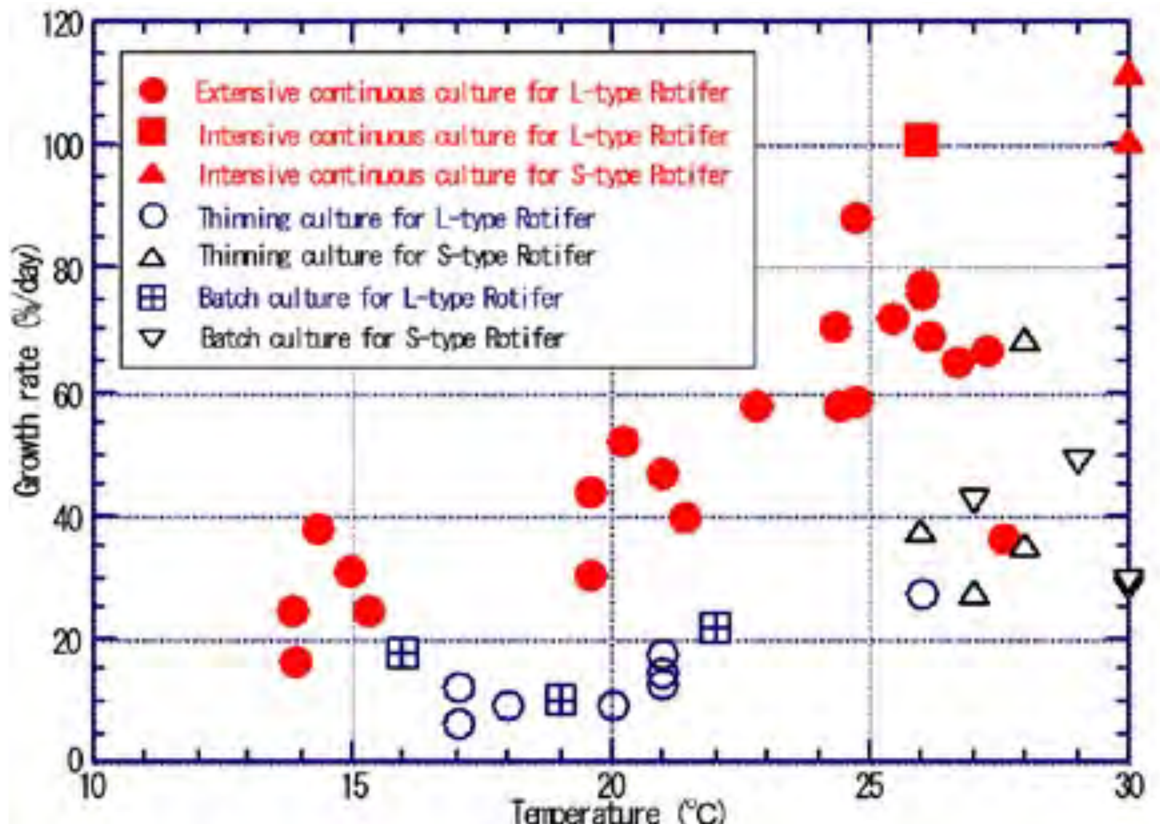

Figure 2. Population growth (%/day) of L- and S-type rotifers in different culture system.

Fundamental rotifer study and future application

Regulation of rotifer size

Size is one of the important biological parameters of *B. plicatilis*, since size-dependent food selectivity with *B. plicatilis* is found in marine fish larvae (Oozeki et al., 1992; Olsen et al., 2000). Some fish species including groupers can ingest only small sized diet of less than 100-150 µm, and, thus, the use of tropical SS-type *B. plicatilis* strains is important.

Figure 3 shows the lorica length and width of 70 rotifer strains of *B. plicatilis* including L-, S- and SS-type. These strains were collected from

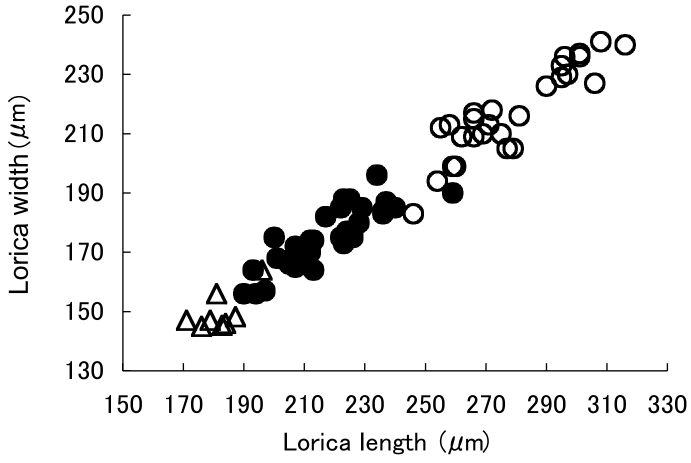

Figure 3. Relationship between lorica length and width of *Brachionus plicatilis*. Open circle, closed circle and triangle indicate data of L-, S- and SS-type *B. plicatilis*, respectively.

different sites in the world (Fu et al., 1991; Hagiwara et al., 1995), cloned and cultured in the laboratory at 23-25°C, maintained at 20-22 ppt salinity, fed *Nannochloropsis oculata*. It is known that the shape of lorica also differs among three rotifer types; S and SS-type lorica is more round than L-type. Rotifer size at sexual maturity (first spawning of parthenogenetic egg) was compared. A large variation in size was detected among rotifer strains within the same type and size distribution overlaps with other rotifer types was also observed. Although the lorica shape is variable, this does not affect the measurement of its length and width, because the plots fit in a linear pattern. It should be noted that the plots on Figure 3 shows only one aspect of rotifer size distribution. Rotifers are known to grow faster during the initial days from hatching; 30-40% growth of lorica length is observed during the first 48 hours from hatching at 25°C. A large plasticity in size is observed in rotifers under variable environment. For example, the lorica length of SS-type ranges between 170-195 µm at 25°C (Figure 3), while it reduces to less than 150 µm at 30°C. An L-type strain with the lorica length of about 280 µm at 25°C can grow to up to 330 um at 15°C (Hagiwara et al. unpublished). Moreover, feeding *B. plicatilis* with *Tetraselmis* results to 12.9% larger (lorica length) rotifers, compared when given *Nannochloropsis* (Okauchi and Fukusho, 1984).

Chemical treatment can also regulate the rotifer size (Table 2). When gamma-aminobutyric acid (GABA), 20-hydroxyecdysone and juvenile hormone were added to rotifer batch culture, a 4.4 to 9.6% increase in lorica length was observed. The mechanism of action of these chemicals in rotiers, however, has not been clarified.

Table 2. Lorica length and width of rotifer *B. plicatilis* after addition of chemicals (after Gallardo et al., 1997).

Chemical used (concentration)	Lorica length	Lorica width
γ-aminobutyric acid (5mg/L)	4.4% larger	n.s.
20-hydroxyecdysone (0.05 mg/L)	3.9 % smaller	4.8 % lower
Juvenile hormone (0.05 mg/L)	9.5% larger	8.9 % higher
Prostaglandin (0.05 mg/L)	5.8% larger	n.s.

Larger and smaller means % increase and decrease relative to the control (without chemical treatment), respectively.
n.s. means no observable difference compared to the control.

Culture diagnosis and treatment

The physiological processes involved in rotifer reproduction are still ambiguous. The effects of addition of some vertebrate hormones and neurotransmitters to the rotifer culture have been investigated in Hagiwara's laboratory. Changes in life history parameters were thoroughly monitored and analyzed. Tables 2 and 3 show the list of these chemicals and their effects on rotifer size, sexual and asexual reproduction. Rotifer sexual reproduction involves a series of processes such as the appearance

Table 3. Increase in population growth and mixis induction in rotifer *B. plicatilis* after addition of chemicals (after Gallardo et al., 1997).

Chemical used (concentration)	Population growth*	Mixis induction**
γ-aminobutyric acid (50mg/L)	2.0 times (on day 4,6,8)	n.s.
Porcine growth hormone (0.025 I.U./mL)	1.7 times (on day 8)	n.s.
Human chorionic gonadotropin (2.5 I.U./mL)	2.4 times (on day 6)	n.s.
Juvenile hormone (0.05 mg/L)	n.s.	1.9 times
Serotonin (5mg/L)	1.5 times (on day 6,8)	1.7 times

*values are compared with the control (without chemical treatment) on daily basis
**values are total increase on the final day of culture
n.s. means no observable difference compared with the control

of mictic females that conduct meiosis, parthenogenetic production of haploid males by unfertilized mictic females, mating, copulation and fertilization, and resting egg formation by fertilized mictic females. Among the chemicals tested, GABA and HCG (human chorionic gonadotropin) doubled the rotifer population growth (Table 3; Gallardo et al., 1997). Addition of GABA is especially effective when rotifers are exposed to stress such as shortage of food and increase of unionized ammonia (Gallardo et al., 1999).

Studies have been conducted to establish techniques to assess the physiological status of cultured rotifers, as well as to predict culture collapse. Table 4 listed possible methods to detect physiological condition of rotifers. Egg ratio means the number of parthenogenetic eggs per female, and has been regarded as an important parameter to assess the status of rotifer culture. This practice started in late 1960's. The flaw of this parameter is that, it takes 24 hours at 25°C until change in egg ratio happens after rotifers are exposed to unfavorable environment. Environmental change rapidly affects swimming speed and ingestion rate of rotifers, but it takes more than 1-2 hours to accomplish the measurements even using computer motion analysis system. In vivo enzyme activity test is also known to be effective in assessing the rotifer culture status, but again it takes 1 to 2 hours to conduct the analysis, and this method needs further refinement before it can be applied in rotifer culture practice.

Table 4. Index used to assess rotifer culture and their characteristics.

Index Used	Features	Reference
Egg ratio	easy; measurement could be done within 30 minutes; takes 1-2 days to reflect egg ratio	Snell et al., 1987
Ingestion rate	easy; takes 2-3 hours to measure; rapid response against environmental change; large variation in data	Ferrando et al., 1993 Juchelka and Snell, 1994
Swimming speed	easy; takes 1-2 hours to measure; rapid response against environmental change	Snell et al., 1987 Jassen et al., 1994
Enzyme activity	simple and easy, needs special instrument such as computer, flourometer, and image analyzer; takes 1-2 hours to measure; rapid response against environmental change	Moffat and Snell, 1995 Araujo et al., 2000

Changes of ingestion rate due to environmental stress provide us an insight of other aspects of rotifer physiology. Mass cultured rotifers need nutritional enrichment, which usually contain fatty acids that could degenerate the water quality. However, nutritional enrichment could not be avoided because it is paramount for fish larvae. It is plausible that culture collapse in mass cultured rotifer is related to the decrease of feeding activity of rotifers. Araujo and Hagiwara (2004) found that addition of GABA can improve the health condition of rotifers when they are exposed to stressful environment (e.g. increase of unionized ammonia, protozoa contamination). Addition of GABA during nutritional enrichment culture could improved survival and swimming activity of rotifers (Gallardo et al., 2001).

Despite the progress of techniques in rotifer mass culture, diagnosis of culture status and treatment for culture for recovery, it is important to have a means of rotifer preservation. To date, techniques have been developed to store live rotifers under low temperature such as 4-12°C (Assavaaree et al., 2001; reviewed by Hagiwara et al., 2001). Resting eggs are appropriate for long-term preservation of rotifers. The advantages are reviewed in several publications (Hagiwara and Hirayama 1993, Hagiwara 1994, Hagiwara et al., 1997)

Rotifers as larval diet

Dietary values of rotifers should not be limited to its nutritional quality. As fish larvae grow, the amount of food they need increases and they prefer to eat larger-sized prey (Ivlev, 1965). It has been generally accepted that the optimal prey size for fish larvae is determined by their mouth size. Feeding regime of fish larvae is

designed primarily based on mouth size data. This has resulted in successful improvement of larval survival and growth in many fish species. Adjustment of the amount of feed depending on larval developmental stage also resulted in better growth and survival. (Kitajima, 1978). Limited information is available, however, on rotifer size selectivity of fish larvae. Some of these, are studies on developmental change of food selectivity in striped mullet (Oozeki, 1992) and Atlantic halibut (Olsen et al., 2000) larvae. In Hagiwara's laboratory, a similar study was done on threeline grunt *Parapristipoma trilineatum* larvae for 15 days posthatch. During the initial 7 days, the larvae grew better by feeding S-type rotifer (90-210 µm in lorica length) than by feeding L-type (160-320 µm). The feeding of L-type rotifer resulted in slower growth during the initial 7 days, but showed better growth than that of S-type after 7 days posthatch. Olsen et al. (2002) studied the food selectivity of larvae when L (>174 µm in lorica length), S (137-174 µm) and SS-type rotifers (<137 µm) were mixed and fed. The 3, 4 days posthatch larvae showed high selectivity with SS-type, 6-9 days posthatch with S-type and 9-16 days posthatch with L-type. On day 17 posthatch, larvae showed highest selectivity with *Artemia* nauplii (ca. 800 µm) (Olsen et al., 2002). Comparison of food selectivity among fish species was also conducted with yellowtail *Seriola quinqueradiata*, spotted halibut *Verasper variegatus* and flathead *Platycephalus* sp. from day 0 to 15-20 days posthatch. The result indicates that among the three species, flathead larvae showed the highest selectivity to larger rotifers despite their mouth size being the smallest (mouth sizes at the onset of feeding were 280, 510 and 260 µm for yellowtail, spotted halibut and flathead, respectively). These results indicate that food selectivity of fish larvae does not only depend on larval mouth size, but also on species-specific characteristics. (Hagiwara et al., 2001a). It is therefore important to establish a rotifer culture stock comprised of many strains of variable sizes in order to have rotifers with sizes appropriate for different species and developmental stages of fish larvae.

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European Aquaculture:
Recent Developments in Marine Hatchery Technologies

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Introduction

The most recent review of the state of world aquaculture from the Fisheries and Agriculture Organization (FAO) 2003 and the FAO Fishstat Plus statistics from 1950-2001 highlights the continuing growth of aquaculture in contributing to the total fisheries catch (Figure 1).

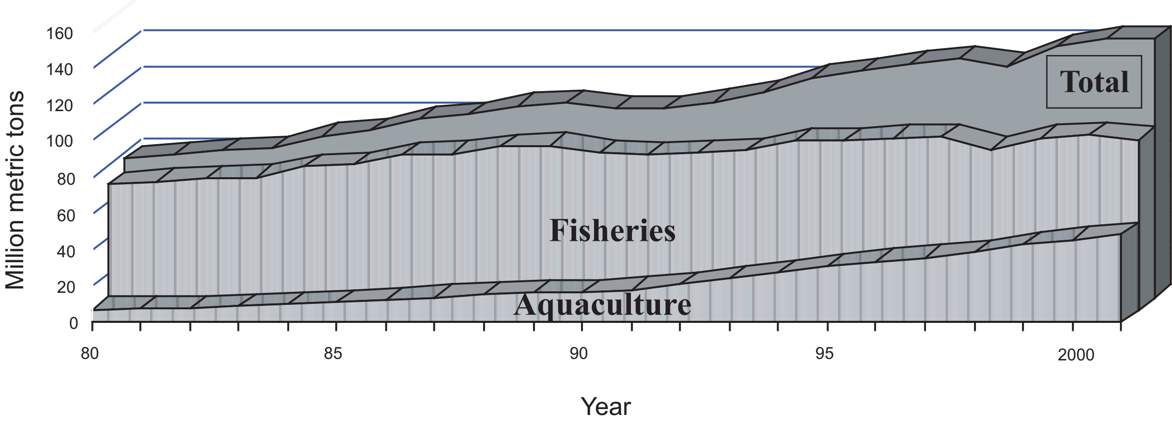

Figure 1. World fisheries and aquaculture production (FAO 2003)

Aquaculture represented 5.3% of the total fisheries landings in 1970 and this had increased to 34% in 2001, i.e. 48.4 million metric tonnes (mmt) of the total fisheries landings of 142.1 mmt. The value of world aquaculture production is now estimated at 61.5 billion US$. Globally the sector has shown an average annual compounded growth rate (APR) of 8.9% pa since 1970 compared to 1.4% for capture fisheries and 2.8% for terrestrial farmed meat production.

Finfish production at 24.4 mmt represents 50% of the aquaculture production and over 130 major finfish species are cultured world-wide. These statistics alone emphasize the scale, complexity, rate of development and diversification of the global finfish aquaculture sector.

European aquaculture

Europe has a total aquaculture production of over 2 mmt, and of the 210 aquaculture species cultured world wide 60 are cultured in Europe with a value of 4.6 billion US$. This means that while Europe contributes 4.4% to global production, it represents 8.2% of its total value. Recently growth from aquaculture has slowed from 7.8% p.a. in the period 1980 to 1990 to 2.3% p.a. in the period 1990 to 2000.

In 2001, 1.34 mmt of Europe's 2 mmt was attributed to finfish production and the break down of production between fresh, brackish and marine environments is shown in figure 2. Salmon, trouts, sea bass and sea bream account for over 1 mmt or 3 billion US$ in value. Salmon is by far the largest activity accounting for 647 thousand metric tonnes (tmt) and 1.86 billion US$. The marine and diadromous finfish species are valued at nearly 3 times the price of the fresh water species according to global FAO statistics and it is primarily in this area that European aquaculture has focused and developed. (Tacon, 2003).

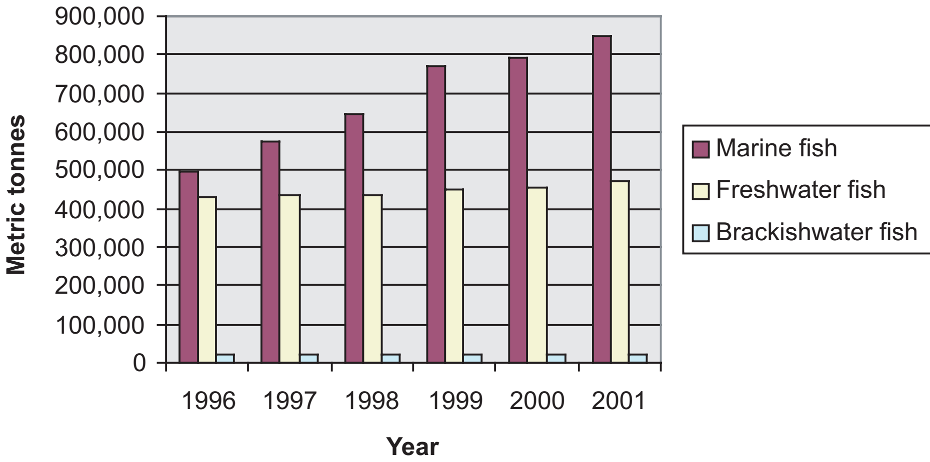

Figure 2. European finfish production by environment (1996-2001)

Table 1 shows the breakdown of the finfish species cultured in Europe and it can be seen that the largest growth is in mariculture with salmon, sea bream, sea bass and turbot. Currently the 25 countries of the European Union produce over 50 % of the finfish total.

Table 1. European finfish production by species (1996-2001) in metric tonnes.

	1996	1997	1998	1999	2000	2001
Freshwater species						
Tilapias	320	200	200	246	180	200
Sturgeon	1,285	1,471	2,022	2,441	3,083	3,087
Eels	8,614	8,696	9,792	10,536	10,713	10,187
Catfish/Perch/pike	19,321	16,389	15,437	19,903	16,583	14,868
Carps and Cyprinids	175,910	172,620	180,015	191,236	197,405	210,667
Trout	279,060	293,142	304,485	301,753	301,371	331,805
TOTAL	**484,510**	**492,518**	**511,951**	**526,115**	**529,335**	**570,814**
Marine species						
Sole	31	25	22	19	23	37
Halibut		2	8	13	34	93
Cod	191	304	199	157	169	763
Turbot	2,663	3,041	3,107	4,113	4,789	4,959
Amberjack & Tunas	78	1	1,959	3,246	3,686	4,453
Misc Marine	740	1,205	2,153	2,733	3,031	2,275
Other Breams & mullets	4,086	3,946	4,051	4,016	4,190	4,137
Sea bass	19,325	24,079	30,168	38,215	41,870	42,600
Sea Bream	23,304	29,139	37626	49,601	58,163	63,370
Salmonids	416,358	473,159	510,059	611,671	617,898	647,043
TOTAL	**466,766**	**534,901**	**589,352**	**713,784**	**733,853**	**769,730**
Total European Production	**951,286**	**1,027,419**	**1,101,303**	**1,239,899**	**1,263,188**	**1,340,544**

Salmon price: a controlling factor

Salmon prices have decreased since 2000 and in 2003 reached their lowest point ever at under 2 € /kg , with a recent average price of approximately 2.5 €/kg in early 2004.

This for many farms is at or below their production costs. Due to the importance of this species in the overall framework of the European industry the repercussions that this has had on the industry, as a whole has been significant. During the same period sea bass and sea bream juvenile production increased from approximately 500 million to 650 million juveniles while sea bream prices decreased from 4.58 to 3.73 €/kg. Many farms

producing only portion-sized sea bream faced sales prices well below their production costs. Overall in the EU their has been a 6.5% APR in production growth but the overall price trend has been negative (-0.5% APR) versus a positive global development (FEAP).

Share prices have fallen sharply, particularly in the salmon industry, investment confidence in aquaculture has been shaken and pressure has been placed on the industry to further improve efficiency and productivity. Acquisitions and mergers have been the order of the day and groups are consolidating in order to benefit financially.

Significantly with salmon being the power house of the European industry diversification into other marine finfish species has been slowed due to the reluctance of financial institutions to invest in a variant of this troubled sector. Further this financial pressure comes at a time when consumer awareness is focusing upon product quality, ease of product use, food safety, and traceability. This is also occurring during a period when the image of aquaculture has suffered by what the industry feels is often unfair treatment in the press.

The industry while weakened has responded with a positive approach. At every level the aquaculture sector is promoting transparency, cooperation and dialogue. The Federation of European Aquaculture Producers (FEAP) initiated the “Aquamedia” project and this is now providing factual, truthful and interesting information about European aquaculture to the public.

Evolution of European finfish culture

The marine and diadromus finfish sector is of vital importance to the European industry and consists primarily of high valued species requiring considerable technological and managerial sophistication to culture in a sustainable manner. The critical developments that were responsible for the commercialization of these species and their subsequent diversification are outlined below. Further areas of future developments and improvements required, to ensure the continued development of the industry, are considered in light of the rapidly evolving requirements of modern day Europe and the consumer.

While the latest FAO/ FEAP statistics described above provide a fascinating insight to the development of the aquaculture industry they obviously lack the benefit of real time information and the regional focus of development promoted by the European Union, National Governments and the industrial sector. This has been achieved through supporting research and development, financial support for the establishment of the industry and by addressing marketing and consumer needs. These driving forces have provided a basis for development and diversification.

Criteria for species selection

The selection of a species suitable for European aquaculture depends primarily on its market value, an understanding of its biology and the ability to produce juveniles in significant numbers for commercial production to take place. Given these characteristics and suitable site availability with the correct environmental conditions for culture the evolution to maturity of a specific species industry can be summarized in the following stages: -

- Identification and selection of high value species together with the R&D necessary to provide an understanding of the species biology and nutrition.
- The development of reproductive technologies to close the life cycle of the species considered together with the acquisition, domestication and manipulation of broodstock to produce eggs year round.
- The industrialization of technologies required for the commercial production of juveniles and their ongrowing.
- Improvement in productivity through economies of scale and the reduction of costs through vertical integration.
- Improvement in productivity through biotechnological solutions such as genetic advancement and nutritional engineering.

- Increased market activity through commercial pressure with the development of quality labeling, processing and other value added activities.
- Streamlining of the industry due to increased production, reduced profits resulting in grouping, mergers and consolidation to remain commercially competitive.
- The maturation of the industry and the species loosing its status of a high value item and becoming a commodity product.
- Species diversification in search of additional profits through a recycling of the above procedure.

Transfer of technology between species.

The transition from salmon to other marine species is of particular interest and importance as it involved the transfer of ongrowing technologies from Norway and Scotland to the Mediterranean for the sea bass and sea bream industry in the 1980's to 1990's. This, however, in itself was not sufficient as the reproductive technologies, zootechnical, nutritional and environmental conditions required for the culture of marine finfish juveniles were significantly different from those of salmon.

A swim up salmon fry (150mg wet weight) is many times larger in body mass than that of a sea bream (0.35mg wet weight) and while the salmon fry is capable, at first feeding, of ingesting and digesting inert particulate feeds this is not the case for many marine species. Due to the rather underdeveloped digestive system of these larvae and their behaviour a sophisticated live food chain was required. Researchers and industry concentrated efforts to develop a species specific nutritional strategy and identify the correct environmental conditions enabling these very small and sensitive larvae to be successfully reared and develop sufficiently to be weaned on to inert diets before traditional on-growing technologies could then be used.

Since the 80's, larval rearing technologies have been continually developing for the sea bass, sea bream and turbot industries and today they are well understood. Survival rates for these species have increased from less than 1% to between 20 and 40% in 10 years. In the last few years these warm water larval rearing technologies have been adapted and introduced to Norway and Scotland enabling the mass production of a marine cold water species the cod (*Gadus morhua*). While production of cod is expected to reach only 12,000 tonnes in 2005, this species, which occupies the same environmental conditions as salmon, offers an alternative or addition to the troubled salmon industry.

Economically the present viability of these two species in the UK can be summarized as shown in table 2. (R. Prickett, Personal communication) and it should be noted that as technology and food conversion rates improve for cod, so should profits. History indicates however that as volume increases so market prices will reduce.

Table 2: Comparative profitability of salmon and cod in the UK in 2003.

	Salmon	Cod
Cost per kg (round)	€ 2.04	€ 2.820
Cost of harvesting/gutting etc	€ 0.423	€ 0.423
Yield (head on gutted)	90%	86%
Cost per kg (head on gutted)	€ 2.735	€ 3.765
Market price (head on gutted)	€ 2.735	€ 4.935
Profit/kg	0	€ 1.170 (31%)
Cost of filleting	€ 0.141	€ 0.141
Yield after filleting (head on gutted)	68%	55%
Cost per kilo (fillet/skin on)	€ 4.23	€ 9.23

It is developments such as these that offer Europe the potential to continue expanding and diversifying its existing aquaculture industry by looking forward to new and more profitable species through the adaptation and development of existing biotechnologies and management strategies.

Reproductive technologies

It has been estimated that less than 3% of the total world aquaculture production is based on genetically improved stocks. Norway started to work on the selection of salmon in the 1960's and it was found that there was a large genetic variability for important production traits between wild and culture stocks offering potential for improvement. The best strains were then used as a base population for a national genetic improvement programme. This has lead to the development of salmon strains that show a genetic gain of around 100% for growth rate as well as other important characteristics such as late maturing individuals, resulting in improved production characteristics and reduced production costs. A similar breeding programme carried out using tilapia in the Philippines has shown more than a 100 % improvement in growth rate over 10 generations.

In both species, selection for high growth-rate can result in a gain of over 10% per generation. Only in the last 10 years has European Union research focussed on broodstock genetics and selection programmes for the sea bass and sea bream industry in the Mediterranean. Prior to this production was still based on wild broodstock and selection from F1 cage production, with little monitoring to avoid inbreeding.

Traditionally, preventing inbreeding has been the largest problem in fish breeding programmes, due to large full sibling groups and the lack of individual identification methods. Today the identification of individual broodstock fish using microchip tagging is commonplace. The cost of microsatelite DNA and AFLP's (amplified fragment length polymorphisms) labelling has significantly decreased enabling the tracking of pedigrees and providing linkage maps to identify quantitative trait loci, such as growth and disease resistance, that have commercial importance (Agresti et. al., 2000). These technologies have enabled individual producers to carry out selection methods and apply them in a practical manner in the industrial environment.

Growth rate is generally the first characteristic of importance for fish farmers as it is relatively easy to select and quantify. Other traits, thought to be genetically dependant, for example disease resistance and flesh quality (muscular lipid content, fat deposition), are difficult to measure and require a more complex approach often beyond the abilities of the individual farmer. Various thematic R&D programmes within the European Union are now addressing many of these issues.

Many species still prove difficult to spawn in captivity particularly in intensive production systems, an example being Sole (*Solea senigalensis*). Triggers for male and female maturation and ovulation and still not well understood for this and other species such as the groupers and some tuna species. Endocrine regulation of reproduction has been effectively applied in some species and hormonal implants are readily available. (Zohar and Mylonas, 2001) More investigation of the environmental and nutritional requirements of many species is required as the production of viable eggs is a prerequisite to the culture of any species.

Egg quality has a significant impact on the viability, survival and growth of marine larvae. The enrichment of broodstock diets with essential fatty acids (HUFA's) and other vitamins and minerals have been shown to relate directly with the levels of these substances in marine eggs and larvae (Watanabe, 1993; Cedra et. al., 1994; Harel et. al., 1994).

Marine larval rearing technologies

The very small and sensitive larvae require the establishment of a reliable chain of live food production consisting of unicellular marine algae, the rotifer *Branchionus plicatilis* and the brine shrimp *Artemia salina*. This is common to both the cold water developments namely cod and halibut and the warm water species such as sea bass, sea breams, turbot and sole.

The most commonly cultured algal species in Europe belong to the 5 taxonomic groups (Table 3) and the choice of species depends on the fish species being cultured.

Table 3: Commonly cultured unicellular planktonic algae.

Bacillariophycaea	Chaetoceros calcitrans
(Diatoms)	Skeletonema costatum
Chlorophycaeae – green algae	Dunaliella tertiolecta Chlorella sp. Nannochloris atomus
Chrysophyceae	Tetraselmis suecica Tetraselmis chuii
Eustigmatophyceae	Nannochloropsis oculata Nannochloropsis gaditana
Haptophyceae	Isochrysis galbana Isochrysis sp. (Tahititan strain) Pavlova lutheri

The use of algae in the larval rearing tanks, the "green water technique" is not limited to the purely nutritional side of rotifer enrichment but has other practical applications:-

- Algae can act as an antibacterial agent. (Austin and Day,1990; Cooper et. al. 1983). In addition specific polysaccharides in the algae cell wall are thought to stimulate a non-specific immune response in young larvae.
- Algae has been reported to act as an in situ biological filter removing potentially harmful metabolites from the water by stripping off nitrogenous substances. It also produces oxygen through photosynthesis.
- Algae acts as a light filter and diffuser facilitating an even distribution of live food and larvae within the tank system.
- It acts as a promoter and background for the location of prey organisms hence playing a particularly important role in the critical first feeding stage of larvae.
- Algae has been shown to stimulate the enzymatic synthesis and onset of feeding in young larvae.

In recent years photo-bioreactor systems have provided an efficient alternative to traditional sack culture systems for the production of unicellular algae. These are labour saving, automated and cost effective. Productivity using these systems can be up to 10 times greater than that achievable with traditional culture methods. Undoubtedly the success of such systems is dependent upon light availability and the Mediterranean climate is particularly suitable.

The search for additional algal species continues and isolates of local species are being investigated in Norway in an attempt to replace some of the traditional non-indigenous species. In addition commercial companies are marketing concentrated algal pastes, delivered either alive with a limited shelf life or cryopreserved, which offer a back up and an alternative to traditional algal production.

The second link of the live food chain is the rotifer. The duration and quantity of rotifers required varies with the species, cod, for example require up to 4 times the number of rotifers per animal produced when compared to the sea bream. Some species such as the sea bass may avoid this stage altogether first feeding directly on Artemia nauplii, the last link in this chain.

Rotifer production methodologies have improved over the years from an algae and yeast based diet giving poor productivity and unpredictable results to improved culture diets. The new generation of diets enables culture densities of 2000 rotifers per ml or more to be achieved over a 4 day batch cycle. Further developments have resulted in improvements, 5000 rotifers per ml can be achieved using concentrated fresh water chlorella algae and automatic dosing pumps and re-circulation systems using protein skimmers, in association with novel

filters enable rotifer cultures to reach and be maintained at densities over 5000 per ml for prolonged periods of time. These developments has been shown to both provide significant economic benefit and importantly improve the microflora of the culture by reducing the incidence of Vibrio sp. (Suantika et.al., 2003).

Ongoing European Union projects have revealed that there is considerable genetic diversity in the rotifer populations in European hatcheries showing considerable difference in performance. It is not yet clear whether it will be necessary to work with selected genotypes cultured over a limited number of generations or how these cultures will be susceptible to changing culture conditions (Sorgeloos, 2004).

A better understanding of the nutritional requirements of the fish species cultured has lead to the development of number of commercially available cultivation and emulsion type enrichment diets for both rotifers and artemia. Dietary research has indicated the importance of the (n-3) highly unsaturated fatty acids mainly eicosapentaenoic acid (20:5(n-3), EPA), docosahexaenoic acid (22:6(n-3), DHA) and more recently the long chain n-6 HUFA arachidonic acid (20:4n-6, ARA) has been implicated as an essential fatty acid for a variety of developing marine species (Estevez et. al., 1999). It is the bio-encapsulation of these and other essential nutrients through the live feed chain in the ratios required by the species concerned that has led to the alleviation of several problems such as pigmentation and some deformity issues in larval rearing as well as improving survival.

These enrichment products today are available together with products, that for Artemia, are capable of altering the microbiological characteristics of the hatching and enrichment environments by reducing Vibrio levels to 30% or less of non-treated environments. This development both provides the farmer with custom nutritional packages and helps to reduce the possibility of disease that may be introduced through the live food chain.

Larval nutrition and feed technologies

Larval rearing technologies are today highly intensive with up to 250 larvae stocked per litre. Until recently the live food chain was entirely responsible for the nutrition of these larvae until weaning commenced at approximately 30 days post hatch. The role of the live food chain is still vitally important for many species but the development of a new generation of sophisticated inert co-feeding and replacement diets have enabled the further intensification of the larval rearing process, and considerably reduced reliance upon the live food chain simplifying production methodologies.

Nutrition, health and performance are concepts that are intimately linked and the industry is placing increasing importance and effort to optimize formulations and improve ingredient quality including the sourcing of fresh raw materials. A cold extrusion spherizer agglomeration system has been used to produce diets that are now aimed at the complete replacement of the live food chain and they focus on high digestibility. Skretting use the above technology together with a patented phospolipid content of 12%. This has been reported to play an important role in the reduction of juvenile deformation and improved growth performance.

It is hoped that further developments of micronised replacement diets will both simplify and standardize future marine fish larval rearing and enable a greater number of species to be commercialized. Possibly given the restriction of the very small mouth sizes of some marine larvae, such as the groupers, and the difficulty in maintaining extremely small strains of rotifers for first feeding highly digestible diets of this type may provide an alternative strategy for the industry.

Consumer confidence and food safety issues are important factors in aquaculture and following the BSE outbreaks, the European Union has banned the inclusion of ingredients derived from terrestrial animal by products. This has stopped the incorporation of hemoglobin, blood meals, meat, bone and feather meals amongst other ingredients. In addition to this many sales outlets and the large supermarket chains require European feeds to be certified GMO free as part of their drive to satisfy consumer demand and perception.

The newly established European Food Safety Authority (EFSA) runs risk analysis and risk management and promotes an integrated approach to the responsibility of feed manufactures, farmers and food operators on the traceability of feeds, food and their ingredients.

These actions while essential to regulate the industry and address consumer and public health issues severely restrict the formulations available for aquaculture. This in turn places a heavy load on the limited resource of fish meal as a protein source and adds to the cost of feed at a time when the industry is striving to reduce production costs by all means possible. Fish meal and oil substitutes are emerging now as viable, economic partial alternatives. The inclusion of digestibility enhancers and organic chelated trace minerals provide better bio-availability of important nutrients.

Health and disease issues

Parasitic, bacterial and viral diseases cause considerable financial loss to the European industry. Disease problems in the marine sector originate from a diverse range of infectious agents which have been reviewed by Le Breton, (1996) and Rodgers and Furones,(1998) a list that has rapidly developed and is continually expanding.

The development of molecular techniques for the identification and screening of pathogens offers the potential to improve disease prevention and control. The sensitivity of nucleic acid probes now enables the detection of sub-clinical carriers of some infections and this is an important development and tool for the establishment of specific pathogen free broodstocks.

Hatchery production units try to avoid the introduction of opportunistic pathogens through treatment of the incoming water supplies using a variety of filtration methods. Sterilization, either by UV and /or ozone, is common practice in both freshwater and marine environments. Production scheduling now includes specific periods where either units of / or the whole hatchery are shut down cleaned and sterilized and all in all out batch production provides regular sanitary control.

Areas within the hatchery environment are kept as discrete as possible with the minimum of interaction by working personnel and equipment from one area to another. Isolated quarantine facilities are employed to prevent the introduction of disease.

In larval rearing various strategies have been proposed for controlling the microflora of this environment. Mature water and the addition of probiotics either through the live food chain or directly in the larval rearing tank have been used. The larval pre-feeding and first feeding stages are critical to the establishment of the microflora of the gut. (Bergh et. al., 1994; Munro et.al., 1993) and the presence of opportunistic pathogens at this stage have been shown to lead to disease (Grisez et. al., 1996).

Fish transfer from the hatchery to the pre-ongrowing or ongrowing facilities necessitates the transfer of fish from a protected to an unprotected environment in which they might come into contact with a variety of different pathogens It is possible to protect against certain disease with vaccines and a limited number are available for commercial use. Vaccination takes places prior to the transfer from the hatchery facilities to the ongrowing but due to the limited duration of protection offered to fish further vaccination may be necessary during the ongrowing cycle.

With the complexity of vaccine licensing and the restricted use of antibiotics and some other therapeutic agents the industry is turning to other methods of prophylaxis and control to improve the fish health status. The concept of nutritional supplementation, the use and blending of selected nutrients, immunostimulants and immunomodulators are rapidly being considered by the aquaculture industry as it learns of their effectiveness in terrestrial animal culture.

Conclusion

European aquaculture is expected to show growth in the marine sector and the success of individual operations will depends on the successful application of a variety of multi disciplinarial activities. Economic viability must be linked to better marketing strategies and food safety, transparency, traceability, quality and sustainability issues are at the forefront of European concerns and actions. Technological improvements are expected to continue to improve cost efficiency and stimulate further species diversification at a time when fisheries production is stagnant and in certain sectors in decline. Simplified legislation and licensing procedures have been called for and continued and coherent policies for research and development are essential.

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Nutrition, digestion and development in marine fish larvae

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Introduction

Until recently, the feeding of marine fish larvae during the first weeks of life depended on live preys whose cost is expensive and which are less and less abundant. The substitution for these preys by a compound diet is now possible as early as mouth opening. The formulation of a compound diet makes possible to study the influence of nutrients on marine fish larvae development in order to adjust each nutrient to the need of the larvae. It is well known that nutrients influence larvae development; growth, survival and malformation rate of larvae. In this paper, we will pay particular attention to peptides, lipids and vitamin A.

Larval development

Marine fish larvae undergo major morphological and functional changes during the first month of life to acquire their adult features. These changes include the maturation of the gastrointestinal tract (Zambonino & Cahu, 2001) which is not mature at hatching. The development of the stomach is nearly complete around day 15 in sea bass and the gastric glands appears approximately at day 25. The exocrine pancreas also progressively develops, its secretory functions become efficient after the third week of life. A global decrease of amylase activity and an increase of the activities of other pancreatic enzymes (trypsin, lipase, phospholipase A2…) are characteristic of this maturation process. The intestine cells, the enterocytes, have two kinds of enzymes: the cytosolic ones (mainly peptidases) localized in the cytoplasm and the brush border membrane enzymes (alkaline phosphatase, aminopeptidase N, maltase…). Around the fourth week of life, the cytosolic activities decrease while membranous activities increase. This reflects the maturation of enterocytes and the establishment of an efficient brush border enzyme digestion represents the adult mode of digestion.

The morphological changes mainly concern the shape of the larvae and the differentiation of the fins. At day 3, European sea bass larvae elongate. At day 5, eyes start to be pigmented and the pectoral fins appear (Barnabé et al., 1976). At day 15, the vitellus is totally resorbed and the larvae only have an exogenous feeding. The vertebrae are visible at day 20. At day 35, the caudal, anal and dorsal fins are visible and around day 43, the pelvis fins start their differentiation. At this stage of development, the larvae present all the adult features. All these differentiation processes are under genetic control involving several families of genes such as the BMPs, IGFs (Solheim, 1998) and Hox genes (Krumlauf, 1994).

Larval nutrition

The formulation of a compound diet for sea bass larvae includes 50% of proteins, 15 to 20% of lipids, minerals and vitamins. The main source of proteins is the fish meal and it has been demonstrated that the molecular form of proteins is determining in larval nutrition. Lipid requirements have been also intensively studied, especially phospholipids and highly unsaturated fatty acids (HUFA) requirements. The main phospholipid source used in compound diets is soybean lecithin.

Peptides

It has been demonstrated for a long time that protein hydrolysates were beneficial to larvae growth. In 1997, it has been shown (Zambonino et al.) that a 20% replacement of fish meal by tripeptides in the diet improved the growth, survival and skeletal formation in sea bass larvae. This can be explained by the presence of specific transmembrane transporters and a high cytosolic peptidase activities in these larvae. Moreover, it also has been shown that the activity of a pancreatic protease (chymotrypsin) was enhanced by short peptides. In juveniles,

the incorporation of protein hydrolysates in formula does not represent an advantage mainly because of the decrease of the cytosolic peptidase activities.

Phospholipids and HUFA

Phospholipids (PL) are the major component of cell membrane and even though larvae can synthesize them de novo, their incorporation in diets improves the growth and the survival rate (Cahu & Zambonino, 2001). It has been suggested that larvae were unable to synthesize enough PL to cover their requirements during a period of intense cell multiplication (Kanasawa, 1993). It has been recently reported that a diet containing 19% lipids with almost 9% phospholipids induced a good growth in European sea bass first feeding larvae (Cahu et al., 2003). These authors suggested that marine fish larvae use phospholipids more efficiently than neutral lipids, since lipase transcription in response to dietary neutral lipid amount was poorly regulated while phospholipase A2 transcription followed dietary phospholipid content in a gradual manner with a greater modulation range in expression.

The n-3 HUFA are essential dietary components for marine fish because these fish can not synthesize them. Two fatty acids, the eicosapentaenoic (EPA = C20:5n-3) and the docosahexaenoic acids (DHA = C22:6n-3) are particularly important as they are present in large amounts in fish cell membranes and are involved in physiological processes. The optimal dietary level of EPA + DHA is 2.7% of dry matter for marine fish larvae and it is 1.5% when EPA and DHA are in PL (Cahu et al., 2003). Actually, these HUFA are more efficiently used when they are brought by the PL fraction (vs neutral fraction).

Vitamin A

The compound diets for marine larvae include in their formulation a vitamin complex containing all the vitamins thought to be essential. As larvae quantitative requirements in vitamin are unknown, a vitamin mixture is commonly added in excess. Our studies paid a particular attention has been paid to vitamin A because it acts on morphogenesis through nuclear receptors. These receptors regulate the expression level of genes, such as Hox (Ross et al., 2000). Recently, we demonstrated in sea bass that these receptors were expressed in the body areas affected by malformations and that their action was modulated by exogenous nutrition after hatching (Villeneuve et al., unpublished data). So, it seems really important to evaluate the optimal dietary level of vitamin A. The only available data was obtained in Atlantic halibut juveniles and was 2.5 mg retinyl esters/kg dry matter (Moren et al., 2004).

Conclusion

All the nutrients described in this paper act directly or indirectly on larvae morphogenesis and they also probably interact together to allow a harmonious development. In order to improve diet formulations for marine fish larvae, it would be very pertinent to study the pathways modulated by nutrition and involved in these morphogenetic processes. Some of these nutritional pathways are common to several nutrients and their study would allow, on one hand, to understand how nutrients influence genes of development and, on the other hand, to evaluate how nutrients interact together.

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Technical Session

Live and formulated feeds: challenges, capabilities and research at the Australian Institute of Marine Science (AIMS)

Mike Hall, David McKinnon, Michael Horne, Paul Southgate (JCU), Samantha Duggan, Alby Steffens, Matthew Salmon and Matt Kenway

The larval phase continues to represents one of the major challenges in the consistent and successful production of many finfish and crustacea aquaculture species as well as in the development of new candidates for captive reared production. Whereas it has proved possible to successfully rear the larvae of many species there is often significant variability between batch runs while in other cases high levels of larval attrition results in poor survival to the juvenile stage. Some aspects of these problems may be due to inadequacies in larval nutrition. As part of several aquaculture projects at AIMS and JCU on crustacea, finfish and molluscs, research has been undertaken on the development of live and formulated feeds. As knowledge is gained in larval rearing technologies of specific species, some of the most likely bottlenecks are tentatively identified. As a result, AIMS and JCU has invested into live and formulated larval feed production capability and equipment allowing a comprehensive approach to aspects of the larval rearing of a range of aquaculture species.

Live feeds are the larval diet of choice in the original development of the hatchery phase of aquaculture candidates and continue to form the mainstay feeds for the major penaeid prawn and finfish hatcheries. Typically, live feeds can be fed less frequently and at lower rates, as feeding to excess does not lead to as rapid deterioration in water quality. In some cases, sensory cues, such as movement, odour and electro-chemical properties, are necessary for acceptance by the larvae and live feeds meet these criteria. However, due to the cost of production of many live feeds there continues to be research and development effort towards the production of formulated larval feeds which are i) economical to produce, ii) have consistent nutritional profiles, iii) are readily digestible at various stages of larval physiological development and iv) have the capacity for medium to long term storage without spoilage. The choice of either live or formulated feeds also determines aspects of tank design, water exchange and flow, and the method and frequency of feeding.

Live Feeds

Microalgae

Microalgae are produced at AIMS in a newly constructed continuous algal production system capable of producing 1,000 litres per day. Microalgae cultures are kept under controlled microbial conditions using 0.22 µm to 1.0 µm filtered and pasteurised seawater and are supplemented with food grade carbon dioxide to increase productivity. As summer temperatures and cloud cover are variable in the Townsville region the unit is indoors as extreme weather bouts can occur which cause microalgae production to temporarily fail. Experience has demonstrated these may occur at critical periods in larval rearing and therefore the unit presently uses artificial lighting and environmental control. Continuous microalgae production is based on a few species chosen from several families held in small-scale stock cultures including *Chaetoceros* and *Skeletonema* (Bacillariophyceae), *Cryptomonas* (Crytophyceae), *Tetraselmis* (Prasinophyceae), *Isochysis* (Haptophyceae), *Pavlova* (Prymnesiophyceae), *Nannochloropsis* (Eustigmatophyceae) and *Heterocapsa* (Dinophyceae), all sourced from either the CSIRO Microalgae Research Centre, Darwin Aquaculture Centre or other collaborators. The microalgae are either used for conditioning of larval tank water, as feeds for *Artemia* and copepods or as live feeds themselves.

Rotifers

Although original considered as a pest due to their rapid proliferation causing deterioration of environmental water quality, rotifers are an important larval feed for marine finfish. As the AIMS Tropical Aquaculture Facility (TAF) concentrates on the larval rearing of various crustacea, rotifers are not regularly cultured.

Artemia

Artemia are primarily cultured as live feeds for prawn and rock lobster larval rearing. Protocols for decapsulation, hatching and enrichment are based on those developed by Inve. A recent change is the use of Virkon S (a strong oxidising agent availably commercially) to disinfect water in hatching tanks prior to the addition of cysts. Ongrown *Artemia*, fed a diet of mixed microalgae species, are also produced for rock lobster larvae. A key objective in *Artemia* production is the management and control of the entire bacterial community, including culturable and the unculturable majority, to minimize the risk of inadvertent transfer of potential pathogens into larval rearing tanks.

Copepods

The brine shrimp *Artemia* (Anostraca) is the mainstay for live feeds for many established aquaculture species. However, as *Artemia* are predominantly found in hypersaline environments they are a surrogate for the natural live prey of the aquaculture larvae. The natural prey of the majority of finfish larvae are copepods, and *Artemia* are a poor nutritional source for the larvae of most aquaculture species. Consequently, *Artemia* require enrichment diets to optimise their nutritionally profile to match the needs of specific aquaculture species. In contrast, copepods typically have a nutritional profile that matches the requirements of marine finfish larvae in particular. For example, the copepod, *Diaptomus (Leptodiaptomus) connexus* (sold freeze-dried under the commercial name of Cyclop-eeze) is considered to be an excellent larval finfish feed. There are, however, quarantine issues with the import of such material from offshore. If the desired prey size is greater than that of the *Artemia* nauplius, *Artemia* may be grown out to the desired size. However, with a massive biodiversity of copepod species, there is a much greater range in size classes that may be selected. Nevertheless, several challenges remain to successfully produce copepods on a continuous commercial scale, including consistent production rates and maximisation of growth.

Alternative Live Feeds

Whereas microalgae and copepods are the dominant form of plankton in the wild, and form the basis of the larval diet for many marine organisms, they are not the only prey of larvae. Gelatinous zooplankton, represented by Hydromedusae, Siphonophora, Scyphomedusae, Ctenophora, Heteropoda, Pteropoda, Thalicea and Appendicularia, typically exceeding >1 mm in size, can form a major proportion of the planktonic community and be larval feeds for some marine organisms. Other soft-body larval feeds include nematodes and various annelids. Various culturing methods have been published for these, some of which have been trialled, and continue to be an area of interest as larval feeds for some aquaculture species.

Formulated Feeds

The primary characteristics of formulated feeds includes very small size of the finished particle, typically in the tens to hundreds of microns, and, as a consequence a very high surface-to-volume ratio and hence a high leaching rate, high palatability and digestibility optimised to the various physiological competencies throughout larval development. Production methods can be generally categorised into microbound, microencapsulated and complex particles.

On-size feeds

Microbound particles are the most widespread and produced as a flake or crumble, typically by fracturing a larger pellet. If a small variance in particle size distribution is required, on-sized feeds are the method of choice but require specialised equipment, most of which are adapted from the pharmaceutical industry. The two-step microextrusion marumerization (MEM) process involves the production of a finely ground damp/wet mash, the production of thin noodles through a cold single screw extruder, followed by forming them into small diameter spheres by marumerization (spheronization) (Barrows F. T. 2000 Global Aquaculture Advocate 3(1):61-63).

Milling

If a small compositionally complex particle of 10-500 micron is required, then the individual components must be significantly smaller than the final pellet diameter. Typical fish meals are several hundred microns in

diameter and hence require further milling to a smaller size. Many milling methods, such as hammer mills, depend on high energy impact with the generation of significant amounts of heat. If the material being milled, such as fish meal, has a high protein (40-70%), oil (>10%) and moisture content, the particle tends to form a paste as it is milled. The most appropriate mill for the production of particles in the 1-100 µm range, is an air classified or air pulverizer mill. As part of the research program on larval feed production, AIMS has acquired an air pulverizer mill capable of reducing particles of fish meals from 572.5 ± 304 µm fish meal particles to 4.22 ± 1.02 µm, processing 0.5 to 7 kg of feed per hour. The greatly reduced particle size allows the incorporation of several hundred particles into a 100 µm microbound spherical particle.

Microextruded diets

AIMS has acquired a lab dome granulator with a range of screens to produce a variety of extruded pellet diameters, including dome (300 to 1,500 microns), radial (600 to 1,200 micron) and frontal (2,000 to 5,000 micron) dies/plates. Marumerization is accomplished with marume plates of groove pitches of 2, 3, 4, 6 and 8 mm, allowing the production of an extensive range of spherical particle size feeds and, more importantly, the ability to produce experimental diets with complex formulations. A modification of the MEM process is the particle assisted rotational agglomeration (PARA) method (U.S. Patent 5,851,574) which produces a wider particle size range of pellets, including a proportion that are smaller than that produced by the MEM method.

Encapsulation

An additional on-size feed production method includes encapsulation by forming a continuous coating around solid particles or droplets of liquids. A common form of encapsulation are spray beadlets which requires a gelling agent, typically with hydrocolloids such as alginates, carregeenans, agar, gum arabic, pectin, and gelatin as well as binders such as starch, cellulosics, chitosan and transglutaminase. A modification of the original technique of Villamar and Langdon (1993 Mar. Biol. 115:635-642) has been used in an attempt to make formulated feeds for the phyllosoma larvae of tropical rock lobsters (see case histories).

Functional properties of diet ingredients

A significant problem in the production of formulated larval feeds is the ability of early stage larvae to efficiently utilise the nutritional content of them, especially as the digestive physiology of marine larvae only develops gradually with time. One partial solution to this problem is adjusting the functionality of proteins in the feed by preconditioning such feed ingredients as fish meals by enzymatic hydrolysis. The large molecular weights of proteins in fish meals can be reduced to peptide or single amino acids sizes which are potentially more readily assimilated by the larvae. AIMS has recently commissioned an enzymatic reactor with pH-Stat titrator to control proteolytic enzyme activity to produce digests of fish meals with known molecular weights. This line of research is directed towards optimising the appropriate molecular weight class of proteins to give to crustacean larvae of specific ages.

An additional significant problem in the production of on-size larval feeds with dimensions in the tens to hundreds of microns is the rapid leaching rate of low molecular weight hydrophilic molecules, such as amino acids and vitamins. A plethora of methods have been developed within the pharmaceutical industry for controlled release technologies. AIMS has trailed the use of interdigital micro mixers which are capable of forming microspheres of immiscible fluids into spheres within the range of 25-500 µm that can be incorporated into microbound and complex particles. This approach holds some promise as one way to incorporate highly hydrophilic compounds of low molecular weight into microcapsules with minimal leaching rates.

Case Histories

Live Feeds

Copepods as live feeds in aquaculture

AIMS has a long-standing collaboration with the Queensland Department of Primary Industries Northern Fisheries laboratory in Cairns. QDPI's reef fish aquaculture program has a significant live feeds component, to meet the requirement to present appropriate live feeds to the first-feeding larvae of grouper species such

as the estuary cod, *Epinephelus coioides*. These fish require sub-100µm live feeds, and rotifers have proved inadequate. Best results have been obtained by the use of nauplii of the copepod *Acartia sinjiensis*, a locally obtained representative of a very widespread genus which has gained currency in aquaculture applications worldwide. However, some aspects of the biology of *Acartia* hamper its development at hatchery scale, and work conducted at AIMS has focused on the culture of small copepods of the family Paracalanidae (*Bestiolina similis* and *Parvocalanus crassirostris* – see McKinnon et al. 2003., *Aquaculture* 223:89-106.). These animals are important natural diets for fish larvae. They are smaller than *Acartia*, and can reach higher densities in culture systems. Coincidentally, the same species have proved to be ideal larval foods for the rearing of tropical snappers in Hawaii, and offer a lot of promise for large scale commercial culture. We are satisfied with the performance of these copepod species as live feeds, but recognise the need to develop reliable low-maintenance culture systems. Consequently, we are now establishing a flow-through continuous copepod culture system to operate in series with the continuous algal culture system.

Formulated Feeds

Spray beadlets as phyllosoma feeds

Efforts towards the development of an aquaculture sector for rock lobsters is primarily being supported by the Rock Lobster Propagation and Enhancement Subprogram (RLEAS) through the FRDC. Successful larval rearing through the entire larval phase of the phyllosomas of rock lobsters has only been reported with the use of Artemia in the first half of the extended larval period (extending over several months and typically 11 larval developmental stages) with fresh minced feeds being used in the latter stages of larval development. Although the natural diet of phyllosomas is not fully known, available evidence suggests that gelatinous zooplankton are the primary prey. Based on such tentative evidence some effort has been devoted to the development of a formulated gelatinous feed for phyllosomas through a MSc thesis by Michael Horne (JCU), co-supervised through AIMS and JCU. The research focussed on the production of microbound and complex feeds and in the first instance examined suitable production methods for spray beadlets of specific dimensions (Fig. 1).

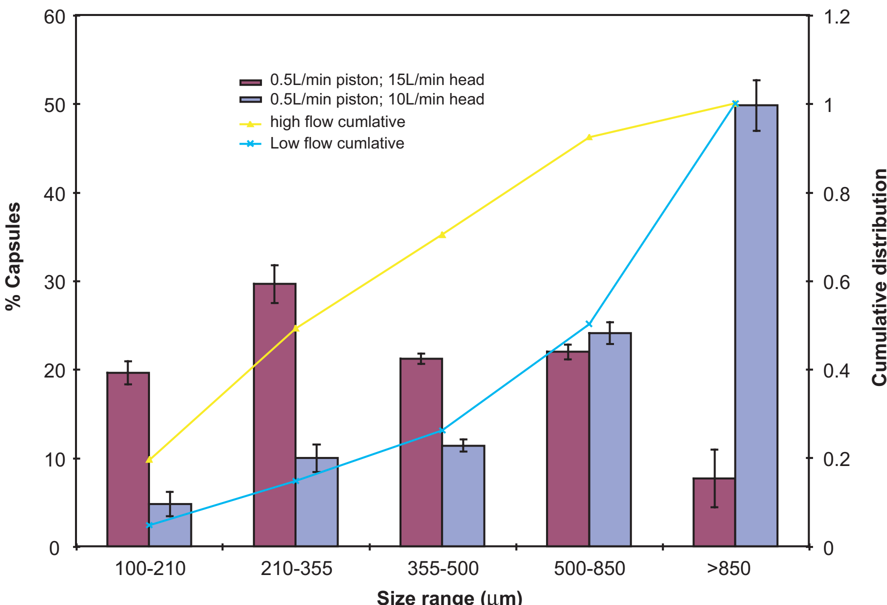

Figure 1 Size distribution of spray beadlets produced under specific flow rates and feeder rates of atomisation nitrogen gas.

Microencapsulated feeds of either homogenised Artemia or pipis (*Donax* spp.) were produced using sodium alginate as the binder (Figure 2). Whereas phyllosomas readily consume homogenized pippies, they were more reluctant to consume them when bound within an alginate capsule. Further work is necessary to identify a more suitable hydrocolloid binder.

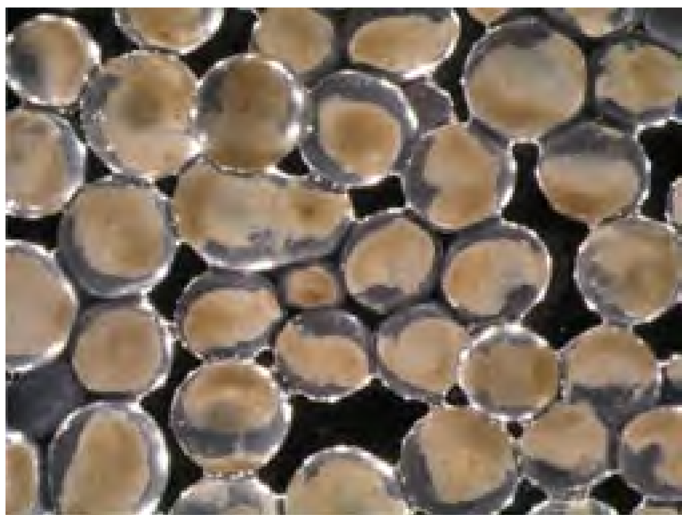

Figure 2. Microencapsulated spray beadlets containing homogenised pipis (*Donax* spp.) surrounded by a solidified sodium alginate coating.

To obtain further data on the ingestion rate of phyllosoma diets of alginate spray beadlets, Artemia where radiolablled by feeding them on carbon-14 enriched algae for set periods. The Artemia were dried and ground to a fine powder and incorporated into alginate spray beadlets. Ingestion of C^{14} alginate beads were tested in Stage 1, 2, 3 and 4 phyllosomas, followed by a ‘cold chase’ of non-radioactive Artemia (Fig. 3).

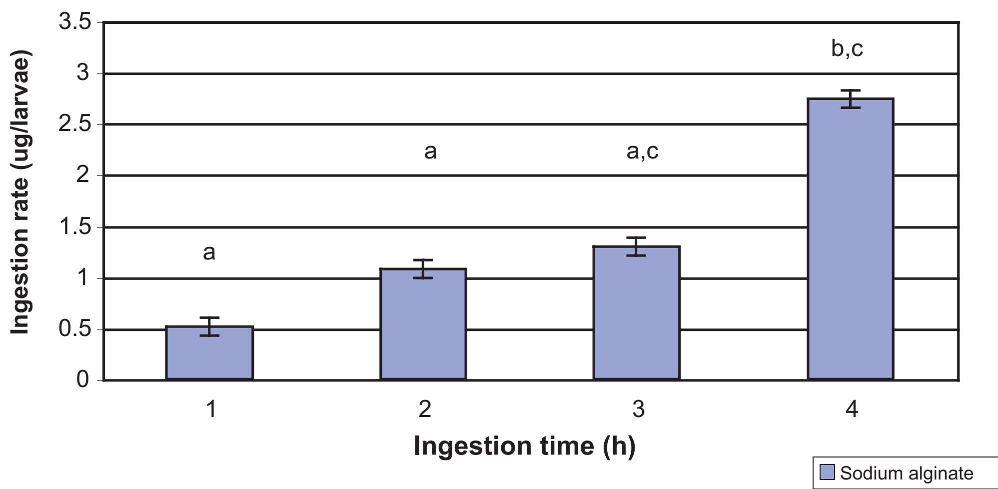

Figure 3. Mean ingestion rate (µg of biet per mg larval dry weight) of stage 4 phyllosomas fed radioactive labelled alginate-Artemia spray beadlets. Means for ingestion rate sharing the same letter were not statistically different (P>0.05).

As with the microencapsulated pipis the encapsulated Artemia were eaten but the phyllosomas appeared to be reluctant to consume the alginate beadlets. Further work continues on digestive physiology of phyllosomas and diets within an ARC-Linkage Postgraduate project of Matthew Johnston and Danielle Johnston (UWA).

Hatchery Capability at NIWA Aquaculture

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Introduction to

NIWA, National Institute of Water and Atmospheric Research Ltd, established in 1992 as one of nine New Zealand Crown Research Institutes (CRI's), NIWA's mission is to provide a scientific basis for the sustainable management and development of New Zealand's atmospheric, marine and freshwater systems and associated resources. As a CRI, NIWA operates as a stand-alone company with its own board of directors and its shares held by the Crown.

The company has a staff of around 650 and annual revenue of more than $75 million derived from competition-based research grants and commercial enterprise.

Spread throughout New Zealand, NIWA has its corporate headquarters in Auckland, main research campuses in Auckland, Hamilton, Wellington, Nelson, Christchurch and Lauder, and field offices in the smaller centres. Research vessels are maintained in Hamilton, Wellington and Christchurch. Subsidiary companies include NIWA Vessel Management Ltd (in Wellington), NIWA Australia Pty Ltd (in Brisbane), NIWA (USA) Inc and NIWA Environmental Research Institute (also in the USA).

NIWA collaborates in the operation of the Institute of Aquatic and Atmospheric Sciences with Auckland University and Centres of Excellence with Otago University, Canterbury University and Victoria University of Wellington.

The core business for the company falls into 5 main areas; Marine (oceanography, ecology, geomorphology etc), Freshwater (lakes and rivers), Fisheries (stock assessment, modelling and management), Atmosphere and Climate, and finally Aquaculture.

NIWA Aquaculture

NIWA staff have been at the forefront of aquaculture development in New Zealand since the inception and NIWA has invested heavily in facilities and expertise to underpin the commercial success of aquaculture in New Zealand. NIWA's latest development has been the construction in 2002 of a dedicated warm-temperate water marine aquaculture production and research facility, Bream Bay Aquaculture Park in New Zealand's North Island. In addition to this new facility NIWA has two additional established aquaculture facilities; Mahanga Bay, Wellington (cold water marine; urchin roe enhancement and seahorse) and Silverstream, Christchurch.

Figure 1. Location of NIWA aquaculture facilities in New Zealand.

NIWA Bream Bay Aquaculture Park has exceeded expectations and continues to attract investors keen to develop new species for aquaculture. The close association with industry allows NIWA to be very commercially focussed and target specific production issue for the development of new ventures.

Currently, NIWA has 4 companies on site working in association with NIWA developing the farming techniques for such species as paua (abalone), eel, mussel spat, Yellowbelly flounder, rock lobster and our own business of kingfish.

Figure 2. NIWA Bream Bay Aquaculture Park.

Kingfish

NIWA currently produce 100,000 kingfish fingerlings per annum with future contracts to produce 250,000 per annum for a commercial client in Northland. Currently there is no R&D on kingfish larvae but efforts are being expended on expanding the broodstock and manipulating photoperiod the enable the spread of the current production season to satisfy current and future customers.

Figure 3. Kingfish broodstock.

Broodstock are maintained in a 70,000l tank and naturally spawn (Figure 3). Fertilised eggs are collected using a surface skimmer drawing water across the surface of the tank by means of an airlift. Collected eggs are incubated under constant artificial light on ambient seawater in flow through systems using conical based 300l incubators[1].

Soon after hatch the larvae are transferred to 10,000l round tanks held under static water conditions using a mixture of *Nanochloropsis* and *C. mulleri* illuminated with natural light. The algae are cultured in bulk in outside tanks with the mature cultures pumped from the external algae culture tanks to the appropriate system within the hatchery (Figure 4).

Figure 4. External bulk algae culture tanks at NIWA Bream Bay.

The hatchery protocol uses the standard green water culture techniques beginning with enriched rotifers and transitioning swiftly through enriched *Artemia* on to artificial diets supplied by INVE.

Rotifers are produced under standard batch culture techniques from sterile stock cultures maintained at NIWA's site at Bream Bay Aquaculture Park (Figure 5).

[1] *All culture water for all hatchery activities 5micron filtered, UV treated seawater*

Figure 5. Stock cultures maintained at NIWA Bream Bay.

The culture diet used is another INVE product, Culture Selco 3000 mixed with "instant algae" which allows the culture of rotifers through several cycles before a new stock culture needs to be used. Standard practice at NIWA is to take the rotifers through 5-6 cycles before renewal (Figure 6).

Artemia are first decapsulated and incubated for hatch standard protocols employing the use of "hatch controller" (INVE). The *Artemia* are enriched using DHA Selco.

Enriched rotifers are introduced to the 10,000l larval tanks just prior to first feeding. Once feeding is established a slow ambient water flow is switched on. Rotifers are fed for a total of 26days post first feeding. *Artemia* are brought in and rotifers fazed out at day 18 post-first feeding. Artificial diets are first introduced at day 25-28 (dependant on the batch) and *Artemia* fazed out completely at day 40 post first-feeding. The first diet the larvae are weaned onto is Proton. Survival of animals once weaned is consistently greater than 95%.

Figure 6. Rotifer culture room at NIWA Bream Bay.

Scope for Improvement

Broodstock and Egg Quality

Much like any other hatchery NIWA seeks to improve the reliability of supply and the quality of eggs produced by the broodstock. In a bid to assess and improve the performance of broodstock NIWA embarked on a programme of DNA testing of all broodstock and egg batches resulting in weaned larvae. Through this process NIWA hopes to weed out poor performing broodstock. Currently all broodstock are from wild stock however, a proportion of all production each year are held and on-grown on site to establish an F1 broodstock with beneficial domesticated traits

First-Feeding Larvae

Total Kingfish larval abnormalities are now down below 10%. Typically, at NIWA we encounter shortened opercula, lack of swim bladder and jaw deformities which manifest themselves as a turned down or crisscrossed jaw. In previous years the instance of larval abnormalities was considerably higher but the improvements achieved were brought about by improvements in the broodstock diet and enhanced live feed enrichment procedures.

Weaning

One of the areas where NIWA feels greatest improvements can be made will be during the transition from live to inert feeds. Hence, significant effort will be directed towards the duration of transition and early inception of the whole process.

Flounder

Initial attempts are being made by NIWA to culture Yellowbelly flounder (*Rhombosolea leporina*) at Bream Bay for a commercial client. The attraction for NIWA is the winter spawning habit of this of this species ensuring the year round use of facilities at Bream Bay.

Broodstock are wild caught and have so far always induced to spawn through injection of Ovaprim (Figure 7).

Eggs produced have fertilised well but are only 0.62mm in diameter (Figure 8).

The larvae at hatch are on average 2.2mm long and have no functional mouth (Figure 9).

Figure 7. Yellowbelly flounder female broodstock at NIWA Bream Bay.

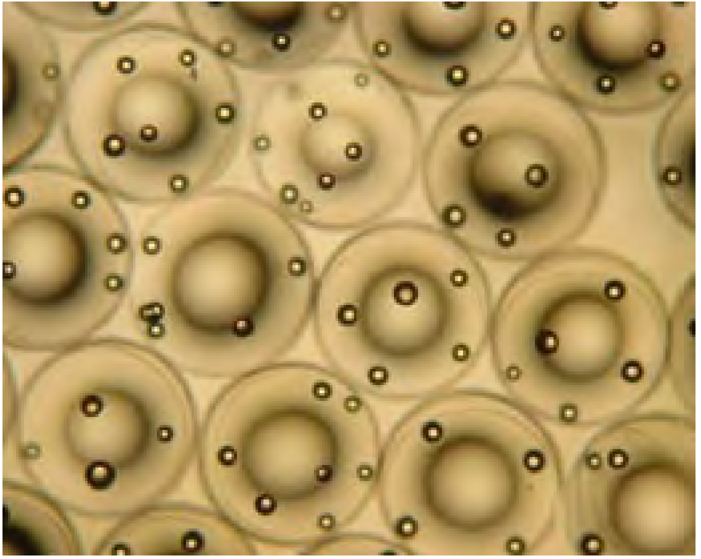

Figure 8. Flounder eggs 24 hours post fertilization.

Figure 9. Yellowbelly flounder yolk-sac larvae.

Larval Rearing

NIWA has found through these early attempts at larval rearing that the process becomes problematic at first-feeding when the size of the first feeding larvae becomes an issue. The main problem is the small size of the mouth. L-strain rotifers are too large. To get round the problem NIWA is trialing the use of mussel trochophores which can be easily produced on demand. In addition this year attempts will be made to initiate effective first-feeding through the use of various temperature regimes to provide a suitable start-feeding stimulus.

Conclusions

Kingfish culture is now well established in New Zealand with NIWA as the sole supplier. The emphasis for future development is to improve and spread the supply of seed and optimise the weaning period.

Yellowbelly flounder aquaculture is still in its infancy and the feasibility of commercial production is still to be determined.

CSIRO Marine Research Capabilities and Research in Larval Feeds

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Introduction to CSIRO Marine Research and its Aquaculture Research

CSIRO, through its Division of Marine Research (CMR; website: www.marine.csiro.au) provides research support across a range of coastal and oceanic industries and clients, including aquaculture. CMR has over 300 staff across three sites (Hobart, Tas; Cleveland Brisbane, Qld; and Floreat Perth, WA). CMR aquaculture research is delivered primarily through the Integrated Sustainable Aquaculture Research Group, which has ≈ 20 staff at the Hobart and Cleveland sites. The major research focus areas are: the genetics, nutrition and production of prawns and salmon, and to a lesser extent abalone, oysters and tuna. We also undertake research in microalgal feeds where there is a direct link to these key aquaculture species.

CSIRO Marine Research's Laboratories at Hobart (left) and Cleveland (right)

Research History in Larval Feeds

CSIRO's initial research and industry support in larval feeds commenced during the mid 1980's, with a microalgal supply service specifically set up to supply algal starter cultures to hatcheries, funded by FRDC. Alongside the supply service we established an R&D project to examine the biochemical properties (and hence likely nutritional value) of microalgae being used, or of potential use for Australian hatcheries. We have examined over 60 strains of microalgae, including many new Australian isolates, and provided detailed biochemical profiles (gross composition, fatty acids, amino acids, vitamin, sugars and sterols) under a range of different culture environments (Publications: Live Algal Feeds).

As an extension of our basic research on microalgal composition, we subsequently assessed the microalgae in feeding experiments and identified species with high nutritional value, especially for oysters (see Publications: Bivalve feeding and diet assessment). Our studies also included a range of alternatives to live microalgae either isolated or developed by CSIRO and our colleagues, that are potentially more cost-effective, i.e. algal concentrates, yeast, bacteria and thraustochytrids (Publications: Alternatives to Live Diets).

The lack of aquarium facilities at our Hobart Laboratories has meant that our larval feed research on temperate species has relied on strong research collaboration with industry (especially Shellfish Culture Ltd) and the Tasmanian Aquaculture and Fisheries Institute (TAFI). CMR has had a strong and productive collaboration with TAFI in projects on the larval production of striped trumpeter and rock lobster (see Publications; and later section on capability), where CSIRO has provided analytical capability, and expertise in live feed enrichment and design of nutritional experiments. At our Cleveland Laboratories, the nutritional requirements of prawn larvae have been assessed using a range of microalgal diets.

Current and Ongoing Research Interests in Larval Feeds

During the last 5 years, CMR has established international collaboration in algal feeds, specifically with development of algal concentrates (IFREMER, France) and high-biomass solutions for algal culture using photobioreactor systems (University of Florence, Italy). This work is now being written up into several manuscripts.

Currently, CSIRO is undertaking a survey to assess novel marine protein and oil sources as potential replacers of fish meal and/or for high value products. The survey will include single-cell protein and zooplankton commonly used as larval feeds. Based on a positive assessment, a fuller proposal may be developed later this year, to be considered as a project within one of the new CSIRO Flagship Programs, i.e. Wealth from Oceans (http://www.csiro.au/index.asp?type=blank&id=FlagshipPrograms).

CMR has had a major role in providing nutritional and analytical expertise to the TAFI-led, FRDC/Aquafin CRC projects (see more in next section) on larval striped trumpeter propagation. The current project is due to end this year, and during this time we will be writing publications from the research with our TAFI colleagues.

CMR has recently undergone a major restructure within its aquaculture projects. As a consequence, research in larval feeds has been scaled down. Nevertheless, despite the current status we plan to maintain our core capability to take up new co-investment opportunities as they arise through future industry needs.

Capabilities and Equipment

CMR houses the CSIRO Collection of Living Microalgae (website: www.marine.csiro.au/microalgae/ collection.html), one of the largest collections in the world, with over 750 strains including representatives from all classes of marine microalgae, some freshwater microalgae, and unusual marine heterotrophic microorganisms (e.g. thraustochytrids). The culture collection specialises in the Australian region, with microalgae from tropical to polar waters, although microalgae from around the world are also maintained. The Collection (through its Microalgal Supply Service) plays a pivotal role in supporting Australian aquaculture, by supplying ≈ 80% of Australian hatcheries with starter cultures of feed microalgae. Specialised skills and capabilities of CMR Collection staff include culture maintenance, species isolation and axenisation, media preparation and knowledge of the specific environmental requirements and growth properties of the different species.

Cultures of microalgae from CSIRO's collection

In addition to the infrastructure for the laboratory scale culture of algae, CMR has 6 x 50 L photobioreactor units for assessing high-biomass cultivation Through our past and current research we have developed expertise in the culture of some key aquaculture strains (e.g. *Nannochloropsis, Skeletonema*) within these systems including optimisation of biomass and omega-3 long chain polyunsaturated fatty acids (PUFA). In the search for cheaper biomass and new sources of PUFA, we

15 L photobioreactor

have also developed expertise in the isolation and culture of thraustochytrids – heterotrophic micro-organisms with high concentrations of PUFA.

During the past two decades, CMR has built up an international reputation of expertise in marine lipids and more recently, in analyses of amino acids and vitamins. We have developed and validated microanalytical techniques to measure the concentrations of fatty acids, sterols, lipid classes, amino acids (free and total), sugars, vitamins (B-group, C and E) and proximate analysis. The Division maintains state-of-the-art laboratories equipped with gas chromatographs (GC), HPLC, GC-mass spectrometers as well as other general infrastructure (eg. freeze-drier, centrifuge, extraction hoods etc.) required for these analyses. This capability has been applied to recent TAFI-led projects on the production of rock lobster phyllosoma and striped trumpeter larvae, where CMR has taken a lead role in the nutritional component of the projects. Specifically, both projects have had a focus on lipid and vitamin nutrition, and their analyses have been undertaken at CMR. CMR has worked closely with TAFI on the design of nutrition-based larval feeding experiment, and also in the interpretation and write-up of the research (see Publications on rock lobster and striped trumpeter).

Based on our lipid chemistry expertise, CMR has also developed protocols for the development of experimental emulsions used and validated for enriching live-feeds in tocopherol and PUFA. These protocols have been applied within the TAFI-CMR striped trumpeter project collaboration. Staff also have expertise in microencapsulation technology, e.g. binding of water-soluble nutrients within abalone diets, and crystalline amino acid encapsulation within prawn diets.

Our Cleveland laboratory has the capability to run multiple treatment x replicate experiments with larvae through the use of its “larvatron” (see Publication: Technology). The larvatron is a computer controlled larval and zooplankton culturing apparatus developed at CSIRO which is capable of maintaining up to 100 x 1 L culture vessels simultaneously, allowing a wide range of experimental conditions (eg. temperature, salinity, food type and concentration, animal stocking density) to be controlled, measured and replicated. The larvatron has been used for experiments with prawn larvae, but could also be used in experiment with other larvae, or to assess the enrichment of live feeds.

LARVATRON at Cleveland Labs

HPLC analysis of vitamins

Summary

CMR has a well-established track record in larval feeds research, especially in microalgae applications for aquaculture. We have a strong capability (staff expertise, state-of-the-art equipment) for the microanalysis of key nutrients in larvae and larval feeds, e.g. fatty acids, vitamins, amino acids. We have developed strong collaborative linkages with local industry, TAFI and overseas institutions such as IFREMER and University of Florence. We have currently reduced our research effort in larval feeds, though we are maintaining our key infrastructure and staff skills, which will enable new co-investment opportunities to be pursued.

CSIRO Publications in Larval Feeds and Biochemistry

*(Descending chronological order within category; Lead CSIRO author indicated by *)*

Live algal feeds

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Early weaning of Barramundi, *Lates calcarifer* (Bloch), in a commercial, intensive, semi-automated, recirculated larval rearing system

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Introduction

The production of live food for larvae of barramundi (Asian sea bass), *Lates calcarifer*, like for most species of marine fish, constitutes a major operational cost and bottleneck in a commercial hatchery.

On one hand, mass culture of rotifers and live microalgae involves high costs and labour requirements, as well as having highly variable nutritional value while also being unreliable. On the other hand, *Artemia* production is also associated with high operational costs and the weaning of the cannibalistic barramundi larvae onto *Artemia* can be highly detrimental to the growth and to the size disparity (variation) of a commercial batch.

We present a commercial system that tackles both issues, with great results, by using two of the latest products available in larval culture methods.

We have developed an intensive, greenwater, recirculated larval system and a continuous, high density, recirculated rotifer system using the HUFA enriched chlorella paste (DHA-enriched Super fresh V12 Chlorella) produced by Pacific Trading Co. Ltd, Japan.

The other major improvement to the system is the use of the micro-diet, Gemma Micro (GM), from Skretting for early weaning. *Artemia* have effectively been replaced in the larval diet by GM making the larval culture process more efficient and economic and producing higher quality weaned juveniles.

Darwin Aquaculture Centre

The Darwin Aquaculture Centre (DAC) is situated on Channel Island in Darwin Harbour some 50km from the city centre. This research and development facility was designed by the aquaculture staff and completed in September 1998 at a cost of $2.3M. This DAC is a showcase of practical design in a tropical environment and currently accommodates 16 staff, and up until recently, 2 post graduate students.

The centre has specific areas dedicated to fish, crustacean, algae, live feeds and environmental control work, in addition to a large dry laboratory, office, workshop and store. A bank of self-cleaning sand filters maintains a supply of suitable sea water all year, a rare commodity from a tropical estuarine environment.

In 2001 the DAC was expanded with the addition of a commercial barramundi fingerling production facility capable of producing up to 2 million advanced fingerlings per year.

Apart from commercial fish production, current projects include: developing barramundi nodavirus control techniques and strategies, refinement of methods for larviculture of mud crabs and a joint venture arrangement with industry for the production of juvenile sea cucumbers for ocean ranching and pond culture. Development and application of techniques for aquaculture in remote indigenous communities is also a key focus area of the DAC and the NT Government.

Some of the finfish broodstock tanks at the DAC. **A modern barramundi nursery was completed in 2001.**

The DAC is designed for research and development of tropical marine aquaculture.

Materials and Methods

Rotifer system

The rotifer system has two 1,000 l tanks with foam fractionator, floc traps (sediment filter), fluidised-bed biological filter and one UV unit for new water disinfection (Figure 1).

The chlorella paste is fed automatically and continuously to the two rotifer tanks 24 hours a day from a standard fridge via two peristaltic pumps (one per tank).

The algal paste offers tremendous advantages, particularly because the rotifers can be produced at high density in small volume cultures. A base rotifer density of less than 100/ml is maintained between larval batches. When rotifers are required the systems are run at a density of 1,000 –1,500/ml with a lead time of 7 days. The algal paste is already enriched with essential fatty acids and no further enrichment is required. The un-consumed rotifers are kept in optimal nutritional value in the larval tanks by maintaining a low algal paste cell density.

The culture system is clean, with low levels of ciliates (free-swimming and attached e.g. *vorticella*) and based on simple technology. Bacteriological studies showed that the recirculating system carries a more stable bacterial population than batch systems and there is an almost total absence of harmful *Vibrio* spp. The rotifers are then continuously and automatically pumped directly (no rinsing) from the rotifer tanks to the larval tanks via two standard diaphragm pumps. Forty to sixty percent of the rotifer cultures are harvested per day (over 1 billion rotifers).

The use of the chlorella paste has been costed at A$ 0.17 per million rotifers produced. The daily labour requirement to maintain the system is less than 1 hour and the construction of the system only requires small tanks, standard pumps and PVC and plastic welding.

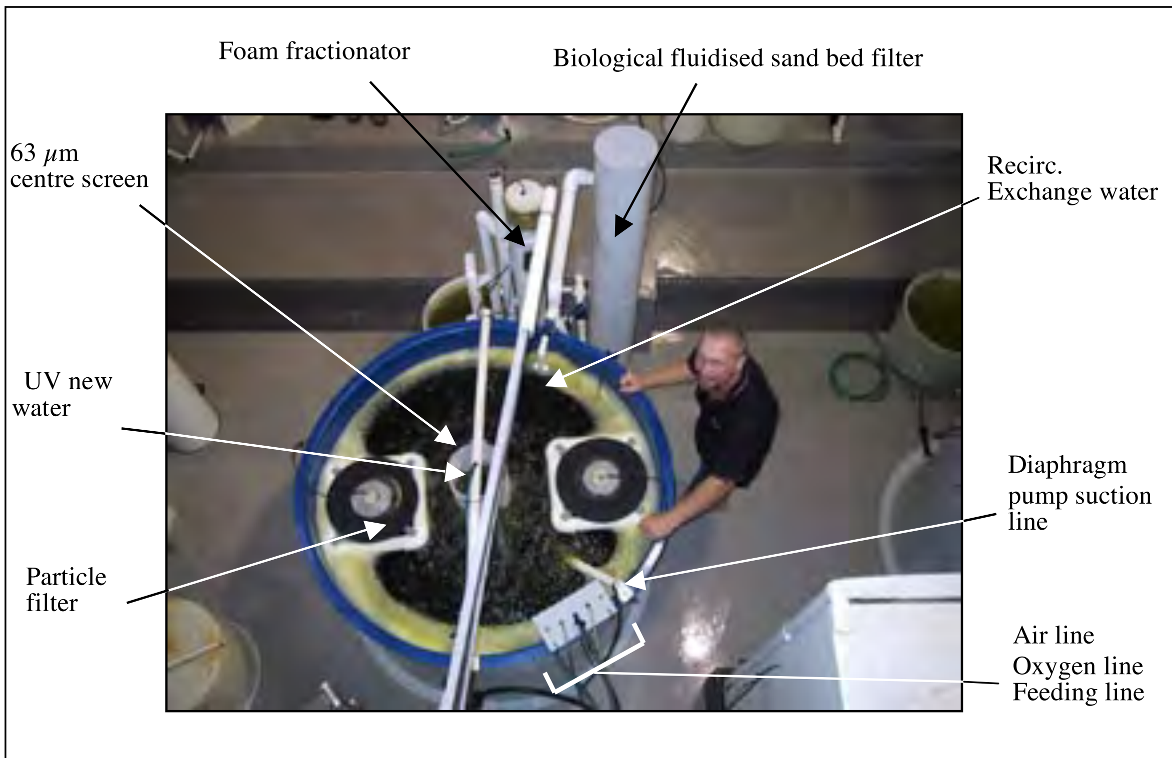


Figure 1. One of the two, 1,000 litre, high-density, rotifer culture tanks.

Larval rearing system

The larval system consists of two 6,000 l tanks in parallel (Figure 2), a rapid sand-filter, foam fractionator, fluidised-bed biological filter, degassing column, UV disinfection and a temperature control unit.

The larval tanks are stocked at 100 larvae per litre (a total of 1.2 million larvae) and the larval rearing regime is described in Figure 1. As per the rotifers system the chlorella is continuously and automatically fed to the larval tanks to keep a constant low algal density. The rotifer density is adjusted by changing the size of the outlet self-cleaning screens (63 to 500 µm) and/or by adjusting the pumping rate to the larval tanks.

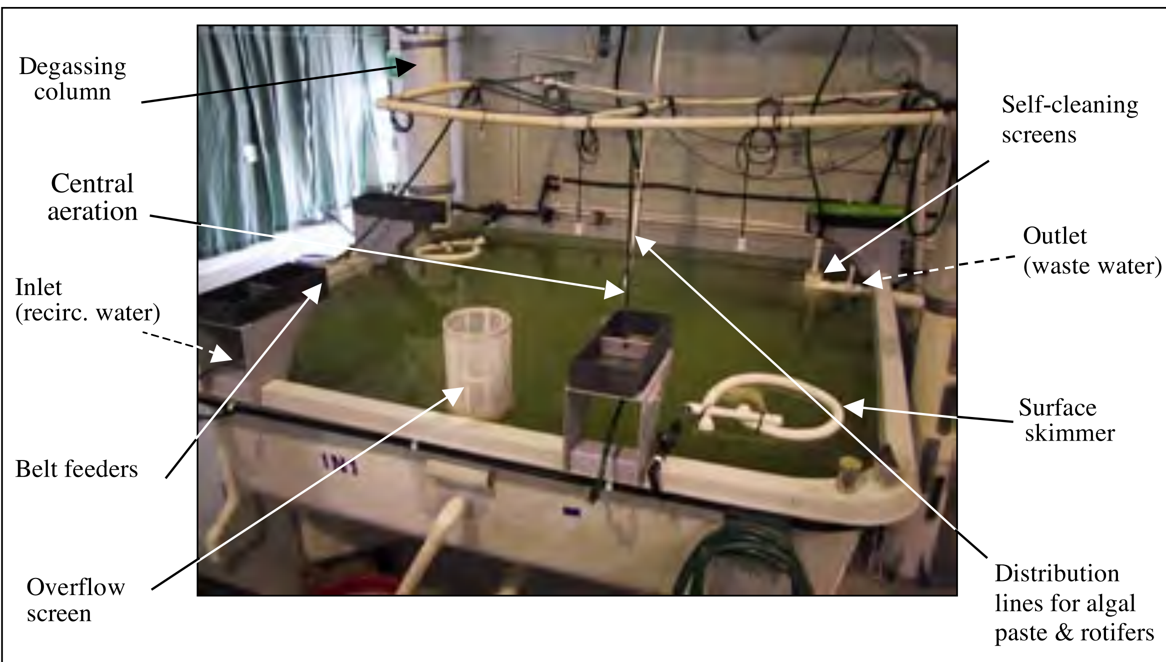


Figure 2. One of the two 6,000 litre intensive larval rearing tanks.

The other major improvement to the system was the use of the microdiet, Gemma Micro, from Skretting (Nutreco) for early weaning. The latest batch of barramundi was produced without the use of any *Artemia*. The larval tanks were stocked at 100 larvae per litre, fed only on rotifers for 6 days and then co-fed with GM for 7 days and were weaned by D16 with only 5% weaning mortality. Over 700,000 fry were produced from this batch with a survival of 60%. In the previous batch less than 2 kg of *Artemia* per million weaned fry (Day 18-20) were used with less than 0.5% weaning mortality. This represented a 95% reduction in *Artemia* use compared to previous batches (Table1). For economic reasons, once the larvae are weaned, Gemma Micro is slowly replaced by the microdiet, Proton (INVE), before moving on to the nursery feed, Gemma (Skretting) (Figure 3).

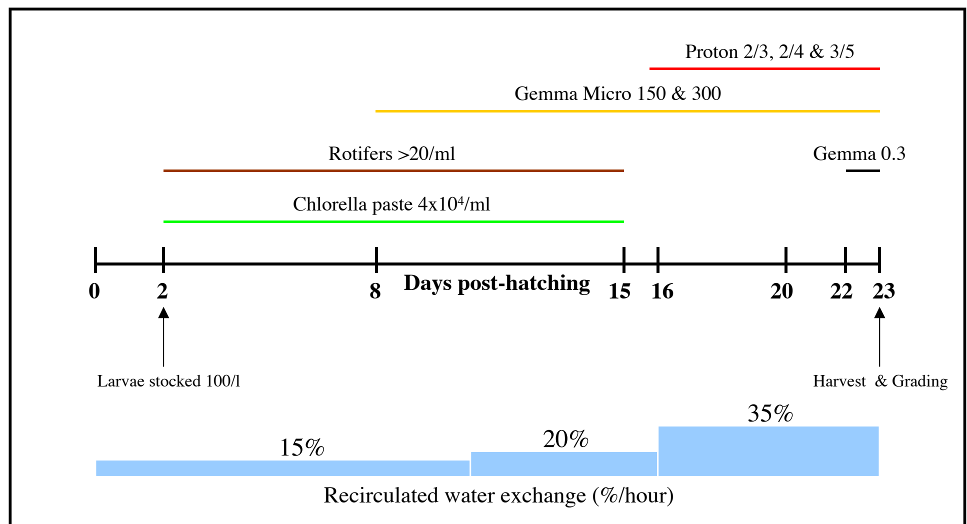


Figure 3. Larval rearing regime for barramundi, *Lates calcarifer*, in the intensive recirculated system from first feeding larvae to metamorphosis. Artificial diets: GM: Gemma Micro (Skretting); Proton (INVE); Gemma (Skretting).

Results and Discussion

Using this system we have routinely achieved a survival rate of 44-58% from 2 day old larvae through to 23 day old weaned fry (15 mm). The deformity rate of the fingerlings assessed at 100 mm was less than 1% (mainly jaw deformity and missing opercula) and was not greater than previous batches reared using *Artemia* (Table 1).

The latest batches of larvae weaned quickly with Gemma Micro and grew to 100 mm 15-25% faster than previous batches (9-10 weeks compared to 11-12 weeks from hatching). There is also less size variation, a great advantage since barramundi are very cannibalistic and need frequent grading on reaching 3-4 weeks old. Once transferred to the nursery, the fish are much healthier and less susceptible to stress (handling, grading…).

The total cost of weaning and post-weaning feeds (including labor) has been summarized in Table 1 and is 20% cheaper, using GM and minimal *Artemia*,, than the standard method. The labour requirement (and operational cost) has been significantly reduced to one person dealing with both the larval system and the live feed system (because of quarantine issues 2 x 0.5 technicians are running both systems). The capital cost of constructing the system was also minimal as the system was mainly built in-house using PVC and assembled using plastic welding.

Table 1. History and production parameters of the intensive larval system and cost of weaning and post-weaning feeds (including labour) per million of barramundi weaned fry produced (15 mm).

	Batch Number					
	03-03	**08-03**	**10-03**	**12-03**	**02-04**	**04-04**
Larval stocking density (/ ml)	54	70	84	100	103	106
Fry produced at D 25 (15 mm)	278,000	450,000	448,000	612,000	577,000	734,000
Survival (%)	44	54	45	51	47	58
End of weaning (Dph)	25-35	20-26	22-24	22-24	18-20	16-18
Weaning mortality (%)	1-2	1-2	0	1-2	0	5
Deformity rate (% assessed @ 100 mm)	< 0.1	<0.8	< 0.1	< 0.2	< 1.0	< 0.5
Artemia (kg) / 10^6 weaned fry	20.5	6.6	12.5	10.1	1.2	0
Cost of enriched *Artemia* (inc. labour) ($A)	5,700	2,500	3,900	3,300	700	0
Gemma Micro (kg) / 10^6 weaned fry	0	4.6	20.5	14.2	12.1	13.2
Cost of Gemma Micro ($A)	0	1,400	6,500	4,500	3,800	4,200
Proton (kg) / 10^6 weaned fry	11.5	6.2	5.8	10.9	10.2	11.8
Cost of Proton ($A)	500	200	200	400	400	500
Total cost in $A per million weaned fry	**6,200**	**4,100**	**10,600**	**8,200**	**4,900**	**4,700**

More than three million barramundi fry have been produced using this system at the Darwin Aquaculture Centre (DAC) over the last 12 months (5 batches of 600,000+).

We are now doing collaborative work with Dr. Sagiv Kolkovski, Department of Fisheries of Western Australia, to assess microdiets and co-feeding management to further improve the weaning process and to maximise survival and growth rates of barramundi larvae during the larval rearing and early nursery phases.

For the past two years we have also assessed grading and feeding strategies in the nursery stage from 15 mm fry to 100 mm fingerlings. Asian, European and Australian nursery feeds have been compared in these experimental trials.

We will be producing technical and scientific papers early in 2005 describing the larval system and presenting the nursery trials.

Richard Knuckey and Elizabeth Cox

The Aquaculture and Stock Enhancement Facility at Northern Fisheries Centre, Cairns was commissioned in early 2002. This purpose built, marine fisheries facility was designed to enhance Queenslands development of tropical aquaculture. It contains dedicated broodstock, larviculture and live feed areas that support a variety of fish aquaculture.

Broodstock facilities for finfish include 7 x 30T and 6 x 60T tanks. These operate with recirculation through dedicated biofilters. Currently, 5 x 30T and 2 x 60T tanks are equipped with photoperiod and temperature control.

Five marine finfish species are maintained in this system. For the role of stock enhancement, Barramundi (*Lates calcarifer*) and Mangrove Jack (*Lutjanus argentimaculatus*) are held. Barramundi broodstock are spawned throughout the year with periodic replacement of fish. Mangrove Jack are F1 fish spawned in ponds at DPI&F Oonoonba, Townsville and on grown in Cairns from ~2 cm. These fish are now approaching sexual maturity where they may be used as broodstock for captive spawning. Another major project "Tropical Marine Finfish Aquaculture" is focused on the development of aquaculture of reef fish and in particular groupers. Three grouper species are held for this project:

Barramundi cod (*Cromileptes altivelis*), Flowery cod (*Epinephelus fuscoguttatus*) and Estuary cod (*Epinephelus coioides*).

Beside finfish the facility also supports research in the aquaculture of the tropical lobster (*Panulirus ornatus*) and sandfish (*Holothuria scabra*).

The live prey facility supplies food to all areas that require it as well as acting as a supply service to the aquaculture industry. It also has a research component to refine existing culture methods and develop new live food species.

Around 10 microalgal species are routinely held in the collection and grown up to a volume of 2000 L. Microalgae are used to feed rotifers, copepods and to on-grow *Artemia* as well as for "green-water" culture and the conditioning of plates for sandfish larvae. The SS-strain rotifer *Brachionus rotundiformis* and the calanoid copepod *Acartia sinjiensis* are grown for marine finfish larvae. *Artemia* are hatched and enriched for finfish larvae or on-grown in algae for lobster larvae.

Broodstock research

Epinephelus fuscoguttatus broodstock at NFC

The increased broodstock facilities, particularly the increase in the number of tanks with photo-thermal control has enhanced the scope of research that can now be undertaken. Flowery cod and estuary cod research is focussed on assessing the use of photo-thermal control and the application of short-term stimuli to both control and extend reproductive development and to induce spawning. Research into captive populations of barramundi cod is targeting a range of issues including poor spawning performance and spontaneous sex reversal of male broodstock in captive culture systems. The target for all three grouper species is to develop protocols that will allow for extended and predictable spawning of captive breeding populations.

Larviculture research

Epinephelus fuscoguttatus larvae cultured at Northern Fisheries Centre

Increased spawning success as a result of the increased scope of broodstock research has enhanced our ability to address bottlenecks within the larval rearing phase for these species. Poor and inconsistent survival remains an ongoing issue during the early larval rearing phases for many grouper species. The primary larval rearing objective has therefore focussed on identifying the basic physical parameters required to obtain consistent survival during the first feeding stages for each species.

The impact of light intensity on survival, growth and feeding incidence of both estuary cod and flowery cod has been assessed across multiple cohorts. Results of light intensity trials have indicated that pre- and early

feeding stages of larval grouper have a preference for lower light ranges. Morphological data suggest that energy expenditure is used for growth rather than increased swimming activity at the lower light levels tested, however the variability between cohorts require that this data is interpreted with caution until replicated further. In addition, larval survival and condition during more extensive feeding trials, must be undertaken in order to fully assess larval feeding ability in response to light intensity.

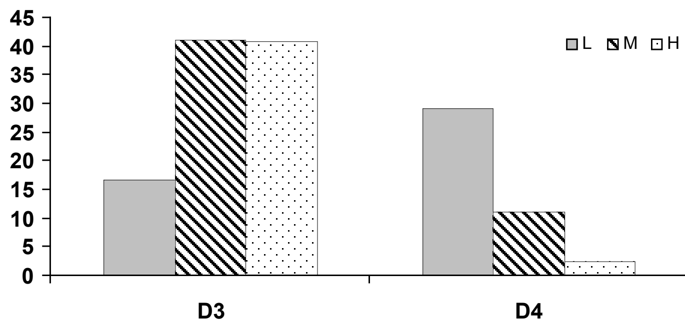


Figure 1. Feeding incidence of larval flowery cod exposed to three light intensities (low 0.05 - 0.085, medium 1.2 - 1.75 and high 6 - 10 µmol.s^{-1}.m^{-2}).

Live prey research

Research in Live Prey production is focused to supply suitable food for the fish species cultured in the facility. Marine finfish such as groupers have a small mouth gape at first feeding. This has necessitated the development of live prey species small enough to be consumed by these larvae. Recent research has focused on:

- Improved nutritional value of rotifers
- Reduced scale-up time and increased production of rotifers
- Increased production of copepods

Outcomes

Improve nutritional value of rotifers

A diet with a high DHA:EPA ratio is preferable for marine finfish larvae. Because of this, the use of Algamac-2000 for routine enrichment of SS-strain rotifers cultured at NFC has been adopted. Rotifers enriched with Algamac 2000 have a higher DHA:EPA (4:1) ratio and a more consistent fatty acid profile than those enriched with DHA Protein Selco. Although the Algamac product states that higher levels of fatty acids are possible if rotifers are enriched for 2 x 12 h periods, we recommend only a single 12 h enrichment. This is because, low rotifer fecundity present after a single 12 h enrichment would likely result in significant mortality during a second enrichment period. To maximise rotifer survival, the use of multiple aeration and additional O_2 is recommended for all rotifer enrichment.

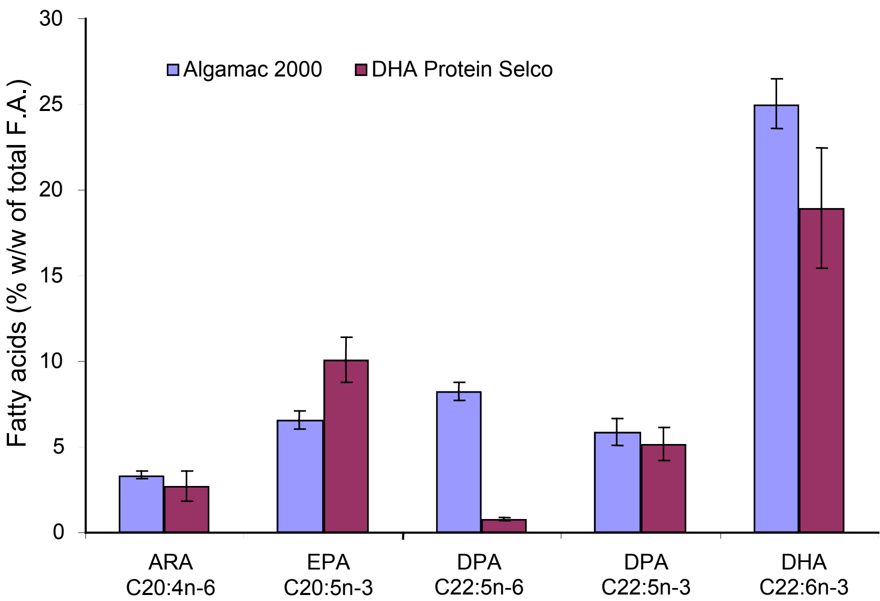
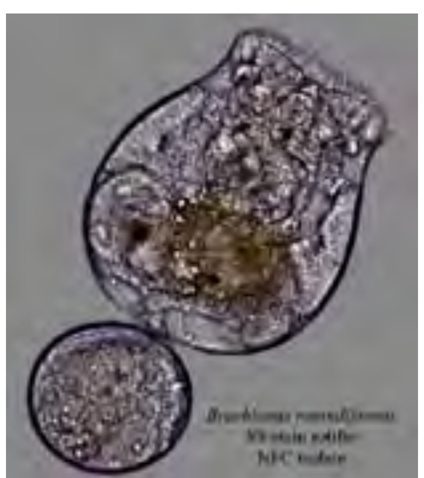


Figure 2. HUFA (% w/w of total fatty acids) content of rotifers enriched with Algamac 2000 and DHA Protein Selco. Error bars are ± SD, n = 3.

Reduce scale-up time and increase production of rotifers



The medium-density, Culture Selco 3000 system is being adopted as a method to increase the capacity of the rotifer culture facility at NFC while reducing the labour required for their production. This system was demonstrated to produce at least as many rotifers as the product and its method stated. Rotifer densities of 1500 to 1800/mL were achieved after 3 to 4 days of culture. Adoption of the method requires strict adherence to the instructions detailed for the product. In particular, the initial rotifer population must to be of a high quality with a fecundity of at least 20% and minimal contamination by protozoa. It is possible that feed rates recommended for the product could be refined and this would reduce the risk of protozoa blooming from excess food. The use of Culture Selco 3000 will reduce the reliance on algal cultures and the time required to scale-up rotifer cultures during spawning events.

Increase production of copepods

When fed on an equal ration (ash free dry weight), Cryptomonad CS-412 is as nutritious as *Rhodomonas* for *A. sinjiensis*. Because of its ease of culture relative to *Rhodomonas*, Cryptomonad is recommended to replace *Rhodomonas* in the diet for *A. sinjiensis* cultured at NFC. A daily diet ration of 1.13 µg AFDW/mL was found to support 99% maximal growth. It is recommended that the current daily feed rate of 1.34 µg AFDW/mL remain, this will ensure satiation with a low impact on water quality.

Increasing copepod production by scaling up of existing methods used in 400 L tanks to 1200 L tanks resulted in 83% of the production per unit volume. With modifications to harvesting equipment, this percentage is likely to approach par. To achieve a production rate to supply the hatchery with 2 nauplii/mL (12 × 280 L larval rearing tanks), it is recommended to increase the copepod culture system to 2 x 5000 L tanks. This would require approximately 150 L of mature Cryptomonad culture per day as feed. The capacity to produce algal species required for the copepod diet, in particular Cryptomonad sp. CS-412 and *Isochrysis* sp. (T. ISO) exists within the current facility.

Hatching of *A. sinjiensis* eggs was effectively (at least 90%) inhibited for 72 h at storage temperatures ≤ 10°C. Eggs and nauplii (N1) could be cold-stored at 10°C for 72 h with no development or loss of viability. Storage at

cooler temperatures (4°C) resulted in the rapid loss of viability of eggs. It is recommended to incorporate cold-storage techniques into the management of the upgraded copepod production (2 x 5000 L) facility. With an expected production of 5 x 10^6 nauplii/ day/tank and the adoption of cold storage, up to 20 x 10^6 nauplii per 5000 L tank could be available for the crucial first feeding stage of finfish larvae.

Results will be discussed in relation to the above points and will cover selection of rotifer enrichment product, use of commercial artificial rotifer diets, methods to increase copepod production through scale-up of existing methods and the development of cold-storage techniques for copepod eggs and nauplii.

Bottlenecks still exist in the supply of suitable numbers of very small live prey items for first and early larval stages of marine finfish. An outline of future work aimed at addressing this issue and selection of potential new live prey species will also be presented.

Tropical Rock Lobster

Hatchery research of the tropical rock lobster *Panulirus ornatus* is also a significant project at the Northern Fisheries Centre, Cairns. The project is primarily funded by the FRDC within the Rock Lobster Enhancement and Aquaculture Subprogram, and has as its collaborators AIMS and MG Kailis Pty Ltd.

Management of *P. ornatus* broodstock, for captive breeding has proven to be quite straightforward. The species is amenable to a captive environment, and will readily breed throughout summer. Environmental manipulation of breeding has been applied to achieve out-of-season breeding. Photoperiod is the primary cue, although temperature and social factors have a significant influence on breeding success.

Larval culture is particularly difficult, primarily because of the protracted larval life which extends to over 6 months in the wild. Too date, the longest lived larvae in our system has persisted to 211 days of age, and stage IX of eleven stages. Recent larval runs with a focus on improved survival of early stage larvae (to stage V) have been increasingly successful. The premise of current research is to achieve consistent survival of larvae to stage V, before realigning our efforts to mid-stage larvae. Hygiene and nutrition are the two most important factors, and research is accordingly focussed in these two areas.

James Cook University

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Species cultured

Molluscs: Pearl oyster (*Pinctada* spp. *and Pteria* spp.), tropical abalone (*Haliotis asinina*), tropical rock oysters (*Saccostrea* spp.) and other tropical species (e.g. *Tridacna* spp. and *Pinna* spp. and *Pectinidae*)

Crustaceans: Mud crab, *Scylla serrata,* sand crab (blue swimmer), *Portunus pelagicus*; ornamental species including the cleaner shrimps (*Lysmata amboinensis* and *L. debelius*) and the dancing shrimp (*Rhynchocinetes durbanensis*).

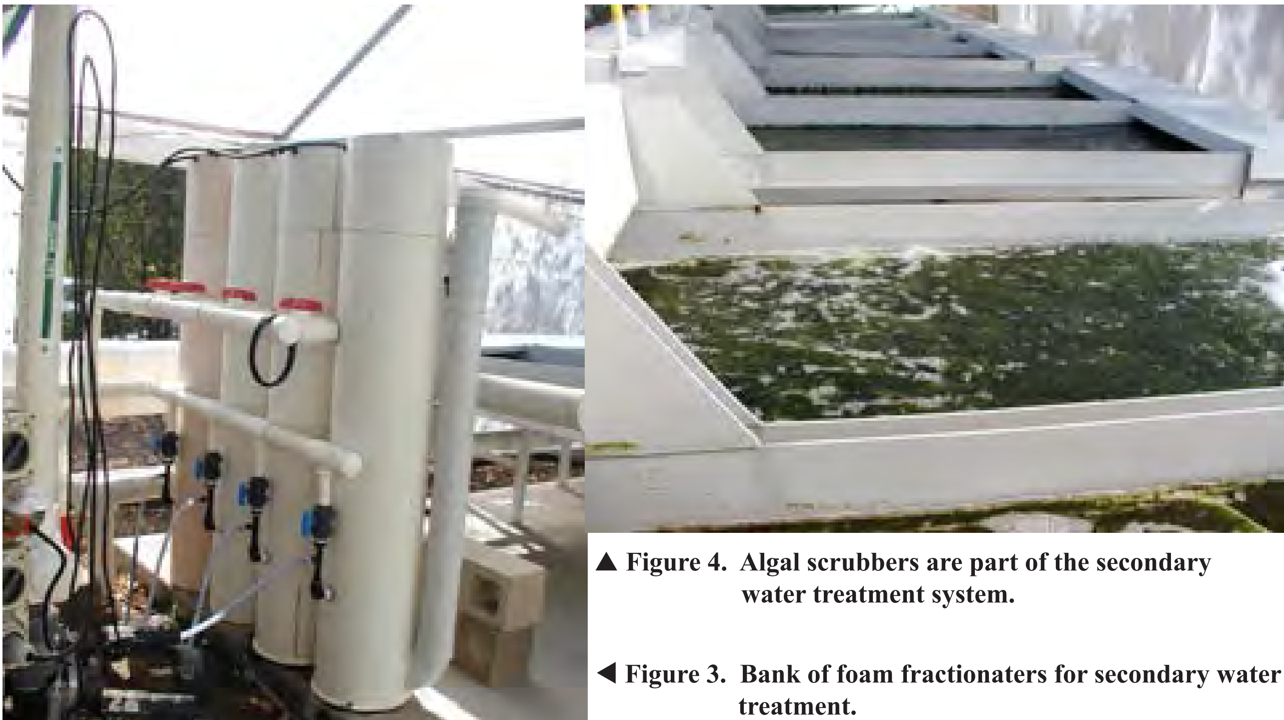
Finfish: barramundi, *Lates calcarifer*; damselfish *Acanthochromis polyacanthus*; clownfish, *Premnas biaculeatus*, *Amphiprion melanopus*, *A. akindynos* and *A. percula*; Coral trout, *Plectropomus leopardus* (new target species for 2005).

Systems

The JCU *Marine and Aquaculture Research Facility Unit* (MARFU) is a facility of the Faculty of Science Engineering and Information Technology and is available to all Schools within the Faculty. All seawater is transported to MARFU from the Australian Institute of Marine Science (AIMS) and MARFU technical staff maintain two seawater systems: primary treated 'new' water from AIMS, (un-chlorinated, UV sterilized, 1µm filtered at salinity of approximately 34‰) and secondary treated 'waste' water (treated using algal scrubbers, UV sterilization, foam fractionation, bag filtration to 20µm at salinity of approximately 31‰). Primary treated water is used for hatchery production of crabs and bivalves whereas secondary treated water is adequate for fish production and crustacean broodstock. A range of indoor, temperature-controlled rooms and outdoor shade-covered aquarium/tank space is available for use. Live feed production is now a MARFU service provided by the centre staff.

Figure 1. Main aquaculture facility area.

Figure 2. Primary water treatment for "new" incoming water including bag filtration, UV sterilizers, heat exchanger



▲ **Figure 4. Algal scrubbers are part of the secondary water treatment system.**

◀ **Figure 3. Bank of foam fractionaters for secondary water treatment.**

Orpheus Island Research Station (OIRS) is another facility of the Faculty of Science Engineering and Information Technology at JCU. Accommodation and research facilities have recently been refurbished and the station now has increased capacity to support broodstock, hatchery and growout research activities.

Molluscs

Bivalve conditioning is conducted in 200 L shallow tanks with either partial flow-through or static water conditions. Small-scale experiments with larvae are conducted in replicate 10 L aquaria. Water is either partially or totally replaced on a daily basis (using a flow-through system incorporating banjo screens), or is totally replaced every 2 days in static systems.

Larger scale bivalve larval culture is conducted in 500 L tanks and 18 tanks allow good replication. Tanks can be run as static water changed every 2 days or flow-through systems. Larger scale bivalve larval culture in conducted in replicate 3000 L tanks at OIRS.

Oyster broodstock are held on long-lines at either White Lady Bay, Magnetic Island, or at OIRS. Broodstock capacity at Magnetic Island is approximately 600-800 and at OIRS is 1200. Nursery and grow-out culture research with bivalves is also carried out at these sites.

Crustaceans

Mud and sand crab larvae are cultured in circular, flat-bottom, 400 -1000 L tanks, using green water (*Nannochloropsis oculata*) and supplied with UV sterilized, primary treated seawater (1 µm) with partial water exchange as required. There is holding capacity for 15-20 broodstock mud crabs at any one time and between 25 and 30 crabs are used for larval production annually. Holding capacity for sand crab broodstock is similar to that of mud crabs.

Figure 5. Mud crab crablet.

Culture of ornamental shrimp species is at an early stage of development, currently using small aquaria (20-50 L), and

recirculated, secondary treated seawater with partial water exchange as required. Production for each larval run is from single female broodstock.

Finfish

Barramundi larval culture is conducted in cylindro-concial tanks in sizes ranging from 250 L to 2,000 L, using green water (*Nannochloropsis oculata*), and supplied with recirculated secondary treated seawater with partial water exchange as required. No barramundi broodstock are held at JCU; larvae 2 days post-hatching are purchased from commercial hatcheries with some provided by QDPI, Northern Fisheries Centre. Both upwelling and downwelling tanks have been used for larval culture.

Clownfish and damsel fish larvae are produced in flat-bottom, cylindrical, 100 L tanks with a collar around the tank perimeter to reduce incident light. Water supply is flow-through secondary treated water with multiple inlet points around the tank perimeter and a single banjo screen at the bottom of the tank for water discharge.

Production capacity

Molluscs

We have the capacity for commercial-scale hatchery production of bivalves, but most is experimental. The production season is September to March but can be extended to June. On average 3-4 experimental runs with pearl oysters are conducted per year producing approximately 40,000 spat each. We do about 2 experimental runs with *Saccostrea* sp., annually each producing approximately 20,000 spat.

Crustaceans

Mud crab broodstock are mostly wild-caught (several hatchery produced mud crabs have reached sexual maturation and started spawning) and are maintained in oval, 2,000 L flat-bottom tanks until spawning. They are fed mussels, squid and prawns every day. After spawning berried females are transferred into indoor 400 L tanks with recirculated, primary treated seawater (1 µm). Larval production can be at any time of the year although it is more common between May and Jan. Production runs are driven by research need and between 10,000 to 50,000 juveniles are produced annually.

Broodstock sand crabs are maintained under similar conditions to mud crabs although berried sand crabs are often collected directly from the field. They are fed mussels, squid and prawns. There is capacity for year-around production; however, this is driven by research need. Currently up to 500,000 juveniles can be produced annually.

Excess production of mud crabs and sand crabs services teaching requirements and the remainder is currently culled. There is capacity for scaling up production of both depending upon demand.

Ornamental shrimp production has just recently been attempted. These species generally spawn twice per month, producing hundreds to more than a thousand eggs per spawn. They appear to have the capacity to produce eggs year-around under controlled environmental conditions. Larva durations are long, often 100 days or longer; however, production trials routinely only achieve survival to between 50 and 80 days. Given the high retail prices achieved for these shrimp species, culture protocols will continue to be a research priority.

Finfish

Ambient production of barramundi occurs from approximately October to February. Blue Water Barramundi have the capacity for photo-thermal manipulation of broodstock and have provided 2 day old larvae from August to March. Production runs are driven by research need and between 10,000-200,000 late-stage larvae or juveniles are produced annually. Excess production is culled or used for teaching.

There is almost year round capacity for damsel fish and clownfish production which occurs as required to meet the immediate demands of research and teaching. Broodstock are maintained on a diet of minced trash finfish and commercial flake. There is capacity for scaling up production depending upon demand.

Live foods used

Currently only S-S-strain rotifers (*Brachionus rotundiformis)* are produced to support crustacean and fish larval culture. Rotifers are produced in outdoor tanks 1000-2000 L in semi-continuous batch cultures using *Nannochloropsis oculata,* without addition of yeast. No additional enrichment products are currently used for rotifers besides micro-algae.

Currently only newly hatched *Artemia* and Artemia instar II are used. A variety of approaches including decapsulation, non-decapsulation and A1 Selco products for enrichment are used.

Figure 6. Hands-on area for undergraduate teaching and outdoor large scale rotifer area.

Crustaceans

Mud crab and sand crab: Early larvae are fed rotifers (Zoea-I to Zoea-II). Larvae are switched to newly hatched *Artemia* at later stages. Depending on the experiment design and particular protocols being undertaken, mixed feeding of rotifers and *Artemia* may occur at Zoea-II to Zoea-IV (mud crab) or Zoea-III (sand crab) stages.

Finfish

Barramundi: D 2 - 12, S-S-strain rotifers; D 8 - 10, newly hatched *Artemia;* D 10 – until weaning, enriched *Artemia* instar II. Fish are weaned starting any time from D 18 - 30, depending upon the particular experiment protocol. Inert diets are sometimes used along with live feeds during the *Artemia* feeding stage. Rotifers are added twice daily at 20-50 ml^{-1} and *Artemia* are added twice daily at 0.05 - 3 ml^{-1}.

Damsel fish: Live feeds, *Artemia* and rotifers, are provided but larvae will accept a commercial flake from hatching.

Clownfish: D1-8, rotifers, gradually reduced from 20 rotifers ml^{-1}; D 5-10, *Artemia,* gradually increased from 1.5 to 2 *Artemia* .ml^{-1}; weaning starting from D 6 or 7.

Inert feeds used

Mud crab: Microbound diets have been developed at JCU that are readily accepted by mud crab larvae and support at least equal development rate and survival as *Artemia* for the megalopa stage. Particle sizes of the microbound diets for Zoea 1, Zoea III, Zoea V and Megalopa are <150 µm, 150-250 µm, 250-400 µm and 400-600 µm, respectively.

Barramundi: 100 µm inert diet if used during the *Artemia* feeding stage; 300 µm starting from D 18. Commercially available microparticulate feeds have been assessed for barramundi larvae and a prototype experimental microparticulate feed has been developed in collaboration with WA Fisheries during a recent FRDC funded project.

Algae

For bivalves: There is a focus on culture of tropical species. Standard species for bivalve culture include: *Isochrysis* sp. (T-iso), *Chaetoceros muellerei*, *Pavlova salina*. Others species used more experimentally include: *Pavlova* spp. (CS63, CS50, CSIRO Catalogue codes) and *Chaetoceros* spp. (CS257, CS256). Axenic stock cultures are maintained continuously in 250 ml flasks under 50-80 µmoles photons PAR m^{-2} s^{-1} at 12:12 light: dark cycle. Most micro-algae production for bivalves is conducted in 20 L carboys with capacity for culture in

100 L plastic tubes and 200 L bags. Autoclaved f/2 medium (0.5 µm filtered, UV sterilized seawater) is used for algae production. Production capacity depends upon experiments being undertaken. Standing capacity of algae for bivalve culture is approximately 2,000 L.

For fish: Large scale production of *Nannochloropsis oculata* for rotifer culture and green water culture of fish and crab larvae occurs outdoors and is produced using a commercially manufactured liquid plant fertiliser and natural sunlight.

Hygiene

Foot baths are provided at the entrance to hatcheries and algal culture rooms; gum boots, and use of ethanol sprays and hand washing are required for entry into all indoor production facilities.

Relevant R&D Activity at JCU (researcher) [projected outcomes]

- Pearl Resource Development in Pacific Islands; *ACIAR* 1993-2005 (Southgate)[Towards cultured pearl industries in Pacific Islands]
- Replacement of *Artemia* with artificial diets; *FRDC* - 2004 (Southgate)[reduce reliance on live feeds]
- Development of pearl resources in Tanzania; *WWF* - 2004 (Southgate)[develop technology for pearl oyster culture]
- JCU mud crab project; *ACIAR* 2004-2005 (Zeng)[reduce rates of cannibalism]
- Mud crab mass larval culture techniques and applying biotechnology to improve production; *China 863 [National High-Tech R&D]* 2004-2005 (Zeng)[development of protocols for commercial scale production]
- Selection of pearl oyster broodstock; *JCU internal grant* - 2004 (Southgate)[improving pearl quality through broodstock selection]
- Triploidy induction in pearl oysters and mud crabs; *JCU internal grant* - 2004 (Southgate, Zeng & Jerry)[improved growth rates and pearl quality}
- Metamorphosis cues in mud crabs; *JCU internal grant* -2004 (de Nys & Zeng)[accelerate, synchronize and enhance settlement]
- DNA pedigreeing *P. monodon*; *JCU internal grant* -2004 (Jerry)[improved selection of broodstock lines]
- Feed technology for temperate fish species; Aquafin CRC PhD project supervision 2005-2008 (Pankhurst and Southgate in conjunction with NSW Department of Primary Industries , Port Stephens Fisheries Centre)[improvement of intensive rearing protocols of larval and juvenile temperate fish species; Mulloway *(Argyrosomus japonicus)* and yellowtail kingfish *(Seriola lalandi lalandi)]*
- Improving growth and survival of cultured marine larvae striped trumpeter: a test case for Tasmania; Aquafin CRC PhD project supervision 2005 (Pankhurst in conjunction with TAFI, Marine Research Laboratories)[optimizing prey consumption through manipulation of the culture environment (green water, light, turbulence)]

Ned and Tish Pankhurst joined the staff at JCU in February 2004, bringing expertise in the areas of finfish larval culture (Tish) and broodstock manipulation (Ned), diversifying the current significant research strength in relation to bivalve and crustacean hatchery production and technologies.

Specific problems and issues

1. **Ornamental shrimp:** prolonged larval duration, undefined mortality at approximately 80 days.
2. **Mud crab and sand crab:** high cannibalism rate from the megalopa stage onwards.

3. **Mud crab:** significant variations in larval quality upon hatching; '*Moult Death Syndrome*' at later larval stages and still need to use antibiotics for the first day of stocking.

Collaboration

JCU hatchery researchers have close links to industry and other researchers both nationally and internationally: TAFI-University of Tasmania, Marine Research Laboratories (Dr Stephen Battaglene) and School of Aquaculture (Dr Marianne Watts, Dr Mark Porter); NSW Department of Primary Industries, Port Stephens Research Centre (Dr Stewart Fielder, Dr Geoff Allan); Fisheries Western Australia (Dr Sagiv Kolkovski); Salmon Enterprises of Tasmania [SALTAS] (Dr Harry King); Blue water Barramundi (Mr David Borgelt); Good Fortune Bay Fisheries (Dr Trevor Anderson); AIMS (Mr Matthew Kenway); QDPI, Northern Fisheries Centre (Dr Mike Rimmer, Ms Liz Cox); Secretariat of the Pacific Community, Noumea (SPC) (Mr Ben Ponia); Xiamen University, China (Prof. LI and Wang); Sematan Fisheries Centre, Sarawa, Malaysia (Dr. Abdullah); Links with Fisheries Divisions in Pacific islands (Kiribati, Fiji, Tonga, Solomon Islands) with CIBNOR and UABCS in Mexico, with SEAFDEC (Phillipines) and Worldfish (Noumea/Penang).

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Applied research and training – the Aquaculture Development Unit approach

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Abstract

The Aquaculture Development Unit (ADU) of the WA Maritime Training Centre (WAMTC), Challenger TAFE is a world-class facility that undertakes applied aquaculture R&D as well as VET aquaculture training activities for marine finfish and other species. The ADU was established in 1992 to stimulate the development of marine aquaculture in Western Australia. A large-scale marine hatchery was established; species selected for commercial aquaculture; grant funding secured to undertake the applied research leading to the development of production technology for the selected species and technology transferred from the researchers to industry.

Projects that the ADU have completed or are currently running include:

Applied research

- Development of commercial production techniques for the culture of snapper and black bream;
- Development of production techniques for the culture of WA dhufish (FRDC) and King George whiting (WA Minister of Fisheries funded);
- Restocking of black bream into the Swan River (1995, 1997) and Blackwood River Estuary (2000 – 2004). Funded by the FRDC;
- Growout of snapper in a recirculation system (2001-current). Industry funded;
- Development of spawning and culture techniques for the abalone *Haliotis scallaris*. Funded by the Aquaculture Development Council (ADC) and industry;
- Demonstration of nutrient stripping from aquaculture wastewater using the sea-lettuce and feeding to abalone. Funded by the Natural Heritage Trust;
- Applied research into fish species and aquaculture systems suitable for use in inland saline water in the WA Wheatbelt (TAFEWA, FRDC, NAC, AusIndustry, AFFA and funded).

Industry Extension

- Publication of a hatchery manual for production of black bream (1999);
- Publication of a hatchery manual in text and CDROM formats for snapper and black bream (2003). Funded by the FRDC;
- Numerous Seminars and Workshops on aquaculture topics;

Training

- Development of a marine prawn culture Unit of Competency under the National Seafood Training Package (2000). Funded by the WA DOT's Science and Technology Innovation Strategy;
- Development of a Unit of Competency for the Environmental Management of Marine Finfish Growout Operations under the National Seafood Training Package (2003). Funded by the WA DOT's Science and Technology Innovation Strategy;

- Provision of a short course for the culture of temperate marine finfish that provides training for industry and research personnel in 'best-practice' culture methods for hatchery operations.
- A Graduate Diploma in Marine Fish Hatchery Management. This course is similar to a medical 'internship' where students qualified in the theory of aquaculture will further develop and extend their practical skills and knowledge. This course can only be effectively taught in a commercial-scale marine finfish hatchery such as at the ADU where VET can be combined with industry scale activities.

The ADU is a unique 'experiment' in the VET field: a successful combination of applied research, development, extension and VET training for the WA industry. This paper details some of these activities; and highlights some of the problems that could be expected in the VET sector in transferring new technologies to industry based on our experiences over the past ten years.

Key words: finfish, aquaculture, training, Western AustraliaBackground

The Aquaculture Development Unit (ADU) of the Western Australian Maritime Training Centre (WAMTC), Challenger TAFE is a world-class facility undertaking applied aquaculture R&D associated with VET aquaculture activities for marine finfish and other species. The ADU was established in 1992 with the charter to:

- 'Conduct applied research and development projects to stimulate development of marine aquaculture industries in WA'; and
- 'Transfer developed technologies, knowledge and skills to industry.'

During 1992, the then Chief Executive Officer of the WA Department of Training began implementing a policy of associating colleges closer to industry. This included the provision of facilities to encourage industry groups to participate in applied research projects and joint ventures. Aquaculture, as the fastest growing primary industry sector in Australia, was identified as an area of need and in 1993, funding was allocated to formally establish the Aquaculture Development Unit (ADU) at WAMTC. It should be noted that the WAMTC has been conducting aquaculture courses in Fremantle since 1989.

The WAMTC is located within the Fremantle Port Authority area on the foreshore between the entrance of the Swan River to the north and Bathers Beach to the south (See Figure 1). The majority of the buildings in which it is located were constructed during the Second World War for Navy support purposes. The WAMTC moved into the buildings in 1988 with a 20-year lease on the premises. The WAMTC has been providing aquaculture courses to Western Australia since 1987 and is the Australian VET pioneer in this area of training.

Figure 1. Aerial photograph showing the WA Maritime Training Centre, Fremantle.

A capital works program of over $2 million was initiated in 1993 to upgrade facilities to provide for the establishment of the Aquaculture Development Unit. An ocean intake, capable of supplying up to five million litres of filtered seawater per day, was installed and commissioned in September 1993. An enclosed area for aquaculture R&D was developed and reticulated with air and seawater. During 1996 two seawater bores were commissioned, supplying filtered seawater to the hatchery. The ocean intake was abandoned at this time, having been technically and operationally problematic and expensive to run and maintain.

The substantial ADU marine hatchery was established and key staff involved themselves in relevant industry associations. The ADU operates with a range of facilities (See Figure 2) and equipment which includes:

- 2000 square metres of enclosed area reticulated with air and ocean water
- 2 saltwater bores supplying 20 litres per second of seawater each
- Hatchery laboratories, aquarium and live food culture rooms
- Controlled environment (photo therm) rooms
- Experimental feed mill facility and testing unit in collaboration with Fisheries
- Quarantine facility

Figure 2. A sample of ADU facilities.

Other specialised equipment at the WA Maritime Training Centre – Fremantle include:

- 7 boats, including the Maritime Image, a purpose built 18.5 metre training vessel;
- Radar and Automatic Radar Plotting Aid Simulator;
- Global Maritime Distress and Safety System simulator;
- Stability Laboratory;

- Global Positioning Systems survival and fire fighting facilities;
- Well appointed air-conditioned classrooms including a computer laboratory;
- Standardised electrical/electronic and hydraulics workshops and laboratories;
- Dedicated maritime library;
- Access to international vessels to assist training;
- Multi media training programs at the Submarine Systems Centre at HMAS Stirling on Garden Island.

Species were selected for commercial aquaculture in conjunction with industry; grant funding was secured to undertake the identified applied research; production technology was developed for the selected species; and specialised training courses were developed to transfer technology from the researchers to industry.

The ADU's research and development work involves it with industry and other research groups, including universities, in a range of projects that benefit industry and the community. The ADU has strong cross-sectorial collaborative arrangements and has a history of successful joint aquaculture projects with Murdoch University, the WA Department of Fisheries and the WA Department of Agriculture as well as with private Western Australian companies. Additionally the ADU provides access to its facilities for these agencies in collaborative projects. Several University students have used the ADU facilities for their post-graduate studies, including for PhD studies in fish genetics, nutrition and molecular biology. Co-location of other government agencies is another strategy the ADU has successfully adopted, ensuring that expensive facilities, equipment and other resources are not duplicated. The Department of Fisheries mariculture section is co-located on the ADU premises and the Murdoch University Centre for Fish Research is co-located on the Fremantle campus. The ADU is an excellent example of leading edge research and development being achieved through collaborative partnerships between education and training, other government agencies and the private sector.

Formal feedback from the participants of the ADU short courses and seminars, as well as from the general industry, demonstrates the effectiveness of the ADU. The ADU rapidly developed a reputation for relevance, effectiveness and efficiency among its clientele. This clientele includes school and University students.

The ADU runs as a business unit and is responsible for generating its own operating budget each year to fund its activities on behalf of industry. A Department of Training grant provides funds towards the ADU staffing and Challenger TAFE provides the facility and power costs. The ADU must generate funds for its activities and additional operational costs. These funds are generated through a diverse range of activities including competitive grants, contracts with private companies, fee-for-service training activities and sale of by-products of training such as fish, live feeds, and publications. The need for the ADU to generate substantial funds each year to provide for its activities ensures that it remains closely aligned with the industry and its needs, and operates in an efficient manner. The business unit is recognised in Australia as the leading organisation extending results of marine finfish research to industry through hands-on training. Its close collaboration with industry and joint approaches to research and other projects ensures the highest standards of customer service.

The history for Challenger TAFE's entry into applied aquaculture research could be seen as 'filling the gap' in the existing market. But, as a sector, VET has much to offer in applied research and should be involved in models of this kind, and not only in areas where no-one else has put in a claim. There are sure to be many models, including Centres of Excellence and Special Research Centres where TAFE could play a legitimate role.

The Systems, Capacity, Species and Projects

The ADU have 5 dedicated broodstock holding tanks, two of 40 tonne capacity with phototherm manipulation capability, one of 40 tonne without phototherm capability, and two of 10 tonne capacity, both with photoperiod capability and one with and one without thermal manipulation capability. Other tanks of sizes ranging from 10 to 25 tonne capacity are utilised for occasional broodstock purposes as required.

The ADU utilises three separate larviculture systems of marine fish. The first is comprised of 9 x 5,000L tanks,

the second of 6 x 1,000 L and the third (which is a joint WA Department of Fisheries and ADU unit) of 24 X 100 L tanks. All systems contain cylindroconical fibreglass tanks. The first two systems will be described here. Dr Sagiv Kolkovski of the WA Department of Fisheries will describe the joint larviculture facility and its capacity in a separate paper.

Figure 3. ADU 5 tonne larval tanks.

The ADU has continuous production of algae and rotifers throughout the year, with a capability of producing a maximum of 0.5 billion rotifers per day utilising the current production systems. This quantity of rotifers will support 4 of the 5 tonne larval tanks at any one time.

The ADU operates it's arrays of 5 tonne and 1 tonne larviculture tanks on a semi – intensive green-water basis (See Figure 3). This entails static green-water culture for the rotifer stage followed by flow-through for the *Artemia* feeding stage and beyond.

The ADU operates the hatchery on a high volume flow-through basis. The ADU draws its water supply from the two salt-water bores which are located at 18 metres depth and approximately 10 metres inland from the Bathers Beach side of the South Mole (see Figure 4). This water supply is free of contaminants and sediments as it is substrate-filtered to approximately 10 microns. Despite an intake water pH of approximately 6.8 due to the limestone substrate, the water is of a sufficiently high standard to raise the larval stages of marine fish and abalone.

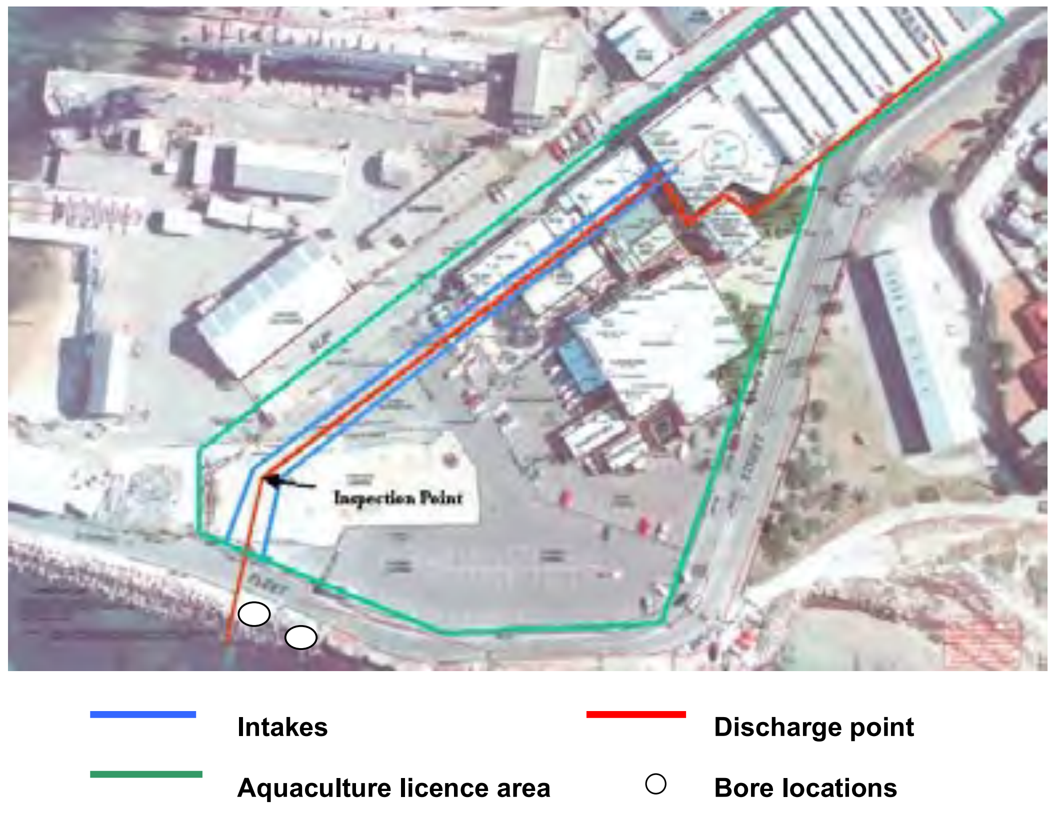

Figure 4. Plan showing location of the ADU, two seawater bores and discharge point at Bathers Beach, Fremantle, WA.

The 5 tonne larval tanks are contained within rooms comprised of black/white 'hydroponics' plastic sheeting (Figure 3). Each of the 5 tonne tanks has lighting above comprised of dual 36 Watt fluorescent (cool white) lights and single 400 Watt Metal Halide lights. The 1 tonne tanks have single 36 Watt fluorescent lights. Lighting systems have an auto-dimmer function. Banjo outflow screens retain the larvae.

The maximum manageable stocking rates in these tanks is 100 newly hatched larvae per litre. This stocking density enables minimal input during the rotifer feeding stages and results in excellent survival. The larviculture techniques are fully described in Partridge et. al. 2003 (See Figure 5).

The production capacity of these tanks is defined by the capability of the ADU staff to maximise the survival rates of the species under culture. Typical survival rates from newly hatched larvae to weaned juveniles are 80-90 % for black bream and 30-55 % for snapper. There have been insufficient opportunities to culture the yellowtail kingfish and therefore survival rates have not been ascertained in these two systems.

During weaning, and as the juveniles grow, additional space is required to maximise survival. Numerous tanks of sizes to 25 tonne capacity (6) are available for this purpose.

The ADU is permitted within its Act to function as a 'pilot' commercial hatchery for a discreet period of time if required by industry. However, one of the limitations of the Fremantle site for commercial production is a restriction placed on the ADU's Department of Environmental Protection licence to hold no more than 5 tonnes of fish at any one time. This certainly limits the facilities capacity as a growout site, but does not limit it's capacity to be a short-term hatchery for a commercial industry with juveniles exiting at 5 grams. This restriction, in combination with two major (or more smaller) larval runs per annum would enable the facility to produce a theoretical maximum of 1.8 million 5 gram juveniles per annum. This capacity however, would require a regular egg supply and an upgraded ADU rotifer production, something that could be achieved relatively easily with the advanced rotifer culture systems currently available.

Research is currently underway or completed at the ADU for a range of species for aquaculture in Western Australia including the following:

Snapper (*Pagrus auratus*)

The ADU and NSW Fisheries are the pioneers for the culture of snapper in Australia. The ADU recently published the 'Hatchery Manual for the Production of Snapper (*Pagrus auratus*)' for the Australian aquaculture industry. This manual and its accompanying CD-ROM multimedia presentation is the first in Australia to describe the techniques required for the culture of this marine fish species and follows on from the very successful publication in 1999 of the ADU manual for black bream. The project was undertaken in collaboration with

NSW Fisheries and the CRC for Aquaculture.

Black bream (*Acanthopagrus butcheri*)

The ADU are the recognised Australian experts for the culture and restocking of the estuarine Australian species the black bream. The ADU (in collaboration with Murdoch University and the Molloy Island Residents Group) are currently undertaking a program to restock the Blackwood River Estuary in the South West of WA with black bream. The Blackwood River has a well-documented stock decline of black bream and the project has so far been very successful. Over 200,000 fish have been restocked with very good survival. The fish will be monitored until 2005.

WA Dhufish (*Glaucosoma hebraicum*)

The ADU pioneered the techniques required to culture this deepwater fish species. Although the WA Dhufish has a single haemoglobin type that restricts its commercial aquaculture potential, the techniques developed are suitable for future restocking projects for the species.

Yellowtail Kingfish (*Seriola lalandi*)

The yellowtail kingfish is a fast growing marine species that is popular in the eastern states of Australia and

overseas. The ADU has captured specimens of this species and is growing them for brood stock purposes in collaboration with the WA Department of Fisheries.

Mulloway (*Argyrosomus japonicus*)

The mulloway is a fish that tolerates a wide range of salinities and grows rapidly. This species is targeted by the ADU for inland saline aquaculture for both commercial and recreational opportunities. The ADU has broodstock and is testing various attributes of the juveniles for their commercial culture prospects in inland saline areas.

Roes and Staircase Abalone (*Haliotis roei* and *H scalaris*)

These are temperate abalone species with aquaculture potential in the warmer waters of the west coast of Western Australia. Research at the ADU has developed culture techniques for the Staircase abalone and highlights its potential for commercial development.

Projects that the ADU have completed or are currently running include:

Training

Curriculum Development

- Development of a marine prawn culture Unit of Competency under the National Seafood Training Package (2000). Funded by the WA Department of Training's Science and Technology Innovation Strategy, this project developed a best-practice VET training course for marine prawn aquaculture in Australia, an industry worth over $50 million dollars in 2001. The project was also successful in establishing prawn VET training infrastructure in WA (at Kimberley TAFE) and in growing the first marine prawn juveniles in WA with the assistance of VET students (Jenkins 2001).

- Development of a Unit of Competency for the 'Environmental Management of Marine Finfish Growout Operations' under the National Seafood Training Package (2003). Funded by the WA Department of Training's Science and Technology Innovation Strategy; this project developed a VET course for the environmental management of the growout of marine finfish in WA and is supported by the MG Kailis Group of Companies, the Conservation Council of WA and the Esperance Marine Institute. The project involved identifying and documenting environmentally sustainable national and international aquaculture best practices for marine fish farming. The project also established guidelines in conjunction with industry, conservation groups and the community and set the standards for the future development of the industry in Western Australia in an ecologically sustainable manner (Jenkins 2003).

Training Delivery

- Provision of a short course for the culture of temperate marine finfish providing training for industry and research personnel in culture methods for hatchery operations. This is a direct transfer of knowledge and skills from the ADU staff to industry. The one-week course can be delivered on demand as the ADU have spawning fish available throughout the year.

- A Graduate Diploma in Marine Fish Hatchery Management. This course is unique in Australia, as it combines research and coursework at a Graduate Diploma Level AQF VII, in a workplace environment, providing hands-on, practical training in a fully functioning marine hatchery. This course provides students with skills and knowledge required for hands-on managers in the marine hatchery field. Prerequisites for this course are a University Degree in aquaculture or closely related science or a Diploma of Aquaculture with substantial industry experience (or skills recognition).

Industry Training and Extension

- Publication of a Hatchery Manual for production of black bream (Jenkins et al 1999);

- Publication of a 'Hatchery Manual for the Production of Snapper (*Pagrus auratus*) and Black Bream (*Acanthopagrus butcheri*)' for the Australian aquaculture industry. This manual and accompanying CD-ROM multimedia presentation is the first in Australia to describe the techniques required for the culture of this marine fish species and follows on from the very successful publication in 1999 of the ADU Hatchery Manual for Black Bream. The project was undertaken in collaboration with NSW Fisheries and the CRC for Aquaculture with funding support from the FRDC (Partridge et al, 2003). See Figure 5.
- Completion of an AusIndustry project with McRobert Aquaculture Systems (MAS) to develop a novel new tank system for the culture of fish. The company was successful in winning a R&D Start grant from AusIndustry during the latter part of 2000 and subcontracted the ADU to trial the grow-out of snapper in the MAS system. MAS have subsequently constructed a $400,000 system in the USA where significant interest exists in this novel Australian technology.
- The ADU has been undertaking a marketing trial for aquaculture snapper for the hospitality industry over the past 24 months. This project involves growing and selling snapper to a local restaurant chain to test the interest in premium aquaculture fish. These fish have been well received and this trial is to continue into 2003.
- The ADU has several areas available for business start-up opportunities by private companies. There are currently several projects being undertaken by private companies at the ADU including the culture of marine aquarium fish and the trial culture of new abalone species for aquaculture.

Figure 5. ADU Hatchery Manual 2003

- Numerous user-pay courses and seminars have been run by the ADU in recent years. These included the ADU short course for marine fish culture, a Murray cod aquaculture seminar, hosting a Victorian Fisheries researcher presenting the most recent interstate data for the industry, and two Recirculation Aquaculture Technology Workshops (2000 and 2003) run by Professor Thomas Losordo, a recognised US world expert.

- Key ADU staff were also instrumental during 2002 in the organisation and running of the Marine Fishfarmers Association's 'Sustainable Development of Marine Fishfarming Workshop' that was held on October 29, 2002. This project was related to the ADU's Science and Technology course development project and was successful in securing the world renown conservationist, **Dr David Suzuki** as the key-note speaker at the event. The event was hailed as very successful by industry, government and environmental groups and Dr Suzuki was very complimentary of the aims of the Workshop. (Jenkins 2003). See Figure 6.

herald earthcare

Seeking common ground

EARTHCARE with ADRIAN KWINTOWSKI

ONE of the world's best-known environmental campaigners, David Suzuki, paid a brief visit to the new maritime museum in Freo last Tuesday to speak at a sustainable fish farming forum.

The event was organised by the marine fish farmers association and the WA conservation council in an effort to set out parameters for a sustainable commercial fish farming industry.

Organisers also hoped to build trust between the industry and conservation groups.

Before the talkfest got underway, Dr Suzuki told the *Herald* the concept was a refreshing approach in an industry which, in other parts of the world, had often put profits before environment safeguards.

"I can't emphasise enough how wise this is," Dr Suzuki said. He said in Europe the industry took on a "gold rush mentality" and only became concerned when depleting stocks and increase in disease began to impact upon business.

But he said it wasn't just the business sector that had a duty of care.

"What astounds me is that the consumer is so uncritical," Dr Suzuki said.

WA had a unique opportunity to set up a sustainable marine fish farming culture before mismanagement, spurred by greed, resulted in fish losses in farms and in the wild.

The seasoned environmental campaigner of 40 years says achieving a common ground was better than winning the war with greedy developers.

"In all these battles there's always going to be a loser," he said.

"We can't afford it now. We've got to stop demonising and work together."

His aim is "the development of trust" between the aquaculture industry and conservationists.

Other speakers at the forum included WA fisheries department chief Peter Rogers and Notre Dame University management professor and MG Kailis director George Kailis.

Dr Suzuki dodged questions from the *Herald* on his thoughts about the Ningaloo Reef marina proposal, which last week was deemed inappropriate in its present form by the WA environmental protection authority.

"I don't know what's going on," he replied, stating he could not be an expert on everything.

• David Suzuki reflects on sustainable marine farming in Freo.

Fremantle Herald, Saturday November 2, 2002

Figure 6. **MFA Sustainable Aquaculture Workshop press**

- Direct assistance was provided by the ADU to several local companies in WA during 2003/04, e.g. maintaining barramundi brood-stock for a private company and live abalone export trials from the ADU premises.

- The ADU Manager is Chair of the WA Sustainable Development of Marine Fishfarming Forum; Vice-Chair of the WA Fish Foundation; Secretary/Treasurer of the WA Marine Fish-farming Association; a Board Member of the WA Fisheries Research Advisory Board and the Aquaculture Council of WA and a member of the National Steering Committee for Inland Saline Aquaculture.

Applied Research

- Development of commercial production techniques for the culture of snapper and black bream (Partridge, & Jenkins, 2002 - a&b, Boarder, S. J & Partridge, G. J. in press);
- Development of experimental production techniques for the culture of WA dhufish (funded by the Fisheries Research and Development Corporation - FRDC) (Cleary and Jenkins 2003): and King George whiting (WA Minister of Fisheries funded).
- Growout of snapper in a recirculation system (2001-current). Industry funded; (Partridge - in press)
- Development of spawning and culture techniques for the abalone *Haliotis scalaris*. Funded jointly by the WA Aquaculture Development Council (ADC) and industry;
- Demonstration of nutrient stripping from aquaculture wastewater using the sea-lettuce and feeding to abalone. Funded by the Natural Heritage Trust; (Boarder 1998, 2000).
- Factors required for the successful aquaculture of black bream for recreational fishing in inland water bodies. This study investigated the requirements to grow-out black-bream, King George whiting and snapper in saline ponds at Northam, WA, and was run in collaboration with Murdoch University and a private company. (Sarre et al 2003), (Partridge et al in press).
- Genetic improvement of black bream. (Doupe et al, in press). Mr Doupe is completing his PhD studies from Murdoch University at the ADU facilities.
- Following on from an AusIndustry-funded study tour of inland saline aquaculture projects in the USA, the ADU and partner, CY O'Connor College of TAFE, was successful in securing a grant from the Science and Technology Innovation Strategy Fund to undertake an Inland Saline Aquaculture Demonstration Farm project in Northam. This project has been further supported by Commonwealth funding through the National Aquaculture Council and the FRDC. This project is utilizing new technology (Semi Intensive Floating Tank System – SIFTS)jointly developed by the ADU and a private company. A sharing agreement for the IP rights to this technology have been negotiated. See Figure 7.

Figure 7. Gavin Partridge of the ADU and Dr Gavin Sarre of CY O'Connor College of TAFE at the ISA Demonstration site.

- The ADU scientist, Mr Gavin Partridge won a Commonwealth Agriculture, Forestry and Fisheries Australia (AFFA) young scientist award during the latter part of 2002 associated with this work. A small grant accompanied this prestigious award and Gavin is investigating the suitability for aquaculture of a CALM pumping site near Narrogin. A related project is also being undertaken with the Dumbleyung Shire (Partridge & Furey, 2002). AusIndustry also provided funding to the ADU in 2002 to investigate the relevance of inland saline aquaculture projects in the USA to the WA Wheatbelt.

- The ADU (in collaboration with Murdoch University and the Augusta community) are leading a Fisheries Research and Development Corporation (FRDC) grant to restock the Blackwood River Estuary near Augusta in the South West of WA with black bream (Jenkins et. al. 2000). This three-year project has been extended for a further year by the FRDC due to the outstanding success of the restocking (due for completion in June 2005). The project has undertaken a stock assessment of black bream in the Blackwood River, has restocked 222,000 fish and is currently monitoring the survival and growth of the fish.
- Indications from the WA Dhufish broodstock capture program were that individuals of the species caught in over 20 metres water depth and released do not survive. Subsequent research by the ADU examined the effects of decompression sickness and focused on the immediate damage sustained by the fish upon capture. Since the majority of WA Dhufish caught by recreational and commercial fishermen are captured at depths of greater than 20 metres, this finding has major implications for the management of deepwater commercial and recreational fisheries.

The Highlights of Technology Transfer

To facilitate technology transfer to industry, the ADU has developed two very specific training courses, both of which are the only training programs of their type in Australia. The first is an intensive, marine finfish hatchery-training course. This one-week intensive course provides 'hands-on' training in all aspects of hatchery technology for temperate marine finfish. Student numbers are restricted to eight to ensure maximum benefit of the 'hands-on' nature of the training. The fish species of interest are the black bream (*Acanthopagrus butcheri*) and the pink snapper (*Pagrus auratus)*.

All students receive a CD-ROM copy of the ADU's hatchery manual for snapper and bream prior to arriving at the course to ensure that they have a basic understanding of the process of marine finfish culture. The number of formal lectures are limited to five during the week with the majority of each day spent in the hatchery where techniques for handling and culture of marine fin-fish are undertaken by each student in commercial-scale systems. By the completion of the course each student has gained skills and experience in the set-up and management of an aquaculture hatchery for temperate marine fish.

The second unique course offered by the ADU is the 'Graduate Diploma in Marine finfish Hatchery Management'. The Graduate Diploma has been designed to address specific training needs identified in the finfish industry and is aimed at students holding a Diploma or Bachelors degree in aquaculture or closely related fields. The course aims to turn theory into successful practice to benefit Australia's rapidly growing aquaculture industry. Similar to a medical internship where doctors spend a year in a public hospital, this course can only be effectively taught in a commercial-scale marine finfish hatchery such as at the ADU where VET can be combined with industry scale activities.

The Graduate Diploma is a highly targeted specialist 12 month long course that offers no more than four student places per year with two intakes per annum. On completion, these students are sought after by industry due in part, to the reputation of the ADU for R&D in conjunction with training.

The Problems of Technology Transfer

The ADU has undertaken many important projects to implement the research and development component of its charter. Advances are irrelevant however, if the new technology is not recognised by and integrated into the industry.

The successful transfer of technology from researchers to industry is a key component of industry development. Impediments to successful technology transfer from researchers to industry include:

- inadequate communication between government agencies and industry to develop effective extension services to the industry, ie to determine what form of training the industry requires;
- the focus of many researchers and agencies on the publication of papers and the consequent lack of experience in more direct technology transfer to industry; and

- the lack of recognition of expertise held in government or quasi-government agencies. This is linked with both the low regard of government employees held by some industry sectors and the often inaccurate perception, that the best expertise is always to be found interstate or overseas.

Collaboration and cooperation, rather than competition, between government agencies and between government and industry are required to ensure that effective technology transfer occurs. Collaboration between TAFE and other agencies is a very effective method of technology transfer to industry. TAFE is a traditional training provider in Australia and has strong links with industry. Challenger TAFE in Fremantle, Kimberley College of TAFE in Broome and Central West TAFE in Geraldton have extended marine aquaculture facilities. This enables effective collaborative applied aquaculture research to be undertaken in these locations. Other TAFE Colleges in WA have forged links with research organisations in order to act as conduits for up-to-date aquaculture technology transfer.

The ADU has developed collaborative research arrangements with Fisheries WA, NSW Fisheries, Agriculture WA, three WA Universities, the National Centre for Mariculture in Israel and numerous private companies. This collaboration has led to the development of synergies that have fast-tracked the applied research progress.

The ADU has also established effective working relationships with the Conservation Council of WA, and is working hand-in-hand to ensure sustainable practices are encouraged and that suitable training programs are available for the industry and the community.

While collaboration and cooperation are important, trust is the key value that is required between government and government and government and industry proponents to develop a new industry. Without trust then it is likely that working relationships will break down and the various proponents and service providers will become frustrated as the development process slows.

Summary

The ADU is a unique 'experiment' in the VET field: a successful combination of applied research, development, extension and VET training for the WA industry. The ground-breaking work of the ADU has been recognised by the industry and by government in recent years:

- In 1997 the ADU was awarded the Premiers Award in the category "Provision for the Future' for its restocking vision in WA;
- In 1999, the ADU was presented with the "Innovation and Development Award for the Community and Industry" as part of the Fisheries Reward and Recognition Program and for "….leadership in practical development of aquaculture species for the Australian market.";
- In 2002, the ADU was a finalist in the WA Premiers Award for 'Environmental Sustainability';
- In 2003, Gavin Partridge, the Aquaculture Development Unit, Challenger TAFE aquaculture scientist, was presented with a Rural Industries Research and Development Corporation 'Science and Innovation Award' from the Department of Agriculture, Fisheries and Forestry – Australia (AFFA). Senator Hon. Ian Macdonald, Minister for Forestry and Conservation, presented the award to Gavin Partridge; and
- The recognition of the TAFE sectors role in Inland Saline Aquaculture Development with the FRDC/NAC funding of the ISA Demonstration Farm in Northam, WA.

The ADU enjoys the wide support and involvement from both the private sector and Government because it provides a focused and targeted approach to applied research and development that, with the direct involvement of industry, is delivering solutions and innovations that have real commercial potential in Western Australia. The fact that the ADU must generate all of its own expenditure ensures it operates in an extremely prudent manner financially and operationally with a keen focus on the future of the industry.

The key to the success of the ADU has been both the focus on the R&D needs of the industry and its associated training needs. The ongoing success of the ADU will continue to be determined by the industry with external funding becoming more important for its continuing operation.

The economic and employment potential of aquaculture in Western Australia is enormous and the ADU, through its training and research programs, is helping establish this emerging and new industry in WA. The future benefit to the aquaculture industry and the Western Australian economy will far out-weigh the investment in the ADU, making it both cost effective and value for money.

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General

The Mariculture Research and Advisory Group (MRAG) is located at the maritime centre run by Challenger TAFE at Fremantle port. Both agencies, TAFE and the Department of Fisheries (DoFWA), have an agreement where many of the R&D activities are on a collaborative basis.

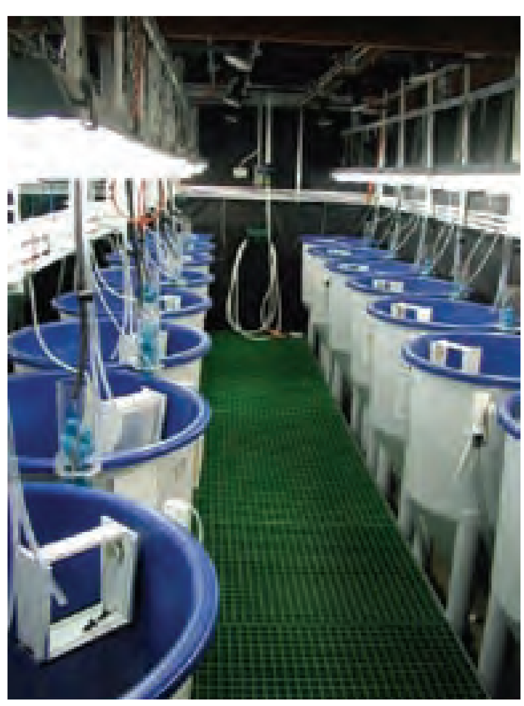
The MRAG includes three major activity centres: finfish, nutrition and abalone. The nutrition activity mainly involves fishmeal replacement (lupin) and environmental impacts of feeds and feeding. The abalone research focuses on improving hatchery and nursery production, and has two main objectives:

- To improve spawning success and 'seed' (settled larvae) performance when conditioned abalone broodstock are used
- To evaluate alternative feeds for the nursery phase when the abalone are between 5 and 15 mm in length.

The finfish group has three main areas of R&D:

1. Systems design and development,
2. *Artemia* replacements, i.e. microdiets development, co-feeding and weaning protocols, and
3. *Artemia* production.

The MRAG activities are strongly linked to industry and all R&D projects are jointly funded with industry.



Finfish Section

Systems

The group has strong technical background that helps with the design of rearing systems and associated equipment.

Larvae system

An experimental larval rearing system was developed to reduce variability amongst tanks (due to manual feeding and other parameters) and enhance control of environmental parameters while reducing the workload. The system includes twenty-four 270 l conical tanks with the option of either an upwelling or bottom-draining flowthrough water delivery system. The inlet water passes through a gas exchange column that saturates the water with dissolved oxygen and stabilizes the pH. The system

was originally designed for nutritional experiments using formulated feeds. The use of an upwelling water inlet method extends the suspension time of inert particles in the water column and also helps to suspend very small or passive swimming larvae. However, when the system is used to grow-on larvae or juvenile fish, it can easily be switched to bottom draining to provide self-cleaning water dynamics for high organic loads.

Although the system was built as an experimental system with relatively small volume tanks (270 l), it can easily produce commercial numbers of fingerlings and can be 'up-scaled' to increase production.

Outlet filter

A unique outlet filter was developed that eases the daily routine of replacing screens when enriched live food is used. This filter can be exchanged with a screened standpipe and outlet surface skimmer when the bottom draining flow characteristics are engaged.

Automatic Control

The system is fully controlled by a single programmable logic controller (PLC). The PLC controls the light intensity, photoperiod, dimming time, live food and algae pumping intervals, substantially reducing labour requirements.

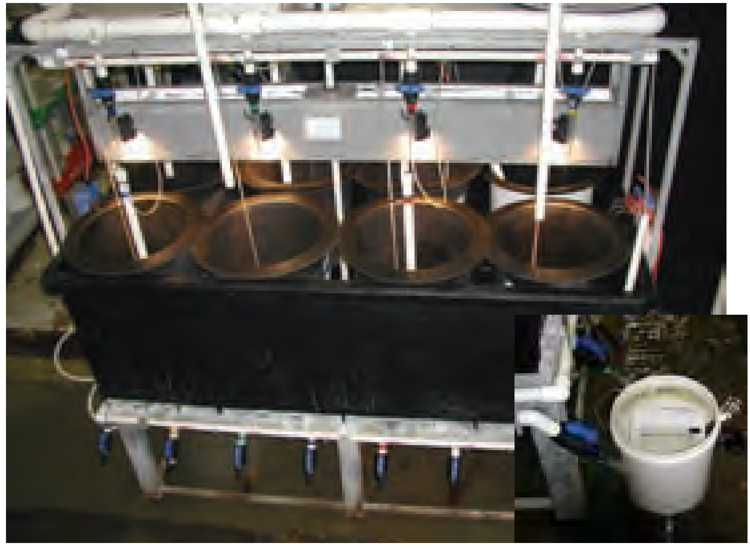
Automatic Feeding System

Automated Microdiet Dispensers (AMD) are installed in a 24 tank experimental system in order to feed up to 8 different experimental or commercial micro diets (MDs) for larval weaning and/or nutrition experiments. The feeders can be operated individually or simultaneously via multiple or single outputs from the Programmable Logic Controller (PLC). Specific PLC output programs are directed to operate particular AMDs within each feeding regime, via a network of 8 input – single output rotary switches. This then connects a 12 V DC power supply via a solenoid switch that is operated by the PLC. The PLC, power supplies and switches are all situated within a single control station.

The AMD is designed to periodically administer a small amount of microdiet to larvae culture tanks, in order to spread the allocation of the required daily amount of feed evenly across the whole day. The feeder can cope with a diet particle size range of 150 microns to 1.5 mm.

Live Food system

A simple, compact experimental system was developed in order to provide a reliable platform for nutritional, growth and other experiments involving live food organisms. The system was built as a compact, all-in-one system with eight 50 l conical tanks in a water bath. The system reduces variation between the replicates (tanks) resulting from individual heaters and aeration. It reduces the manpower time through simple procedures for harvesting, washing and refilling all of the tanks synchronously and allows automated addition of enrichments. The system has been



used for a variety of experiments, such as comparing commercial and experimental enrichments, bacterial monitoring and evaluation of different *Artemia* procedures. Enrichments can be added at predetermined times via an automated dosing system.

Other equipment and systems includes an automatic feeder for grow out fish (large particles, crumbled and/ or extruded) using a PLC controller, a filter for *Artemia* ponds cysts, nauplii and adult *Artemia*, and large-scale *Artemia* harvesting devices.

Artemia replacements

Microdiets

Microdiet development comprises all the aspects involved in microdiet utilisation by the larvae, such as physical and chemical properties of particles including leaching and sinking rates, diet technology – microbound and microencapsulation methods, protein sources based on digestion index of the protein, and digestive system and enzyme development of the larvae.

Rearing and weaning protocols

Development of co-feeding and weaning protocols is aimed at reducing the dependency and use of *Artemia*. A variety of microdiets, which are commercially available in Australia, as well as experimental ones, are being tested and compared. Algae, *Nanochloropsis* or algae paste (*Chlorella*) are being used in a 'green water' method. The algae paste was found to be superior to the live algae used in the past both for rotifer enrichment and green water in the larvae tank. Some of the trials are done in collaboration with other R&D centres (Darwin Aquaculture Centre, NT) and commercial companies.

Artemia

Artemia enrichments

Hygiene of *Artemia* and *Artemia* processing (hatching, enriching, harvesting and growout) are being investigated. Commercial products as well as experimental ones are investigated in terms of bacteria loads and levels during the process.

As part of an FRDC project, 'tailor-made' enrichments and broodstock additives are produced for R&D institutes, commercial hatcheries and public aquariums. Lipids, essential fatty acids, vitamins, immune-stimulants and any other nutrient can be manipulated and adjusted to match required levels and ratios and be added to a stable, oil-based emulation. The 'tailor-made' products are produced in collaboration with a private company (Nutra-Kol, Western Australia).

Intensive culture

An experimental system that includes 18 x 165 l heated tanks has been used for the *Artemia* intensive culture. The system can be operated on a static or semi flow-through basis. The system is currently being used to compare inexpensive, locally available, inert feeds as well as algae and algae products (pastes and wastes from algae extraction). A series of experiments will be used to further determine the types and effects of bacteria associated with culture techniques (static or semi flow-through culture)

and feeds (micro-alga, inert diets). With current prices as high as AU$ 600 per kg of live *Artemia,* it is invisaged that these experiments and data collected from them will soon be applied to develop a commercial intensive system.

Large scale Artemia production

As part of an FRDC project, an initial assessment of commercial production of *Artemia* cysts was carried out in Western Australia. Hut Lagoon at Port Gregory, north of Geraldton in WA, is a natural saline lake where the microalga *Dunaliella salina* grows naturally. At high salinity the algae starts to produce beta-carotene. Besides *Dunaliella, Artemia* also grows in the lake. The combination of high salinity and an abundance of food is an ideal situation for *Artemia* production.

During the last two years the Department of Fisheries, WA and Cognis Australia, a company based at Port Gregory which harvests and processes the *Dunaliella* for the food and the pharmaceutical industry, have commenced an assessment of the potential for commercial scale production of *Artemia* cysts.

A pilot scale grow out experiment was carried out using six 22 m^3 plastic-lined ponds. A specific 'in-line' filtration system was developed to separate adult *Artemia*, nauplii and cysts for population control and cyst harvesting. *Dunalliela* harvested from the main algae ponds was used as a food source for the *Artemia*.

During the next three years, DoFWA together with Cognis will continue to develop the commercial production of *Artemia* cysts and biomass as part of a new FRDC project.

Fish species

The larvae system at Challenger TAFE can supply marine bore water at temperatures ranging between 19°C and 28°C using a heater-chiller to heat the water (ambient temperature is 20°C ± 1°C). Therefore, a variety of species from temperate to tropical can be reared. The main species that the group is working with includes, snapper *Pagrus auratus*, yellowtail kingfish *Seriola lalandi* and barramundi *Lates calcarifer*. Coral trout is now under investigation as a new species together with a private company.

Other marine organisms such as western rock lobster *Panulirus Cygnus* and shovel nose lobster are also under investigation. Both larvae stages (mainly nutritional requirements) and juveniles (grow-out) are being looked at. A black tiger prawn (P*enaeus monodon*) farming project is currently conducted using local broodstock in the north of WA (G. Maguire PI).

R&D Collaboration and Links

The marine finfish group at MRAG is strongly linked with industry partners. Currently, R&D collaboration is established through a joint project with Cognis Australia. The group is advising to other commercial companies such as M. G. Kailis and Marine Farms (mahi-mahi farm), and western rock lobster fishing/holding companies.

The group also established research collaborations and links with many of the research centres in Australia including, Darwin Aquaculture Centre, NT, TAFI, Tas, and James Cook University, Qld. Research links through current projects have also been established with overseas centres in Spain, Portugal, Mexico, Japan New Zealand and Malaysia.

Abalone Section

Improving spawning success and seed performance

Successful conditioning and spawning of farm-grown abalone broodstock are crucial for a selective breeding program in Australia. However, large variability in spawning success, hatchability of the eggs and survival of the larvae and juveniles has been observed between batches and hatcheries. It has been reported that farm-grown female abalone, that were fed with commercial formulated feed, spawn less readily and produce eggs of poorer quality than animals collected from the wild. Formulated diets used successfully for growout are not always an adequate diet to maintain captive broodstock that yields viable, high quality eggs and larvae. Our preliminary results indicated that eggs spawned from wild caught females differ from conditioned females that were fed formulated diets for several years, in their fatty acid composition. Eggs derived from wild caught broodstock feeding on mainly red seaweeds showed about twice as much arachidonic acid compared to eggs from conditioned broodstock, which are feeding on formulated feed low in arachidonic acid. Arachidonic acid is a major precursor of prostaglandins, which influence reproduction in molluscs. The question of whether arachidonic acid is essential to abalone reproduction needs to be examined. A broodstock conditioning trial is now underway with wild-caught greenlip abalone (*Haliotis laevigata*) to determine if low levels of a particular fatty acid (arachidonic acid- ARA) affect abalone reproduction. Two levels of ARA enrichment are being tested and compared to a formulated diet without the enrichment (negative control), and a diet of red seaweeds (positive control). All animals were spawned out and induced to spawn again after a conditioning period of 16 weeks. Egg size and colour, fatty acid composition of unfertilised eggs, fertilisation and hatch rate, larval survival, settlement success and post-larval survival are being determined and compared between batches (1 Female x 1 Male) using replicated groups of abalone.

New microalgae suitable as a food source for juvenile abalone

Currently commercial abalone nurseries rely on diatoms as a food source for post larvae and juveniles. When juveniles reach 5 mm in shell length, the volume or composition of the food on the nursery plate becomes inadequate. One new diatom species (*Cocconeis* sp.) has been isolated and maintained in culture. This isolate is about 3-4 times larger than other species previously kept in culture. Alternatively, and in contrast to *Cocconeis*, chain-forming diatoms offer a 3D structure compared to the 2D structure of non-chain forming, prostrate attaching species. This 3D structure may provide a more continuous food source for the juveniles through grazing of the top cells of the chain leaving the remainder of the chain to continue dividing. Only one chain forming species, *Delphineis* sp., showed ability to attach to substrate and only this species was tested further. Scale-up cultures have been successful and feeding trials are conducted comparing the growth of juvenile *H. laevigata* (4 mm in shell length) when feeding on new and old isolates.

New macroalgae suitable as a food source for juvenile abalone

Feeding macroalgal germlings, which can grow on the nursery plates, can provide more biomass and may be an alternative feed for the later stages of the nursery phase. We developed methods for the spore release, attachment and germination of the green alga *Ulva* sp. Ulva germlings are now successfully grown on PVC nursery plates. Feeding trials indicated that *Ulva* sp. germlings might be a suitable and practical additional food source for advanced juveniles in a commercial nursery. Further experiments are planned to investigate the potential of other macroalgal germlings (eg red seaweeds) as potential food sources.

Tropical molluscs

Enhancement of trochus fisheries project is on-going in the north of WA (Broome) using hatchery stock. A small-scale production of juvenile tropical abalone (*H. asinna*) is also carried out.

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Species cultured

Snapper	*Pagrus auratus*
Mulloway	*Agyrosomus japonicus*
King George whiting	*Sillaginodes punctata*
Yellowtail Kingfish	*Seriola lalandi*

Systems

SARDI R&D Hatchery facilities are located at the South Australian Aquatic Science Centre (SAASC), West Beach, SA. This building is 29 x 16m with 4.5m high work areas constructed of 75mm –100mm thick metal clad polystyrene panels within an external frame and roof. R&D and culture systems available include broodstock egg incubation, larval rearing, microalgae and live feed production areas together with an office and laboratory.

Figure 1. SARDI R&D Hatchery at the South Australian Aquatic Science Centre, West Beach

Broodstock

Finfish broodstock holding systems used to support SARDI R&D Hatchery operations include;

1. Outdoor ambient tanks (5 x 40,000L) to provide eggs during the natural spawning season.
2. Indoor tanks (4 x 10,000L and 2 x 22,000L, Figures 1a and 1b) are located within an insulated controlled

environment room to provide eggs at alternated spawning times during the year (Figure 1a and 1b). These broodstock tanks are supplied by recirculated seawater treatment systems that incorporate mechanical and biological filtration, foam fractionation and control of water temperature and light cycle control.

Figure 2 a) Four 10,000L broodstock holding tanks in a controlled environment room within the R&D Hatchery facility at the SAASC. b) Side view of the broodstock tanks, 2 x 10,000L front and centre and a 22,000L tank at rear.

Egg incubation

Eggs collected from tank spawning or manual stripping of broodstock are incubated in a room housing 8 x 220L tanks. Each tank is supplied with 5µm filtered seawater and aeration and has a central 300um screen with internal standpipe. Eggs are stocked at up to 1500 per litre and maintained until larvae can be assessed for quality as determined by hatch rate. Selected batches of larvae are transferred into larval rearing tanks in another room.

Figure 3. Eight 220L egg incubation tanks adjacent to the broodstock room in the SARDI R&D Hatchery.

Larval rearing system

Larval rearing facilities in the SARDI R&D Hatchery are installed as a system of 12 x 1800L fibreglass tanks (Figure 3) supported by 3 recirculated seawater treatments systems (Figure 4). These can be operated as separate systems each supplying 4 tanks, or joined together to provide common water to all tanks. Water treatment systems provide mechanical filtration and biological filtration, with additional foam fractionation and disinfection using ultraviolet irradiation on side streamed plumbing lines. Tanks have been allocated at random to each water treatment system and plumbed accordingly for water supply and return. All tanks are provided with independently switched overhead lighting.

Figure 4. Larval rearing tanks (12 x 1800L) in the SARDI R&D Hatchery.

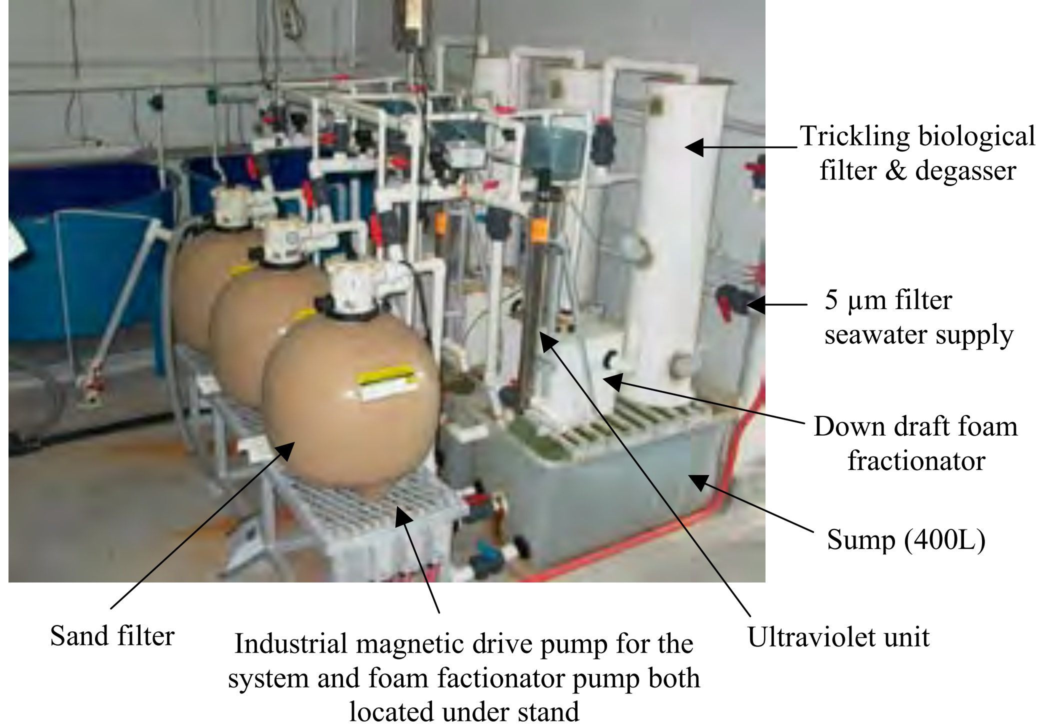

Figure 5. Components of the recirculated seawater treatment systems supplying the experimental larval rearing tanks in the SARDI R&D Hatchery.

Live foods used

Rotifers and *Artemia* are the only live prey organisms cultured for marine finfish larval rearing research or production in the SARDI R&D Hatchery. Generally only large L-strain rotifers (*Brachionus plicatilis*) are cultured, although small S-strain rotifers (*Brachionus rotundiformis*) have been used when culturing King George whiting. Rotifers are cultured at 24 – 28°C in 3 x 600L conical bottomed fibreglass tanks with heating and aeration, and an additional 3 x 2,000L polyethylene tubs if required (Figure 5). Rotifer production capacity can be increased to 200 million per day, but is typically in the order of 50 – 100 million per day during normal operations with cultures maintained at 200 – 400 rotifers/ml. During larval rearing rotifers are fed microalgae once per day with 2-3 additional feeds of yeast (*Saccaromyces cerevisae*) based upon the rate that feeds are being cleared from culture tanks. Future improvements to rotifer cultures that may be considered include use of specific rotifer culture diets, feed dosing systems and use of microalgal concentrates.

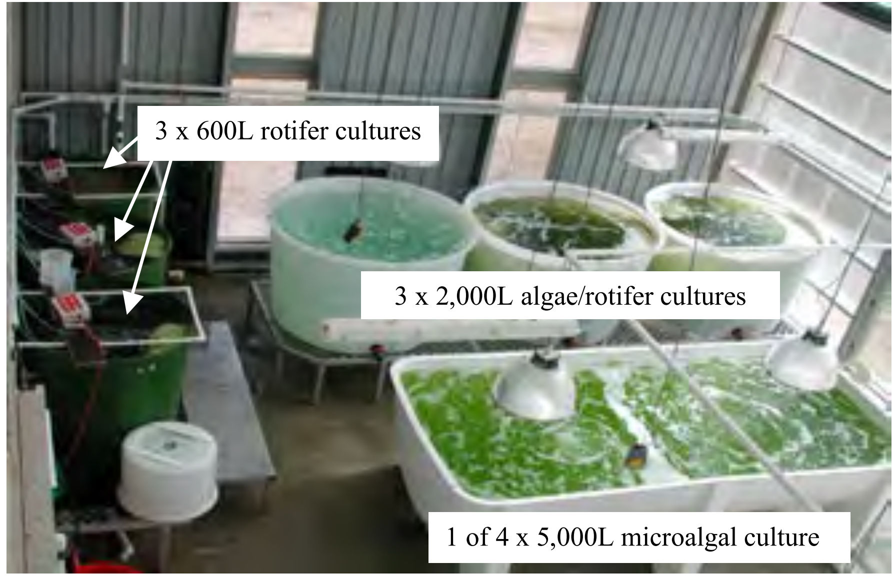

Figure 6. Rotifer and mass microalgal production tanks in the SARDI R&D Hatchery.

Artemia

Artemia production facilities consist of an insulated room adjacent to the larval rearing area that can be maintained at 30°C. Two rows of 3 x 240L white conical bottom tanks are used to hatch *Artemia* cysts at densities up to 2g/L. Each tank has an overhead light with separate switching (Figure 6). Two 450L tanks are used to provide extra capacity during overnight enrichment. When more than 1.5kg of cysts/day need to be hatched the rotifer culture tanks are used to provide capacity of up to 8.0kg/day. All seawater is chlorinated and dechlorinated before use and all cysts are decapsulated before addition to hatching tanks. Hatch controller (INVE) is used to minimise bacterial build up during hatching. Enrichment products used for *Artemia* include DC DHA Selco and DC Super Selco (INVE), although a range of other enrichment products are available for consideration.

Figure 7. Artemia room in SARDI R&D Hatchery.

The live feed use schedule for each species cultured (Figure 7) is varied according to the growth rate of the fish larvae and their ability to ingest and utilise different sizes of live feeds. Major influences on the outcome of hatchery production have mostly been attributed to the time that different size and quality of inert feed is introduced to the larvae.

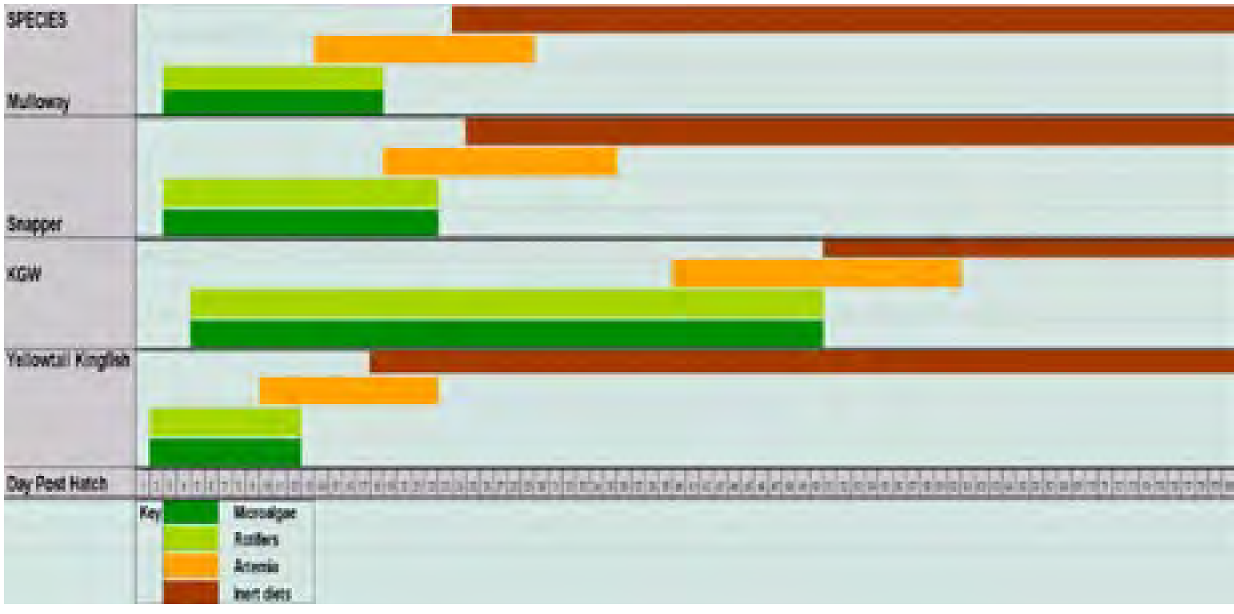

Figure 8. Standard feeding schedules used at the SARDI R&D Hatchery for different species of finfish larvae cultured.

Production capacity

The SARDI R&D Hatchery was designed to be able to produce batches of up to 100,000 weaned fingerlings for pilot scale stocking of commercial ongrowing systems. In the past, clients have been supplied with batches of up to 40,000 snapper and 440,000 mulloway fingerlings for R&D purposes, although in-house R&D has involved much smaller batches. This required temporary installation of extra seawater supply and oxygenation of 5,000L microalgal mass culture tanks that were converted for use as extra nursery tanks (Figure 8).

Inert foods

SARDI has not pursued larval nutrition research as a number of international feed companies (e.g. Kyowa feeds, Nippai ML range; Higashimaru – Minami; Nutreco – Gemma; INVE – Proton, Lansy and NRD; Dana – Larviva Start, Wean-Ex and Dan-Ex; Ewos – Promal and AgloNorse; Salt Creek – Progression) have dedicated research teams working on this area. These efforts are aligned to either large marine finfish culture industries for species such as European sea bream (*Sparus auratus*), sea bass (*Dicentrachus labrax*) or developing industries such as Atlantic cod (*Gadus morhua*). The expansion in weaning, Atremia replacement and nursery culture diets that are now available to finfish aquaculture suggests that Australian hatcheries can now take advantage of improvements in the production and application of these products in their facilities. It is expected that small variations in the timing for use and selection of product will be required as these are applied to the culture of Australian species (i.e. barramundi, snapper, yellowtail kingfish and mulloway). Typical inert feeds that have been used for culture of finfish species in the SARDI R&D Hatchery include (in order of use):

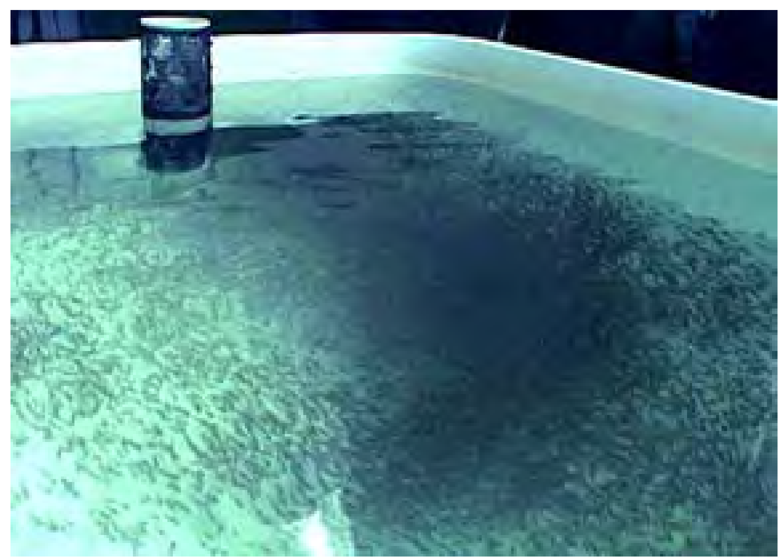

Figure 9. Typical density of weaned 1-2g mulloway fingerlings in 5,000L nursery tanks in the SARDI R&D Hatchery.

King George whiting	Proton 2, ML 300 Proton 3, ML 400 NRD 4/6 NRD 5/8, ML 800
Mulloway	Proton 4 (300 – 500 µm) NRD (500 – 800 µm) EPAC 800/1200
Snapper	Gemma Micro 150 Gemma Micro 300, Proton 3 (200/400), Lansy W3 Proton 4 (300/500 µm), Gemma 0.3 NRD (500 – 800 µm), Gemma 0.5, Gemma 0.75 EPAC 800/1200, Gemma 1.0, Gemma 1.2
Yellowtail kingfish	Proton 2 Proton 3 NRD 4/6 NRD 5/8 EPAC 800/1200

Microalgae

A range of microalgal stock cultures are maintained at the SAASC to support bivalve and finfish aquaculture research. Species used for marine finfish culture are restricted to *Nanochloropsis oculata*, *Pavlova lutheri*, *Isochrysis* Tahitian strain T. Iso and *Tetraselmis suecica.* Stock cultures are used to inoculate 250ml working cultures that are scaled up to aseptic 3L then 16L cultures in 20L polycarbonate carboys. Carboys are used to start 400L bags in a continuous harvest system of 10 bags (Figure 9). Alternatively carboys are transferred to the R&D Hatchery where they are used to start 2,000L (1 carboy per tank) or 5,000L (2-3 carboys) open

mass cultures. Mass microalgae culture facilities comprise 3 x 2,000L polyethylene tanks and 4 x 5,000L rectangular fibreglass tanks with parabolic ends. Open mass cultures and bag cultures are supplied with a carbon dioxide enriched air supply to increase cell density and maintain stable pH.

Microalgae are used in finfish culture operations for rotifer feeding (*N. oculata*, *T. suecica* and T. Iso), rotifer enrichment (T. Iso and *P. lutheri*) and to provide green/brown water (*N. oculata* T. Iso and *P. lutheri*) during the rotifer feeding phase of larval rearing.

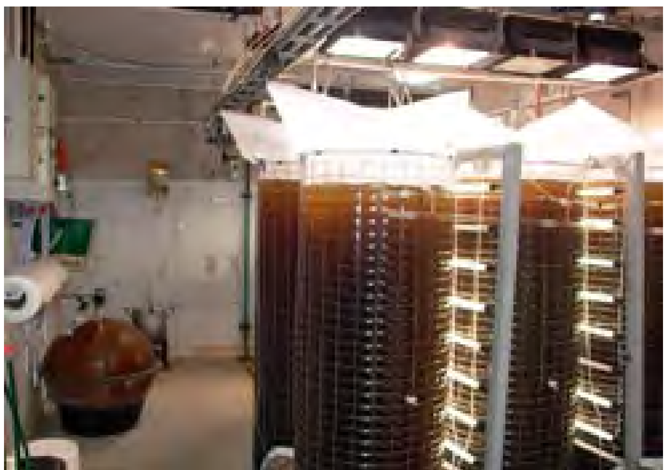

Figure 10. Continuous microalgal production system (10 x 400L bags) at SAASC.

Hygiene

A number of steps are followed to manage the level of micro-organisms during larval rearing operations. These include;

- Culture vessels and fluids that are cleaned and disinfected before use. Algal culture tanks are thoroughly cleaned, refilled with 5um filtered seawater then chlorinated and dechlorinated prior to inoculation.
- The use of batch production methods for rotifers and microalgae so that these live feeds do not accumulate high levels of micro-organisms over long periods. Each rotifer culture is used over 1-3 days depending on demand, then thoroughly rinsed and a proportion used to restart a new culture in a clean tank. Microalgal cultures are used completely over 1-3 days while in the exponential growth phase. Cultures are not restarted from old cultures, rather, new aseptic carboys are used to start each mass culture.
- Thorough rinsing of all rotifer and Artemia additions to larval rearing tanks.
- Daily siphoning of all larval rearing tanks and repeated spot siphoning as required.
- Recirculated seawater is UV irradiated within the water treatment system that supports each larval rearing tank.

R&D Activities

Initial marine finfish research undertaken by SARDI from 1992 – 1996 was done in conjunction with a group of regional small business people in the Whyalla and Port Augusta region. Research was conducted to assess the suitability of snapper for aquaculture in the hypersaline Upper Spencer Gulf. In addition to salinity trials SARDI provided small batches of up to 10,000 snapper fingerlings and assisted with grow-out assessments and transfer of hatchery technology required to provide the confidence needed for local investors to commit to a commercial hatchery at Port Augusta.

SARDI completed an FRDC funded project to evaluate the aquaculture potential of King George whiting during the period that the R&D Hatchery was under construction and for a period of 2 years after completion. This project identified a number of issues related to egg supply and quality, and difficulties with larval rearing. It was concluded that although King George whiting have an excellent market price and their propagation and rearing is possible, commercialisation of this species would require a significant commitment to further research upported and there would still remain an issue with the slow growth of this species to market size.

Mulloway aquaculture development in South Australia was initiated using the SARDI R&D Hatchery facilities in a project conduct for industry. Since the production of over 400,000 fingerlings by SARDI in 2000/01 the industry partner has been provided broodstock and conducted successful hatchery production of up to 750,000 mulloway fingerlings for stocking in commercial seacages at Arno Bay. In 2003 a batch of 20,000 mulloway was produced to stock inland saline aquaculture R&D systems at Cooke Plains.

Recent research has been confined to trials on *Artemia* replacement during snapper production as part of an Aquafin CRC project with NSW Fisheries. The R&D Hatchery also provides ongoing support for research conducted by Aquafin CRC PhD student Bennan Chen who is conducting research on the development of the digestive system in King George whiting and yellowtail kingfish.

Specific problems and issues

The most significant hatchery related issue in South Australia remains the amount of deformities that occur during culture of yellowtail kingfish, although the degree and significance of deformities varies between years and batches of fingerlings. This research problem may be complex but an understanding of the factors that contribute is constrained by the willingness of the commercial hatcheries to share information and work with researchers. While fingerling requirements from commercial hatcheries remains moderate (200,000 – 500,000 pa) it is expected that excess production in the early stages can largely compensate for the percentage of deformed fish discarded at the nursery stage prior to transfer into sea cages.

Both SA commercial hatcheries are continually varying different elements of hatchery operations (i.e. physical conditions, nutritional enhancement through enrichments and testing of modern weaning and *Artemia* replacement diets, etc). These ongoing evaluations provide incremental improvements to production through improved quality of fingerlings and cost reduction within the hatchery.

Mulloway can be produced in numbers in excess of grow-out capacity and the survival and performance of this species does not support any further investment of Hatchery R&D effort. Commercial snapper aquaculture in South Australia has ceased due to the developments that have occurred with yellowtail kingfish and mulloway, which are both faster growing.

Collaborations

Aquafin CRC, marine finfish companies in South Australia, the SA Marine Finfish Farmers Association and other National marine finfish researchers including, Dr S Fielder (NSW Fisheries) and Dr S Battaglene (TAFI).

Tasmanian Aquaculture and Fisheries Institute

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Species cultured

Fish: striped trumpeter (*Latris lineata*), pot-bellied seahorse (*Hippocampus abdominalis*), spotted pipefish (*Stigmatopora argus*), Barbour's seahorse (*Hippocampus barbouri*), White's seahorse (*Hippocampus whitei*), greenback flounder (*Rhombosolea tapirina*), damselfish (*Acanthochromis polyacanthus*), Tasmanian whitebait (*Lovettia sealii*), galaxids (*Galaxias maculatus*), blenny (*Parablennius tasmanianus*), black bream (*Acanthopagrus butcheri*)

Crustaceans: southern rock lobster (*Jasus edwardsii*).

Shellfish: Pacific oyster (*Crassostrea gigas*), blue mussel (*Mytilus edulis*).

Squid: southern dumpling squid *Euprymna tasmanica*, Pygmy squid *Idiosetius sp.*

Systems

Striped trumpeter eggs are incubated at 14 ºC in 250 L upwelling tanks. Yolk-sac larvae are reared at 5 to 50 per L. A temperature and photoperiod controlled system of 24 replicate 300 L black hemispherical tanks is available for experimentation at MRL. Each 300 L tank has a central screened outlet and is typically supplied with recirculating seawater and ozonated flow-through seawater. Production runs are conducted in 3000 L black conical-bottomed tanks using downwelling and upwelling at different stages in the production cycle. Early culture is better in greenwater (*Nannochloropsis* sp.) usually to at least D 20. Post-larvae are reared in clearwater and are weaned using automated feeders and modified light background environments.

Seahorse broodstock are held in 1 m^3 tanks in which courtship, egg transfer to the males, egg incubation and juvenile release occur. Juveniles are removed and reared in smaller 60 L conical based cylindrical tanks supported by recirculation systems and light- (12:12 LD) and temperature-control (18°C). Experiments are undertaken in 18 x 3 L, 20 x 25 L or 12 x 60 L tank systems. Juveniles are fed enriched *Artemia* instar II metanauplii until they are weaned onto frozen mysids or amphipods.

Phyllosoma larvae of southern rock lobsters are cultured in 10 L circular tanks at 20-40 per L. They are reared at 18°C, at a photoperiod of 12 h light: 12 h dark with the light phase of low intensity to minimise stress. The culture water is flow-through which has been filtered to 1 µm and disinfected with ultraviolet irradiation. The culture tanks have four jets positioned near the base at the outer perimeter and another two jets at the base near a central cylinder to achieve circular, steady flow. A screen is fitted to the wall of each tank to allow escape of water while retaining live *Artemia* in the tank.

Shellfish broodstock conditioning is carried out in a 270 L recirculating system. Experimental larval runs are conducted in triplicate 200 L static tanks at around 10 per mL (decreasing to 1-2 per mL at set), with aeration and water changes at approx 2 day intervals. Around day 14, larvae are either set chemically and transferred to a 160 L recirculating spat upweller system, or set onto billets and on-grown for 2-3 weeks prior to movement to a farm-based nursery.

Production capacity

Reliable supplies of broodstock striped trumpeter, seahorses and southern rock lobsters are available for research in Tasmania. At MRL over 100 striped trumpeter broodstock are held in five 25 000 L tanks on photoperiod and temperature control to provide a consistent source of eggs from February to June and August to December. One 4000 L tank of greenback flounder broodstock provide eggs during the ambient season from June to September. F1 generation broodstock striped trumpeter are available and F3 generation greenback flounder. Broodstock are fed moist pellets incorporating commercially available ingredients, fresh fish and squid and vitamin supplements. At SOA around 50-100 seahorse F2-F3 broodstock are held in two 1 m^3 tanks. Although these fish spawn year-round, the SOA also acquires significant numbers of juveniles from Seahorse World at Beauty Point for replicated research trials. Broodstock are fed frozen (and live) mysids and amphipods.

Ovigerous female lobsters naturally hatch their phyllosoma larvae in late September for a period of 6-8 weeks. At MRL, wild-caught broodstock are also held at photoperiods and temperatures to hatch out-of-season, ensuring the production of larvae for up to 8 months each year (April-November). The lobster diet consists of mussels, squid and Kuruma prawn pellets, with each component fed on rotation on separate days of the week. They are housed in 12 x 4000 L tanks with concrete shelters and supplied with filtered, recirculated water.

Conditioned Pacific oyster broodstock are currently maintained from around August to February. Several small larval rearing runs of oysters (1-10 million larvae per run) and/or blue mussels are carried out during this period to support research projects and molluscan biology teaching.

Live foods used

Rotifers, L-strain rotifers *Brachionus plicatilis* and S-strain *B. rotundiformis* are available. At MRL, rotifers are intensively cultured using ozonated seawater at densities up to 1000 per mL on a diet of microalgae *Nannochloropsis* sp. and bakers yeast *Saccharomyces cerevisiae* in recirculation systems. Stock cultures are kept in test tubes and 20 L carboys. Intensive systems of 50, 250 and 1800 L are used and they are comprised of a reservoir tank, foam fractionator, circulation pump and dosing system for liquid algae. Larger production systems hold up to two billion rotifers and provide a harvest 200 million rotifers daily. Rotifers are enriched with vitamins, algae and commercial products depending on the nutritional needs of the target species. Algamac (Aquafauna Biomarine) enrichment is the standard hatchery practise at MRL with enrichment of 0.2 g per million rotifers over 12 h. Batch cultures or semi-continuous cultures of *B. rotundiformis* and *B. plicatilis* fed yeast and microalgae in 100 or 500 L containers is practised at SOA. Rotifers are normally enriched on both micro-algal and Selco (INVE) products.

Brine shrimp, *Artemia* (early stage nauplii through to adults) are an important component of most larval diets, and especially for striped trumpeter, sea horses and southern rock lobster. The newly hatched *Artemia* nauplii are reared using ozonated seawater from decapsulated cysts. Hatch Controller from INVE (Primo Aquaculture) reduces bacterial loads in 70 L conical tanks. Care is taken in selecting brands of *Artemia* with good nutritional profiles and in matching the appropriate sized *Artemia* to each development stage. There is a need for large-scale on-growing of *Artemia* to around 12 mm in total length for feeding to southern rock lobster phyllosoma larvae and sea horses. At MRL, *Artemia* are ongrown in 600 L tanks using flow-through seawater on a diet of rice pollard and algae (*C. muelleri* and T. *Iso*). Considerable effort is expended to reduce external and internal bacterial loads in on-grown *Artemia* using a series of algal purges incorporating formalin. At SOA, *Artemia* instar II and adults are mainly used to support seahorses and squid. *Artemia* instar II production uses a variety of approaches including decapsulation, non-decapsulation, A1 Selco products, microalgal enrichment, and a range of other commercial product enrichments.

Copepods (e.g., harpacticoid *Tisbe,* cyclopoid *Apocyclops*, and calanoid *Acartia*) have been used as a supplementary feed to rotifers and *Artemia* in the culture of "difficult" marine fish. However, mass-culture of cold-water copepods has been problematic. *Tisbe* has been cultured in Launceston using batch, semi-continuous and recirculation systems on diets of microalgae, fortified yeast, pelleted feeds, vegetables, macroalgae and associated microflora. Striped trumpeter have been reared successfully using harpacticoid copepods as a supplementary feed. Seahorse juveniles have been reared successfully on cultured copepods (*Tisbe* sp.) and a range of crustaceans (copepods, amphipods, caprellid shrimps) collected as net biofouling. Research during

these trials has documented the relationship between seahorse size and prey size, gut enzyme development and digestive tract development.

Amphipods have been used to feed sea horses and the endangered handfish *Brachionychthys hirsutus.* The techniques for mass-culture of amphipods are still being developed in collaboration with aquaculturists in the seahorse industry. Frozen mysids are used routinely in seahorse cultures starting at about 3 months of age while live mysids are fed to dumpling squid held for behavioural experiments. Whilst the culture of mysids is possible, at present it is more effective to collect them from the wild under licence.

Striped trumpeter: D 5 to D 20, L-strain rotifers; D 18 to D 50, 48 h brine shrimp; copepods as available to D 30. Rotifers are added twice daily at 5 to 10 per mL. *Artemia* at 0.1 to 2 per mL.

Greenback flounder: D 5 to D 20, L-strain rotifers; D 18 to D 35, 48 h brine shrimp.

Seahorses: *Artemia* instar II metanauplii are used on pot-bellied seahorses from day of birth onwards. Seahorses consume around 35-45% BW/day (wet weight basis) at the early juvenile stages. Current trials aim to identify a suitable replacement feeding regimen starting at copepods, followed by amphipods and mysids. In the smaller seahorse species (*H. barbouri* and *H. whitei*) and pipefish (*S. argus*) both *Artemia* and rotifers are added for the first 1-2 weeks. Copepods also are used as a supplementary feed.

For newly-hatched rock lobster phyllosoma larvae, *Artemia* ongrown to 1.5-2.0 mm are fed at 1.5 per mL. As phyllosoma grow, they are fed at a relatively lower rate with larger *Artemia*. For late stage larvae (≥ Stage 9), 6-9 mm *Artemia* are fed.

Inert feeds used

Striped trumpeter are fed formulated diets from as early as D 30. Recent weaning strategies have centred on the use of *Artemia* replacement diets like Gemma Micro (Skretting). Striped trumpeter are generally weaned by D 100. Japanese ML diets (Nippon) have also been used successfully. A recent experiment has shown that greenback flounder can be reared from first feeding on Gemma formulated diets with only one day of rotifer feeding. At this stage, trials using a range of commercially available and manufactured formulated diets on early seahorse juveniles have not proved successful with ingestion levels being sub-optimal. Such trials are continuing on other products.

Algae

At SOA Launceston, a wide range of marine and some freshwater axenic stock cultures are maintained in 250 mL Erlenmeyer flasks in autoclaved f/2 medium at 18°C under 50-80 µmoles photons PAR m^{-2} s^{-1} with a 12:12 light dark cycle. Aquaculture feeds species such as *Chaetoceros muelleri, Chaetoceros calcitrans, Isochrysis* sp. (T. *Iso*), *Pavlova pinguis, Pavlova lutheri, Tetraselmis suecica, Nannochloropsis oculata* are on-grown in f/2 medium (0.2 µm filtered seawater) in batch and semi-continuous cultures with CO_2/air injection, under metal halide lamps in 10 L carboys (axenic), 500 L poly bags, at high cell density in 10 L "mini-bags", or in 1000 L fibreglass tanks. Microalgae are batch cultured at MRL. Autoclaved 20 L carboys provide high quality axenic cultures and microfiltration (0.2 µm) and chlorination are used in batch culture in tanks up to 1000 L, using metal halide lamps, CO_2 and aeration injection. *N. oculata* is the main alga used in green water culture of marine fish. *C. muelleri, I. galbana,* and *P. pinguis* are used for enrichment and bacterial purging of *Artemia.* There is increasing reliance on liquid algae from Reed Mariculture (Proaqua), particularly liquid *N. oculata* as a rotifer feed and for greenwater culture.

Hygiene

Eggs of striped trumpeter are disinfected with ozone prior to hatching at 1 ppm for 1 min to control nodaviruses. Eggs are less sensitive to shock and ozonation after the embryo is well developed, around three days post-fertilisation. Seawater used in the fish and rock lobster hatcheries at MRL is filtered, foam fractionated, heated, ozonated, UV treated and charcoal filtered. Seawater is sterilised in the fish hatchery to remove parasites and disease organisms by ozonating at an ORP of over 850 for 10 min. Water baths are provided at the entrance to

both hatcheries, gum boots, laboratory coats, ethanol sprays and hand washing are required to enter the fish hatchery. Seawater for seahorse and live feed culture at SOA is filtered and UV treated. Seahorses occasionally display protozoan, annelid or anemone infestations of the skin and are treated by short freshwater bath or chemical bath.

R&D Activity

Projects include:

1. Aquafin CRC: Improving growth and survival of cultured marine larvae striped trumpeter: a test case for Tasmania
2. Aquafin CRC: Enhanced hatchery production of striped trumpeter in Tasmania through system design, microbial control and early weaning
3. Rock Lobster Enhancement and Aquaculture Subprogram: Advancing the hatchery propagation of rock lobsters
4. Rock Lobster Enhancement and Aquaculture Subprogram: Propagation of southern rock lobster (*Jasus edwardsii*) in Tasmania
5. TAFI: Manipulating genetic variation in Pacific oysters
6. TAFI: Optimising juvenile oyster growth and condition in spat upweller systems
7. TAFI: Novel microalgal feeds for bivalve culture
8. TAFI: Bacterial flora replacement and probiotic delivery via *Artemia* and other live feeds

There are three main planned outcomes from the research on striped trumpeter:

1. Australian aquaculture will be provided with a more systematic way to evaluate and match the nutritional profile of live feeds with the needs of new species of marine fish larvae leading to improvements in growth and survival of a wider range of cultured juveniles. For example, survival has increased from <1% to >10% for striped trumpeter over the last three years.

2. Australian aquaculture will be provided with more systematic ways to control microbial communities. Methods of water treatment, system design, and techniques to evaluate, identify and produce probionts for use in improving hatchery survival rates in finfish, especially in relation to the needs of new 'difficult' species of marine fish larvae, leading to improvements in growth and survival of a wider range of cultured juveniles.

3. There will be a greater choice of new marine fish species available for culture through the efficient technology transfer between research agencies and industry of new products and systems for culturing marine fish larvae.

The industries to benefit from the planned outcomes include the Atlantic salmon aquaculture industry and the emerging marine finfish culture industry throughout Australia. In particular, the development of novel techniques for water treatment using ozone, probiotics and enrichment of live feeds is of direct relevance to barramundi, dhufish, kingfish, snapper, whiting and grouper culture.

The successful outcome of the research on rock lobster larval rearing will be a strong foundation on which to close the life cycle and has application to temperate and tropical species. A reliable and consistent supply of high quality Stage 5 phyllosoma and the development of suitable culture protocols will assist the development of lobster aquaculture. Effective probiotic techniques will control the microbial environment of *Artemia* and larval cultures leading to a significant improvement in the survival of phyllosoma. It will also have wider application elsewhere due to the ubiquitous nature of *Artemia* as the live food of choice for many aquaculture species.

The seahorse culture program has five main aims in relation to live feeds:

1. Use of alternate live feeds to replace or partially replace *Artemia* in the production cycle. Trials currently show the use of copepods, mysids and net biofouling organisms to be promising replacements to reduce costs and avoid reliance on a product which can be difficult to obtain and expensive to use as a feed.

2. Trial greenwater culture on newborn juveniles to assess if there is any benefit over clear water culture. Preliminary experiments suggest that there is little or no benefit to *H. abdominalis* but possible benefit (in the absence of copepods) to other seahorse species.

3. Trial the use of formulated diets to further reduce the reliance on live feeds. Seahorses are visual feeders which places a heavy reliance on live prey so weaning is not easy. However, it is possible to wean fish from live to frozen diets and a focus on formulated diets soon after birth will be examined.

4. Nutritional needs of seahorses at various ages to be assessed. Regardless of whether *Artemia*, live, frozen or formulated diets are used there is a need to quantify the requirements of the fish to optimise growth and survival. Coupled with nutrition, feeding behaviour and activity patterns are also indicators of the success of various diets and feeding regimes.

5. Rearing protocols for new syngnathid species. The existing protocols for *H. abdominalis* will be transferred to other species and further refined.

Shellfish and microalgal research aims to improve larval nutrition and growth rates through improved nutritional profile of microalgal feeds and a better understanding of larval/juvenile raising technologies. Work on oyster genetic variation aims to develop reliable methods for producing genetically consistent shellfish broodstock. These broodstock lines can be used develop oyster lines with consistent growth rate and desirable shape, colour and flesh quality characteristics for the Australian and international market.

Specific problems and issues

Four key areas of research are being examined with striped trumpeter through the Aquafin CRC:

1. Improving the health of larvae through better control of disease and parasites. Control of myxosporeans and nodavirus will be undertaken in companion projects outside the Aquafin CRC.

2. Further development of live feed enrichments, weaning, and grow-out diets, leading to a better understanding of nutritional requirements and other factors.

3. Further improvements in tank design, management and operation to improve post-larval quality, particularly malformations possibly aggravated by "walling behaviour".

4. Contingent on above, assessment of survival and growth of post-larvae and juveniles under semi-commercial conditions.

The overall objective of the research on the larval rearing of southern rock lobsters is to understand the essential requirements for culture of early-stage phyllosoma by:

1. Identifying and assessing broodstock conditioning protocols that permit routine production of high quality, viable larvae.

2. Developing pilot-scale larval rearing systems which result in high survival of large numbers of Stage 5 phyllosoma.

3. Better understanding the role of water quality and controlling microbial contamination as one of the key bottlenecks to survival and growth of phyllosoma.

4. Developing diets and feeding protocols which optimise the survival and growth potential of phyllosoma to Stage 5

The key areas to be researched in relation to seahorses are:

1. Optimising feeds and feed utilisation, including a better understanding of adult and juvenile feeding

behaviour and a cost analysis of replacement diets.

2. Gaining a better understanding of the source, prevention and treatment of seahorse diseases including a range of protozoan, bacterial, annelid and anemone infestations.

3. Although seahorses breed year-round some research is required on the manipulation of breeding cycles, including the effect on courtship behaviour, re-conditioning of broodstock and the social interactions of broodfish within rearing tanks.

4. Seahorses are used in the aquarium trade and while size is an important determinant of price so too is colour. Preliminary research suggests that colours can be changed in *H. abdominalis* using food and environment. Further research would better define how colour can be manipulated and which conditions can be used to optimise this character.

5. Tolerances of newborn seahorses to environmental conditions such as temperature, salinity, ammonia, nitrite and nitrate need to be quantified to assist with the development of management plans for high density culture in recirculation systems. Further, the viability of low density cage culture of large juveniles and adults needs to be explored.

Microalgal production research is focused on:

1. Optimising the use of 10 L hanging "mini-bags" high density algal cultures as a flexible and space-efficient alternative to 500 L bags. Preliminary work indicates a 10-fold improvement in algal biomass per litre of medium (3-4 x 10 L bags = 1 x 500 L bag) due to more efficient utilisation of light and nutrients in the smaller diameter 10 L bags.

2. Examining the nutritional value of small hardy dinoflagellates as supplemental feeds (10-20% of cell biomass) for larval bivalves. This group or microalgae are a major feed source for bivalves in natural systems that contain a range of potentially important long-chain fatty acids and novel sterols. They are currently not utilised in intensive aquaculture due to slow growth rates and poor growth in standard algal systems.

Collaboration

TAFI researchers have close links to industry and other researchers both nationally and internationally. The striped trumpeter and rock lobster teams have worked closely with the CSIRO Marine Research scientists including Drs M. Brown, P. Nichols and K. Williams on aquaculture nutrition. Collaborating departments of UTAS include, Fish Health Unit (Drs J. Carson and J. Handlinger), Agricultural Science (Dr J. Bowman) and School of Human Life Sciences. Other institutions include Curtin University (Professor Bruce Phillips), QDPI Cairns (Dr Clive Jones), James Cook University (Dr T. Pankhurst), the Ludwig Institute for Cancer Research (Dr A. Trotter), and Department of Fisheries Western Australia (Dr S. Kolkovski). Recent working groups and review teams have included leading scientists from SA (Mr W. Hutchinson, Mr S. Clarke), NSW (Dr S. Fielder) and WA (Dr S. Kolkovski). International links include collaborations with the United Kingdom on marine finfish culture, Stirling University (Dr G. Bell, Prof J. Sargent) and the Northern Atlantic Fisheries College (Dr L. McEvoy), and in Spain at the Centro de Acuicultura -IRTA (Dr A. Estevez). Other close associations include researchers working on probiotics in New Zealand, NIWA (Drs E. Maas and Dr M. Bruce) and on sea cucumbers in New Caledonia, Worldfish (Dr S. Purcell). Industry collaborations include M G Kailis Pty Ltd, major food manufacturer Skretting and the salmonid industry through the Tasmanian Salmonid Growers Association. The SOA has close industry links with Seahorse World (Beauty Point, Tasmania) who support general syngnathid research and PhD research. The School also has informal links with seahorse researchers in New Zealand, NIWA (Chris Woods) and Singapore, TMSI (Dr K. Reddy, Dr J. Walford, Dr B. Sivaloganathan).

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Hatchery Technology on the Breeding and Fry Production of Marine Finfish in Indonesia

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Interest in culturing the various species of marine finfish has always been around, fuelled by high on-farm price. In Indonesia current ex-farm price ranged from US$ 9-12 for the tiger grouper *(Epinephelus fuscoguttatus)* whereas prices for Barramundi cod *(Cromileptes altivelis)* reach US$ 30-38. The recent upward trend in production is attributed to advancement in hatchery seed production and improvements in nursery techniques. The interest in marine finfish farming has not only provided jobs for many coastal communities, but also established secondary servicing industries such as hatchery technology, feed manufacturing, product processing, transportation and marketing. This paper describes the present status of hatchery technology for the breeding and fry production of marine finfish in Indonesia, such as Tiger grouper *(Epinephelus fuscoguttatus),* Estuarine grouper *(Epinephelus coioides)*, Barramundi cod *(Cromileptes altivelis*), Coral trout *(Plectrophomus leopardus),* Napoleon wrasse *(Cheilinus undulatus*) and Red snappers *(Lutjanus sebae and L.argentimaculatus,* based primarily on the work have been carried out in Gondol Research Institute for Mariculture and Lampung Mariculture Development Center. Broodstock are maintained in tanks (100-150 MT capacity) on land and fertilized eggs are obtained by natural spawning. Broodstock are fed by mixed fresh squid and trash fish *(mainly Sardinella sp*.). For larval rearing, eggs instead of hatched larvae, are mainly stocked in the rearing tank. Feeding of super small (SS-type) rotifers for very early larvae and feeding of artificial diets for late larvae and juveniles improved larval survival. The recent advances are in the seed production of the Tiger grouper, estuarine grouper and Barramundi cod, hatchery breed grouper fry are well accepted by farmers. There are now 75 groupers hatcheries (55 small/backyard hatcheries, ten medium, nine large/complete and one intensive hatchery which hold broddstock. The total production of grouper fry in 2003 was 4.20 million juvenile for tiger grouper, 1.13 million for Barramundi cod and 350 thousands juveniles for others groupers. In addition to the groupers mentioned above, corral trout, napoleon wrasse and red snappers being targeted for development.

Introduction

Marine finfish culture is expanding in many areas of Indonesia. While there is lack of statistical data available on marine finfish culture in Indonesia, national statistic aquaculture shows cage culture growing at 16 per cent during the 1990s. The primary areas for grow-out culture are Aceh (Nias and Sibolga), Riau Islands, Bangka-Belitung Islands, Lampung Bay, Seribu Islands, Karimun Jawa Islands (Central Jawa), Teluk Pengambetan (North-West Bali), Teluk Saleh (West Nusa Tenggara), South Sulawesi, South-East Sulawesi, North Sulawesi and Gorontalo. Marine fish culture is generally characterized by the use of wild caught seed and use of trash fish for feed. There is limited use of hatchery reared seed, although this is growing especially for groupers.

There has been a good deal of research on hatchery production of groupers, snappers and other marine finfish. This has been stimulated by the development of a large number of private milkfish hatcheries by applying the technology that has been developed in GRIM.

Species Cultured

At Gondol Research Institute for Mariculture has been doing research and development on seed production of Milkfish *(Chanos-chanos)*, Shrimp (*Penaeus monodon, P.vannamei, P.stilyrostris)*, Mud Crab *(Scylla*

sp.), Swimming *Crab (Portunus sp)*, Yellow fin Tuna *(Thunnus albacores),* Tiger grouper *(Epinephelus fuscoguttatus),* Estuarine grouper *(Epinephelus coioides)*, Napoleon wrasse *(Cheilinus undulatus*), Red snappers *(Lutjanus sebae and L.argentimaculatus* Barramundi cod *(Cromileptes altivelis*) and Coral trout *(Plectrophomus leopardus),*. In this paper present only marine finfish seed production.

Systems

Broodstock

Broodstock tanks are used not only for rearing of broodstock but also for spawning. Based experiences in Gondol Research Institute for Mariculture (GRIM), ideal size of broodsock tanks is 60 MT for Barramundi cod *(Cromileptes altivelis)* and Coral trout *(Plectrophomus leopardus)* and tanks of 100-150 MT for other groupers *(Epinephelus fuscoguttatus, E. coioides and Plectrophomus* sp), Red snapper (*Lutjanus sebae and L. argentimaculatus)* and Napoleon wrasse (*Cheilinus undulatus*). Since broodstock swim around on the tank during spawning activity, it is recommended to use round shape tank with the depth of 2-2.5 m. Each tank is equipped with a water inlet and outlet and an aeration system. The source of water supply is direct from sea using sea water pomp. It had a flow through by changing 200-300 % of the tank water daily. An overflow pipe is connected with an egg collection tank where a net to collect spawned eggs is placed. The size of tank for collecting egg is 2x2x1 m depth Fig.1. The water temperature and salinity is ranged from 26.8-28.9°C and 33-34 ppt, respectively. The broodstock were usually fed to satiation once a day by mixed trash fish (mainly *Clupeidae* and *Scombridae*) with squid. The feed is supplemented with 1% of vitamin mix.

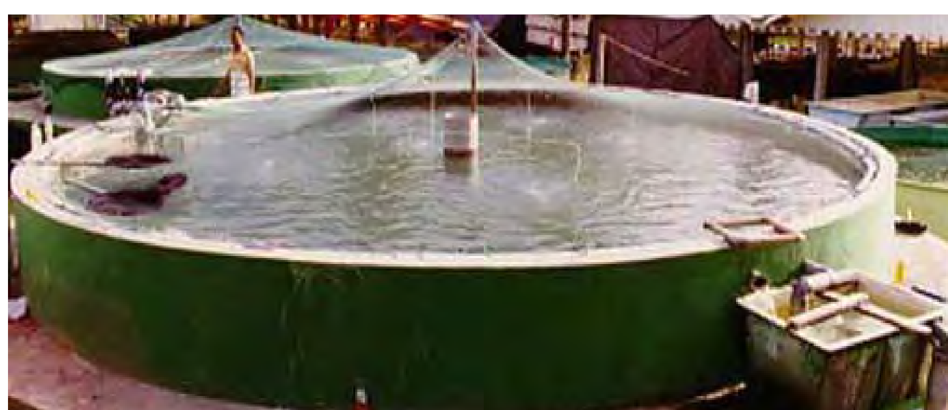

Figure 1. Broodstock tank for Marine finfish in GRIM .

Larval rearing tanks

The size of larval rearing tanks is approximately 10 MT with 1.2 m depth and equipped with aeration system, this size is enough to produce 10,000 juvenile of groupers and snapper seeds. Both circular and rectangular shape tanks can be used for larval rearing. For rectangular shape tank, corners should be rounded to avoid larval aggregation at the corners. The sea water used in the larval rearing tank is pre-treated using sand filters. The water temperature ranged from 27.8-30.0 °C and water salinity ranged from 34.0-34.5 ppt. Based on experiences, a light blue color is preferred for larval rearing in GRIM. Larval rearing tanks should be roofed to avoid direct sunlight and water rain, and to avoid fluctuation of water temperature. (Fig.2).

Figure. 2. Larval rearing tanks for marine finfish.

Larval rearing

Larval of groupers shows drastic changes in their morphology as they grow from the larval stage to juvenile stage, therefore, careful management is necessary. The factor contributing to larval mortality are floating death, sinking death, entanglement with the spine, nutritional deficiency , cannibalism and diseases. For larval rearing, eggs, instead of hatched larvae, are mainly stocked in the rearing tanks with initial stocking density

10 ind./L. The larva rearing is carried out in the same tank for 45-50 days. Live food for the larval rearing consisted of micro algae, *Nannocloropsis sp*, SS type rotifer (Size 80-120 micron), S-type rotifers 140-200 micron) and *Artemia* nauplii. Artificial diets were introduce prior to feeding *Artemia* nauplii. The larval raring protocol is shown in Fig.3.

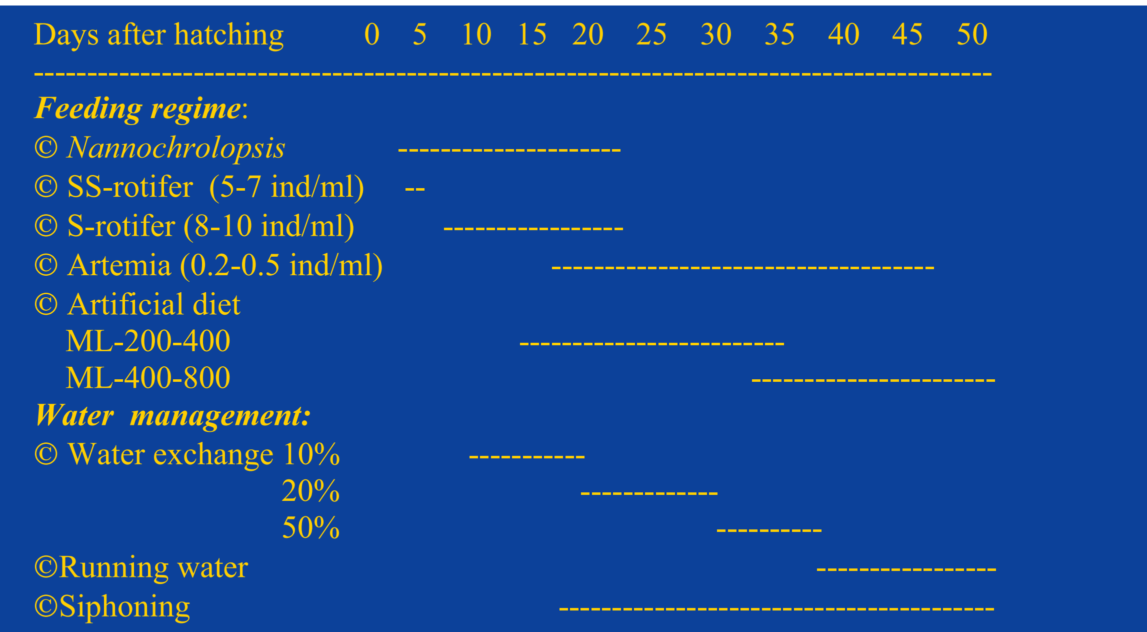

Figure. 3. Larval rearing protocol of groupers.

The *Nannochloropsis* was introduce in the larval rearing tanks after 24 hours of stocking the newly hatched larvae (1-DAH), the Nannochloropsis density was maintained at 300 thousand cell/ml. The SS-type rotifer, are introduce on day two when the larvae partly absorbed their yolk. The SS-type rotifer density is maintained at density 5-7 ind/ml during 2-5 DAH. The S-type rotifer with a density of 8-10 ind/ml was maintained during 6-20 DAH and the density gradually decreased as the rate of rotifer consumption by the larvae increased and eventually rotifer disappeared at day 25. From days fifteen onward, small size commercially formulated diet (ML Powdered) with a particle size of 200-400μwas used. The feed size was gradually increase from 400 t0 800μ from day 30 to day 50. From day seventeen onward, newly hatched *Artemia* were introduce with a density of 0.2-0.5ind/ml. Before introduction to the larval rearing tanks, one day old *Artemia* were treated or enriched with “Super Selco” to increase their nutritional value. One day after the fish were fed artificial diets and *Artemia*, 20-50% of the rearing water was changed once daily. At day 30, running water at an exchange rate of 100 % were done to avoid water quality problem. By applying to these protocol improved larval survival with survival rate ranged from 30-50 %.

Main Problem

The major cause mortalities are due to diseases especially Viral Nervous Necrosis (VNN). Once VNN break out during larval rearing, a high mortality occurs and some time total mortality was happen. Up to now, there is no effective treatment has been developed for VNN. Dead juveniles caused by VNN are shown in Fig.4

Figure. 4. Dead juveniles caused by Viral Nervous Necrosis.

Production

By applying the technology mentioned above, the total production of the tiger grouper in 2003 was 4,300,000 juveniles of Tiger grouper, *Epinephelus fuscoguttatus* (Size 5-8 cm in total length), 1,130,000 juveniles of Barramundi cod, *Cromileptes altivelis* and 350,000 juveniles of Estuarine grouper, *Epinephelus coioides*, Red snappers, *Lutjanus sebae and L. argentimaculatus.* Hatchery bred seeds are now well accepted by farmers, There are now 75 marine fish hatcheries (55 small/backyard hatcheries, 10 medium hatcheries, 9 large hatcheries which hold broodstock and 1 hatchery super intensive and several more under development. In addition to those fish other marine fish under propagation are Napoleon, *Cheilinus undulatus,* Coral trout , *Plectrophomus leopardus and* Yellowfin Tuna. *T albacore* In GRIM currently hold the brodstock of Tiger grouper 83 fish, Barramundi cod 127 fish, Coral trout 42 fish, Napoleon 23 fish, Red snapper 70 fish, Yellowfin Tuna 42 and Cobia 8 fish. With the production capacity 6-9 million seeds per year. GRIM has also run the supper intensive hatchery, this hatchery built by Denmark Government and now under observation weather can be use to produce grouper seed or not. The Super Intensive hatchery or Close system hatchery is shown in Fig. 5.

Figure 5. Super intensive hatchery at GRIM .

Research Collaboration

The Government of Indonesia has formulated several research programs that help address main problem faced the marine finfish farmer or industry. Research and development will focus on study related to broodstock genetics, feed formulation with low pollution, improve of seed production technology, production of free diseases broodstock and seeds, produce vigor seeds and vaccine. GRIM currently doing research collaboration with ACIAR-Australia on improvement of hatchery technology of grouper, especially Coral trout, *Plectrophomus leopardus*, Research collaboration also with OFCF Japan for yellow fin tuna propagation.

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Industry Perspectives

Commercial practices for the production of barramundi, *Lates calcarifer*, fingerlings: An industry summary

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Introduction

Barramundi fingerlings have been produced commercially in Australia for over 15 years. Early industry development was assisted by the activities of the Qld Department of Primary Industries who developed an extensive culture technique described essentially in Rutledge and Rimmer (1991). This technique is still used to some extent in commercial practice accounting for 25% of fingerling production.

Fingerlings produced in intensive systems were considered to be inferior to those produced in extensive systems being smaller for age, likely to show more frequent deformities and thought to be less robust. Recently, the establishment of hatcheries in regions outside of the natural range of barramundi in Australia and the development of improved live food production processes, enrichment media and weaning diets has led to increased use of intensive hatchery production systems.

In 2003/4, the majority of barramundi fingerlings produced in the 7 major hatcheries in the Australian commercial sector were produced in intensive systems. In 2003/4, approximately 12.5 million barramundi fingerlings were produced using intensive larval rearing methods compared with 4.5 million fingerlings using extensive methods. Intensive systems allow much more predictable survival of larvae through to fingerlings, more reliable quality of fingerlings and production out of the natural breeding season.

In view of the nature of this workshop, this presentation will be restricted to the methods and issues of intensive production of barramundi fingerlings. Most procedures used in the barramundi industry are not greatly different to those of other intensive commercial marine fingerling production systems. A significant modification of the traditional method developed recently is described by Bosmans *et al* (2004) in this Proceedings. Since that method varies in nature and timing from the traditional method and is described elsewhere, it will not generally be included in this discussion. This presentation is general in nature and does not describe the practice of any individual company and some of the methods used in commercial hatcheries are proprietary information and will not be discussed.

Production capacity

Capacity for production of barramundi fingerlings is not fully utilized in Australian hatcheries for a variety of reasons. These include the fact that some companies are in the development phase while others are unable to sell all of their production. It is estimated that approximately 85% of the capacity is utilized.

The production season is largely dictated by the capacity to subsequently rear fish through nursery culture. Fingerling production occurs throughout the year and most companies have temperature controlled hatcheries that allow them to produce fingerlings when required. However, as heated nursery facilities are limited, most northern hatcheries suspend production for some part of the winter period and take advantage of this time to perform maintenance and spend a period of time in dry out.

The number of broodstock held is in great excess to that actually required for spawning purposes. Generally, 50 to 100 broodstock (including both male and female) are held per hatchery. The major constraint on broodstock performance is poor fertilization of eggs. Poor fertilization results from insufficient numbers of developed males in the spawning tank, early onset of sex inversion with consequent loss of males from the spawning

population and a stress response to handling in male fish that results in regression of gonadal development. The relatively large number of broodstock allows for variable performance and some capacity for selection. Broodstock turnover is at a relatively high rate as hatcheries seek to respond to these challenges, particularly the effects of early sex inversion and as they seek to improve the genetic characteristics of their stock.

Larval feeding regime

Larval feeding regimes incorporate a mixture of rotifer (*Brachionus plicatilis*), Artemia and formulated feeds.

The larval feeding regime is shown in Figure 1. Rotifers are provided from Day 1 after hatch (ah) and may continue up to Day 14 ah if available in sufficient numbers. Artemia as newly hatched nauplii are introduced between Day 9 ah and Day 12 ah and continued until fish are fully weaned or are determined to be too slow to grow and are consequently sacrificed. Weaning diets are introduced at some time on or after Day 12 ah.

Rotifers

Artemia

Weaning diet

Formulated feed

Typical daily water exchange													
20-25% Top up		25% exchange		50% exchange		100% exchange		200% - 500% exchange					
2	4	6	8	10	12	14	16	18	20	22	24	26	28
Day after hatch													

Figure 1. Diagrammatic representation of the typical feeding regime and water exchange used in barramundi larval rearing in Australian commercial hatcheries.

Traditionally, rotifers are produced using a culture of *Nanochloropsis* sp. supplemented by enrichment media and yeast. Relative levels of algae, enrichment media and yeast can be varied to achieve higher or lower densities of rotifers as required for the stage of the culture system. The INVE product *Culture Selco* appears to be the enrichment of preference for rotifer culture and densities of over 250 animals/ml can be easily achieved cost effectively using a combination of relatively low amounts of *Culture Selco*, yeast and *Nanochloropsis* in a batch culture. Some hatcheries in the past have used the published method of INVE involving super high levels of *Culture Selco* only but, as far as the author is aware, are currently not doing so.

Recently, some work has been undertaken by the Darwin Aquaculture Centre in the Northern Territory using an algal paste as the only production and enrichment ingredient for rotifers. Those authors (Bosmans *et al.*, 2004) claim great success with that method, described elsewhere in this proceedings, and the use of algal paste in comparison to culturing algae has much economic merit.

Artemia production follows standard procedures. The enrichment product of choice for Artemia appears to be the INVE product *DC DHA Selco*.

Weaning diets vary according to availability and price. Proton (INVE) 3-5 is the most prevalent product used. Some hatcheries use Nippai although availability and price prevent more widespread use of this and Skrettings Gemma Micro is becoming more widely used. Weaning can be a period when large losses of fingerlings occur. Weaning techniques incorporate continuous delivery of feed using automatic feeders and careful monitoring of feed delivered to ensure enough feed is provided. A high degree of technical skill on the part of the person weaning the larvae is important in ensuring good survival through this phase.

Larval Culture System

Greenwater culture, usually utilising *Nanochloropsis*, is almost universally used for intensively rearing barramundi larvae. Water exchange varies from zero at the start of the culture to 100% by Day 14 ah. Water exchange may be driven to a large extent by the concentration of available algae but, by preference, it follows a pattern similar to that in Figure 1. Larvae are stocked at between 12.5 and 37.5 larvae/L. Stocking density used is dependent upon the number of larvae, the number of available rotifers, the number of fingerlings required and physical constraints such as water supply, tanks available and labour. Fish grow more quickly and show fewer deformities at lower stocking densities than at higher densities and risk management dictates that larvae are divided between a number of tanks.

Figure 2. Photograph of the GFB Fisheries Ltd hatchery showing typical tank design and operation of a greenwater culture.

Tank size used varies between 2000 L and 10000 L but a tank of 5000 L provides a good commercial combination of numbers produced/unit labour combined with adequate control of the biological and physical constraints.

Tank design usually incorporates a slight cone in the bottom to facilitate removal of waste during water exchange. Keeping the inside of the tank free of structure facilitates cleaning, sterilisation and limits surfaces for bacterial growth. Internal screens of 500μm to day 10 followed by 1 mm prevent egress of fish from the tank. Additional aeration is provided by normal aquarium air stones at a density determined by the surface area of water rather than volume. A tank of 2.5 m diameter would have 5 to 7 air stones providing aeration. Layout of a typical hatchery is shown in Figure 2.

Hygiene

Losses of larvae to disease occur from either infection by Viral Nervous Necrosis (VNN or Nodavirus) or by bacterial infection. While it is possible with excellent husbandry, low stocking densities and particular attention to hygiene to rear larvae infected with VNN, it is undesirable. The resulting fingerlings may infect other fish in the production system, may cause a residual infection in the farm particularly those farms where complete sterilisation is not possible and may not perform with regard to growth rate and food conversion as well as uninfected fish perform later in the growth cycle. Therefore, it is important to prevent infected larvae entering the hatchery. This is easier said than done, however. Determination of VNN infection in broodstock is unreliable with a reasonable probability of a false negative result. A prudent management regime involves testing broodstock for carrier status and removing those that are positive. As a secondary barrier to transfer, eggs are treated with an iodine bath which provides some protection in the event that the screening of broodstock failed to identify a carrier. All larvae in Queensland are tested for VNN using histological techniques according to State determined protocols prior to shipping interstate. Most hatcheries test all batches of fish in order to prevent distribution of infected fish through the industry.

Bacterial infections occur occasionally. Although it is difficult to determine the precise cause of these infections, a *Vibrio* species is the most likely agent. Prevention of bacterial infections involves using strict hygiene protocols. Tanks are cleaned using various protocols incorporating detergent, chlorine, acid and sterilized water. Incoming water to hatcheries is sterilized with chlorine subsequently neutralized by sodium thiosulphate or by ozone. Sterilised water is then usually filtered through 1 μm filters prior to distribution to larval tanks. Bacteria can be treated upon emergence of a disease or prophylactically with a fresh water bath at about Day 10 ah, or with the use of antibiotics. Most hatcheries operate using a minimum of antibiotics in order to prevent development of resistance.

Introduction of disease can also occur with introduction of algae or rotifers. As well as bacteria, it is possible to introduce ciliate infections by this route. Prevention of ciliate transfer is achieved by passing algal and rotifer cultures through filters but is limited by the need to use filtration that allows the food organism to pass.

Deformities

The prevalence of physical deformities in fingerlings, particularly spinal deformities, is known to be greatly affected by dietary vitamin C levels during early rearing (Fraser, 2002). Therefore, it is important that whatever rearing technique for rotifers is used ensures that the level of vitamin C delivered to the larvae is adequate to allow cartilage development and ossification to prevent deformities. Incorporation of sufficient levels of *Culture Selco* appears to have achieved this with spinal deformities in larvae being less than 1%. Swim bladder deformities determined by histological section may or may not impact on subsequent performance of the fish appear to be about 5% of larvae produced. Use of surface skimmers to prevent build-up of surface oils on the water up to Day 6 ah is important to optimize swim bladder inflation.

Constraints

While techniques for rearing barramundi larvae are reasonably well defined, industry constantly strives to improve survival and reduce costs. This must be done in an environment where changing infrastructure design can be very expensive, both in terms of capital cost and in terms lost production. Prevention of disease particularly VNN and providing alternatives to, or less expensive methods for, culturing algae and live feeds would provide opportunities to improve profitability in commercial hatcheries.

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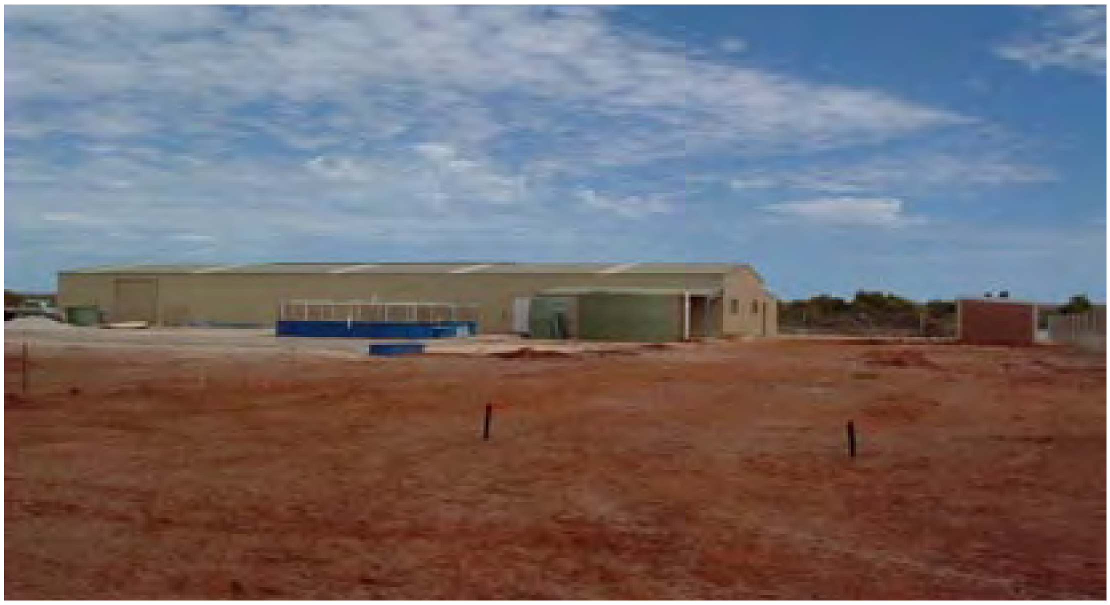
Company profile

Marine Farms Limited ("MFL") is an unlisted public company that was formed to develop a commercial mahi mahi farm. The company has well-established R&D and commercial credentials in its main area of expertise: the culture of marine fish.

Over the past three to four years, Marine Farms conducted an R&D programme at a facility it constructed in North Fremantle. The main purpose of this work was to develop the culture technology needed to proceed with the commercial development. The company is now in the process of constructing its first commercial aquaculture facility at a site it has secured near Exmouth, in the North-West Cape region of WA. Before selecting this WA site, the company identified and evaluated several other sites in New South Wales, Queensland and the northern Territory, as well as overseas in New Caledonia.



The commercial development is being staged through an initial pilot farm with a production capability of approximately 50 tonnes per year. Production will then be increased through successive increments and Marine Farms anticipates reaching an output of approximately 500 t/yr within three to four years. The ultimate yield from the farm has yet to be determined but will be governed by factors such as the availability of high-quality sea water and the market demand.

The mahi mahi farm is vertically integrated, in that it has its own dedicated hatchery to produce seed stock as well as growout facilities. The farm will use above-ground tanks for growing the fish to a market size of approximately 4.5 kg.

Initial production from the farm will be sold in domestic markets. The company has also identified and targeted several export markets for the fish.

Production capacity

The farm is currently under construction. The hatchery is operational but not quite completed. The first few growout tanks are being constructed. The growout tanks represent a new design for land-based marine aquaculture and are also under development.

The project will be developed in stages, the first of which will have a production capacity of 250 tonnes per year. Through ensuing development stages, we expect to continuously increase the production capability of the farm. The ultimate yield from the site will be governed by factors such as the quantity of sea water that can be extracted from the aquifer (we are using beach wells for sea water supply) and the ecology of the receiving environment. We are also focusing on developing a treatment system for used sea water. The project is being developed to be consistent with the principles of ecologically sustainable development.

We generally hold a group of five to six spawning stock, comprising one male and four to five females. The mature fish spawn perennially.

Live foods used

Artemia is the principal live food used.

Inert feeds used

We use inert diets starting from a particle size of 500 microns.

Systems

The project employs an intensive, land-based system using sea water pumped from an aquifer. There is no recirculation. Larvae are reared in cylindroconical tanks using clear water, flow-through techniques. After weaning and a pre-growout period, the fish are transferred to the growout farm.

The growout farm uses a series of above-ground tanks and a flow-through sea water supply system. The loading (kg fish per litre per minute water flow) varies according to the size of the fish. Some R&D is still being carried out in the growout area, but we anticipate growing fish to a market size of 4.5-5.0 kg.

Hygiene

The sea water supply is pumped from an aquifer, so is relatively clean and free of parasites and potential pathogens. The production system does not rely on the routine use of any chemical substances. Standard hatchery hygiene procedures are stringent.

Specific problems

It is very difficult catching wild broodstock and getting them back alive. Fortunately our F1 stocks are nearing maturity. We will have to introduce wild fish periodically. Larval rearing is at an acceptable level right now, but we plan to do additional R&D to improve survival. Weaning: similar comments to larviculture. We do get a little cannibalism and tail biting but nothing too serious.

There are many unknowns about the growout of the species in intensive land-based tanks. This is the main area on which we will be focusing our R&D efforts over the next two years.

MG Kailis Exmouth Hatchery

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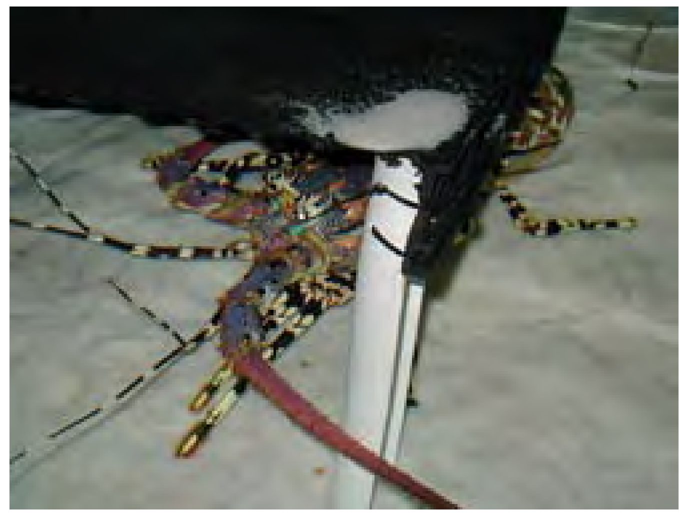
Background

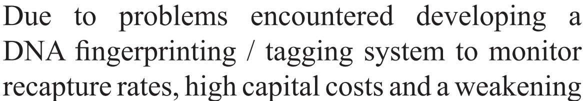
The MG Kailis Group is involved in tuna farming in South Australia, lobster fishing in WA, tropical rock lobster fishing in the Torres Straits and Great Barrier Reef, prawn fishing in Exmouth and pearl oyster farming from Exmouth north to Darwin in the NT.

The Exmouth Gulf is just north of the Tropic of Capricorn on Australia's West Coast. The MG Kailis Group (MGK) has a long history in the Exmouth Gulf – from a time even before the town of Exmouth existed. Against the odds posed by a remote and inhospitable location, sometimes severe cyclone seasons and reports that commercial quantities of prawns did not exist, the Group's founder Michael Kailis pioneered the Exmouth Gulf Prawn Fishery in the early 1960's. MGK now owns and operates over 90% of the catching vessels in the Exmouth Gulf fishery.

When in the early 1990's the MGK group purchased some prawn fishing licences from NorWest Seafoods, they got a prawn processing facility thrown into the deal. Around the same time they bought out a pearling company's pearl quota and got a pearl oyster hatchery near Broome as part of that deal. As they already had their own prawn processing facility in Exmouth it was decided to convert the old NorWest Seafoods factory into a pearl oyster hatchery. The equipment from the Broome hatchery was trucked down to Exmouth and the MG Kailis Exmouth Hatchery started operating in 1995.

Initially solely pearl oyster spat were produced then later in 1999 the hatchery started to diversify and began trials into the propagation of tropical abalone (*H.asinina*). Next in mid-2000 a Prawn Fishery Enhancement project commenced as a joint venture with CSIRO and Fisheries WA. MGK built a super-intensive juvenile prawn raceway production system on a semi-commercial scale. During trials growing Brown Tiger prawns (*P.esculentus*) MGK achieved the highest prawn production densities anywhere in Australia at the time.





Due to problems encountered developing a DNA fingerprinting / tagging system to monitor recapture rates, high capital costs and a weakening prawn price, MGK put the prawn fishery enhancement project on hold. At this point the company decided to concentrate it's R & D efforts into lobster propagation. It had already funded the QDPI to do research into Tropical Rock Lobster (TRL) propagation and grow-out trials in Cairns for the previous three years. MGK now entered a three- year FRDC project as the commercial partner in a TRL propagation project, together with QDPI Cairns and AIMS Townsville.

Whilst doing R & D work with various species (currently lobsters) MGK Exmouth Hatchery remains predominantly a commercial pearl oyster hatchery.

Species cultured

Pearl Oysters (*P.maxima*), Prawns (*P.esculentus*) (project on hold at present), Lobsters (*P.ornatus, P.versicolor*)

Production Capacity

Pearl Oysters – 2 batches per year; 3-5 million spat; spawning October – March;

Prawns – 1 batch per year; 1 million juveniles (0.5-1.0g) spawning September; R & D - Fishery Enhancement

Lobsters – 6 batches per year; R & D – Lobster Propagation

Live Feeds used

Algae (*C.calcitran, T.isochrysis, P.lutheri, C.muelleri, T.suicica*), *Artemia*

Inert Feeds used

Prawn artificial feeds, 50-1000 micron. Various fresh feeds including squid, mussels and other molluscs

Systems and Rearing Methods

Pearl Oyster Larvae: 500 – 15,000 litre tanks - static, semi-continuous flow-through and continuous flow-through. Prawns: parabolic larval tanks 10,000 litre - semi-continuous flow-through; raceways 60,000 litre – continuous flow-through, re-circulation closed system.

Lobsters: tanks 100 – 5000 litre – re-circulation closed system. All species on clear-water / Galveston type system

Algae

All algae (*C.calcitran, T.isochrysis, P.lutheri, C.muelleri, T.suicica*) batch-fed for pearl oysters and for Artemia enrichment

Hygiene

Pearl Oysters – sterile seawater rinsing of eggs only. Prawns – iodine and seawater rinsing of eggs. *Artemia* – Hatch Controller DC, freshwater rinsing. Water (plus nutrients, fittings etc.) for algae – autoclaved. Water for animals – one micron filtered and UV treated (plus ozone on re-circ. system).

Specific Problems and Issues

Inadequate artificial diet for larval, grow-out and broodstock lobsters – fresh diets too variable in quality and expensive / labour intensive

Overview of lobster aquaculture research

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Introduction

Lobsters (family of Palinuridae and Scyllaridae) are highly prized seafood with a ready market. The Western rock lobster (*Panulirus cygnus*), the Southern rock lobster (*Jasus edwardsii*), the Eastern rock lobster (*Jasus verreauxi*), the Ornate rock lobster (*Panulirus ornatus*) and Slipper lobsters (eg., *Thenus* spp., *Ibacus* spp.) together comprise the highest value fisheries in Australia with over $460 million annual production value (ABARE, 2003). In most cases the wild stocks are highly utilised with management controls being applied to assure successful year-by-year recruitment. Aquaculture development is one of the keys to expanding production levels of these lobster species.

The major unsolved problem for developing aquaculture techniques of lobster species lies in the successful maintenance of the planktonic larval (phyllosoma) stages, where the phyllosoma stages are longer-lived (150 to 300 days) and oceanic in natural habitat. Although significant efforts for advancing rearing methods of rock lobster phyllosomas have been made in recent years, mass-culture of rock lobster phyllosomas is still not feasible at this stage. However successful results have been reported from a recent study of the slipper lobster species (*Thenus* spp.), where phyllosomas of *Thenus* spp. pass through only 25 to 30 days with high survival rate (>80%), and juveniles can grow to market size (250g) within 400 days in the growout phase (Mikami, 1995). This paper summarizes current rock lobster research and examines the potential to apply information and techniques obtained from larval rearing of *Thenus.*

Life cycle of lobster species

The families of Palinuridae (rock lobsters) and Scyllaridae (slipper lobsters) exhibit five major phases within the life cycle: adult, egg, phyllosoma, puerulus/nisto and juvenile. The male lobster deposits a spermatophoric mass (spermatophore) on the female's sternum during mating, and fertilisation of the eggs occurs when the female spawn the eggs onto the abdomen and pleopods. The time between mating and fertilisation of eggs differs depending on the species. For example, in *Panulirus japonicus* it is less than an hour, whereas *J. edwardsii* carry the spermatophore for months before fertilisation. Phyllosomas (derived from the Geek *phyllos* meaning leaf and *soma* meaning body) are planktonic larvae, dorsoventarally flattened with a transparent body and long appendages. In the case of palinurid lobsters, the phyllosoma period extends for a number of months (9 to 14 months) with many moulting stages, resulting in large late stage phyllosomas (often >35mm). After a number of months in the ocean, planktonic phyllosomas metamorphose to the briefer, non-feeding, free-swimming post-larval phase (puerulus for palinurid lobsters and nisto for scyllarid lobsters), the transaction stage between planktonic phyllosoma and benthic juvenile stages. The puerulus/nisto apparently navigates to the juvenile nurseries by complex receptor systems formed by the antennae and a pinnate setal system, and once in the nursery grounds, the puerulus/nisto settles into preferred habitats. The understanding of larval recruitment of many lobster species is fragmentary due to the complexity of the oceanic habitats of phyllosomas, and much information concerning the biology of oceanic phyllosomas, such as food, behaviour, predation and physical environmental requirements, is still missing.

Overview of phyllosoma rearing attempts

There have been a number of successful rearings of scyllarid lobsters phyllosomas (eg., *Scyllarus americanus* (Robertson, 1968); *Ibacus ciliatus and Ibacus novemdetatus*. (Takahashi & Saisho, 1978); *Scyllarus demani* (Ito & Lucas, 1990); *Ibacus perionii* (Marinovic et al., 1994); *Thenus* spp. (Mikami & Greenwood, 1997)) due to the relatively shorter phyllosoma periods (a month to a few months). On the other hand, the complete phyllosoma rearing of palinurid lobster species has been recorded by only four institutes, three from Japan and one from New Zealand (see table). However these successful rearings have been limited in scale, often using systems of less than 10l, and with less than 1% survival to the juvenile stage.

Successful records of palinurid lobster phyllosomas

Species	Duration	Temperature	Source
Southern rock lobster (*J edwardsii*)	300-324 days	10-20ºC	Kittaka *et al*. (1988), Illingworth *et al.* (1997)
Cape rock lobster (J. lalandii)			Kittaka (1988)
European rock lobster (*P. elephas*)	132 days	17-19ºC	Kittaka and Ikegami, (1988)
Japanese spiny lobster (*P. japonicus*); White-whiskered rock lobster (*P. longipes*); Double-spined rock lobster (*P. penicillatus*)	app. 200 days (shortest)	24-28ºC	Matsuda (pers. comm.) Murakami (pers. comm.)
Scalloped spiny lobster (*P. homarus*)	app. 180 days	24-28ºC	Murakami (pers. comm.)
Eastern rock lobster (*J. verreauxi*)	184-341 (av. 341) days	15-20ºC	Kittaka (unpublished) Tong (pers. comm..)

To date (Sep 2004), the most advanced research groups are perhaps the National Centre for Stock Enhancement (NCSE, formerly known as JSFA) at Izu, Japan and the Mie Prefectural Science and Technology Promotion Centre, Fisheries Research Division (MPSTPC), at Hamajima, Japan. These groups are capable of producing up to a few hundred juveniles per year. The keys for their success may be the highly sterilised rearing environments and the use of antibiotics (bacterial control). However, their previous methods have been unsuitable for large-scale production (>1,000 juveniles) due to difficulties in expanding and maintaining sterilised facilities and the heavy use of antibiotics. In fact, though they achieved yearly production of a few hundreds juvenile several years ago, no further major achievement has been reported in last few years.

In Australia there are four institutes studying palinurid lobster phyllosoma rearing. Tasmanian Aquaculture and Fisheries Institute (TAFI) is rearing *J. edwardsii* (Southern rock lobster) phyllosomas, and the Queensland Department of Primary industries Northern Fisheries Centre (QDPI NFC), AIMS and MG Kaillis are rearing *Panulirus ornatas* (Tropic rock lobster) phyllosomas. So far, no successful mass rearing of these phyllosomas to the juvenile stage has been reported.

Three key issues have now been identified for the successful rearing of oceanic phyllosomas; 1) nutrition, 2) hygiene (bacteria/virus control) and 3) hydrodynamics (tank design). Although Artemia and mussel gonad have been used a food source, poor survival and growth rate records indicate that they may not be ideal in terms of quantity and quality, particularly for late stage phyllosomas. Zooplankton (eg., *Sagittas*, Copepod), hydromedusa and fish larvae have also been tested previously, but have not been successful due to difficulties in maintaining a continuous supply. The development of artificial diets is one solution for overcoming the nutritional problem, and NIWA and CSIRO Cleveland are currently working on this.

Bacterial/viral control is another key issue for the rearing of long-lived oceanic larvae under controlled environments. Traditionally, a number of antibiotics (eg. OTC, Streptomycin, Chloramphenicol) and chemicals (eg. formalin) have been heavily used for controlling bacterial colonies in the rearing water. Though antibiotics and chemicals are strong agents for minimising bacterial growth in the laboratory, they are not considered a long-term solution for the large scale rearing of lobster larvae. Alternative disinfection methods, such as UV and Ozone (O_3), should be considered.

Tank design with consideration of hydrodynamics is crucial for the maintenance of fragile phyllosomas. Because of the phyllosomas' unique morphology (flat body and long appendages), strong aeration can damage body segments. No water movement, or only gentle water movement, can be used in the tank. Movement of rearing water also needs to take into consideration the phyllosomas' behaviours (swimming, feeding and phototactic), food distribution and maintenance of tank system.

Development of Thenus aquaculture

Bay Lobsters (*Thenus* spp.), commonly known in Australia as Moreton Bay bugs, live on the sandy or muddy sea floor in coastal waters up to 30m deep. In Queensland, Bay lobsters represent an estimated 4% of the annual commercial catch by weight, however they are becoming increasingly sought after as a valuable seafood product, emphasizing the need for aquaculture to meet these demands. Females spawn as many as 60,000 eggs per individual during the summer. Phyllosomas hatched from eggs go through 4 moult stages, then metamorphose to the benthic nisto stage. After 19 moults, juveniles can reach a typical market size of about 250g. The time taken from eggs to market size depends on temperature and food supply, but generally ranges 400 to 450 days. Despite the advantage of a short growout phase, there has been no record of commercial production of *Thenus* anywhere in the world. The major hurdle in the commercialisation of *Thenus* aquaculture has been the difficulty in maintaining the phyllosoma stages. Recently however, the scientific riddle of growing Bay lobsters in a laboratory from eggs to juveniles was solved, with consistent survival of over 80%. A pilot system for growing Bay lobsters has been operating successfully, and the establishment of a commercial scale operation is now underway.

Pilot systems for larval rearing of Thenus (at BIARC)

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Development of marine fish larval diets to replace *Artemia*

PART 2

Paul Southgate

FRDC Project No. 2001/220

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Development of marine fish larval diets to replace *Artemia – Part 2*

Paul Southgate

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1.0 JAMES COOK UNIVERSITY

SUMMARY

Research at James Cook University focused on developing more appropriate feeding regimes for barramundi larvae in four areas: (1) evaluation of currently available commercial foods during larval culture; (2) development of more appropriate weaning protocols for larvae; (3) investigation of dietary protein and energy requirements of larvae; and (4) assessment of the characteristics of food particles prepared and processed using different methods.

Commercially available inert foods were assessed for their nutritional value for barramundi larvae. The best of these could be used to replace 30% of a standard ration of live food (*Artemia*) without affecting growth when fed to larvae from 12 days after hatch or older. An experimental diet developed during this project supported similar larval growth rates to the best of the commercial foods when used at the same level of *Artemia* replacement. Larvae fed diets where 25%, 50% and 75% of a standard *Artemia* ration was replaced by the commercial diet or the experimental diet grew at a similar rate to larvae fed a full *Artemia* ration. Both inert foods were of high nutritional value for barramundi larvae and performed similarly in weaning trials where larvae could be fully weaned by 23 days after hatch. This is a more rapid weaning protocol than used by some commercial barramundi hatcheries and may offer significant cost savings.

Protein is the largest and most expensive component of formulated feeds for fish. Experiments were conducted to determine qualitative and quantitative protein requirements of barramundi larvae from 14 days after hatch. Feeding trials identified a mixture of 9:1 fishmeal:squid powder as a superior dietary protein source for use in inert larval foods. Feeding trials also identified a dietary energy content of 21 MJ/kg to be optimal and thyroid hormone analysis indicated a 50% dietary protein content to be optimal. The data generated will allow fine-tuning of the experimental diet developed during this project based on specific nutritional requirements of barramundi larvae.

Regardless of diet formulation, the performance of inert food particles is influenced to a large extent by physical and behavioural characteristics such as buoyancy, stability following immersion and digestibility. The size, shape and surface texture of diet particles varied considerably according to the preparation (crumbled vs. marumarisation) and drying (moist, oven-dried and freeze-dried) technique. Freeze-dried particles had a greater surface area to volume ratio compared to oven-dried particles and moist marumarised particles. The majority of amino acid leaching (38% and 71% of potential total) from diet particles occurred 1-5 minutes after immersion. Crumbled particles leached more than marumarised particles which also maintained the greatest degree of structural integrity. Particle settlement rate depended more on drying technique than method of preparation with marumarised particles maximising the quantity of diet particles available to fish larvae. Drying method did not affect rates of ingestion or assimilation of diet particles. Such data are useful towards development of a suitable particle type for presentation of formulated diets to marine fish larvae.

1.1 ARTEMIA

1.1.1 The effects of various enrichment protocols on growth and survival of Barramundi (*Lates calcarifer*) larvae

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1.1.1.1 Introduction

The nutritional value of rotifers and *Artemia* is largely determined by their food. Generally, these live foods have naturally low levels of essential fatty acids (EPA and DHA) that are required for growth and development of marine fish larvae. In order to overcome these deficiencies, live foods must be 'enriched' prior to feeding to fish larvae (Southgate, 2003). Several enrichment materials can be used including microalgae, oil suspensions, microencapsulated diets and yeasts (Leger et al. 1986), and many fatty acid enrichment preparations are available commercially. Two experiments were conducted to determine a baseline feeding protocol for barramundi larvae as a basis for future experiments. The first determined whether it is beneficial to enrich rotifers fed to barramundi larvae and which of two commercially available *Artemia* enrichment products (Super Selco or DHA Selco) supported the greatest growth and survival of barramundi larvae. The second experiment determined the effects of various *Artemia* enrichment products on growth survival and physiological condition of barramundi larvae.

1.1.1.2 Materials and methods

Experiment 1

Rotifers were reared at a density of >350 mL^{-1} in 140 L flat-bottomed round tanks. *Nannochloropsis oculata* was added to the tanks twice daily as the sole food source. Rotifers selected for further enrichment were placed into a 20 L carboy containing 10 L of 1-µm filtered seawater at 25‰. The enrichment mixture consisting of DHA Protein Selco which was prepared by blending 2.5 g of DHA Protein Selco with 0.75 L freshwater at high speed for three minutes was then added to the 20 L carboy in two equally measured and spaced rations.

Artemia cysts were hydrated and decapsulated before being placed into a 20 L carboy containing 10 L seawater at 25‰. Vigorous aeration was applied along with constant light. *Artemia* were harvested 24 hours later and nauplii were divided among three 1.5 L conical beakers, two of which were enriched with either Super Selco or DHA Selco whilst the third (control) was untouched. The enrichment procedure followed manufacturer's instructions by offering one ration in the afternoon and leaving until the following morning. After the morning feed, remaining *Artemia* were placed in the refrigerator at 4°C with aeration until required.

Barramundi larvae (at 2 days after hatch [DAH]) were stocked into 20 L aquaria at a density of 8 L^{-1}. Six diet treatments were assessed:

1) Micro-algae enriched rotifers and non-enriched *Artemia.*
2) Micro-algae enriched rotifers and Super Selco enriched *Artemia.*
3) Micro-algae enriched rotifers and DHA Selco enriched *Artemia.*
4) DHA Selco enriched rotifers and non-enriched *Artemia.*
5) DHA Selco enriched rotifers and Super Selco enriched *Artemia*.
6) DHA Selco enriched rotifers and DHA Selco enriched *Artemia.*

At the start of the experiment, larvae were fed rotifers twice daily at a density of 10 rotifers.mL^{-1} until 15 DAH. At 8 DAH, larvae were offered newly-hatched *Artemia* twice daily at a density of 0.5 nauplii.mL^{-1}. From 11 DAH until the end of the experiment at 19 DAH, larvae were fed *Artemia* at 1.5 nauplii .mL^{-1} twice daily. Each treatment was replicated three times. Uneaten food was flushed from the aquaria overnight through either a 250 or 500 µm banjo sieve.

Larval growth was assessed by measuring total length (TL) and dry weight (DW). TL was measured from digital images taken with a JVC300 camera mounted on a Leica 12.5 dissecting microscope and measured using Leica IM50 measurement module. DW was determined by first washing the larvae with distilled water before oven drying them at 50°C to constant weight. Larvae were then weighed on a Cahn 33 microbalance to 0.1 mg. Data were analysed using a one-way ANOVA and Tukey's HSD.

Experiment 2

Artemia cysts were hydrated and decapsulated and placed into a 20 L carboy containing 10 L vigorously aerated seawater at 25‰. *Artemia* nauplii were harvested 24 hours later. Four enrichment products were assessed:

1) AlgaMac 2000 (source: Aquafauna);

2) Super Selco (source: Primo);

3) Super HUFA (source: Aquasonic); and

4) Experimental enrichment preparation (source: Sagiv Kolkovski, WA Fisheries).

A control treatment of unenriched *Artemia* was also included. Enrichment materials were prepared according to manufacturer's instructions. Algamac was prepared by blending 0.4 g of product with 400 mL water. The remaining enrichments were prepared by blending 1.2 g of product with 400 mL water. The enrichment procedure was performed in 4 L aquaria containing 2 L of 25‰ seawater and 200,000 newly hatched *Artemia* nauplii. Enrichment suspensions were added to respective aquaria at 16:00 h. Vigorous aeration with supplemental oxygen was applied to aquaria to maintain oxygen levels in the cultures. Unenriched (control) *Artemia* were treated identically without addition of enrichment product. *Artemia* were harvested at 08:00 h the following morning and washed thoroughly before being counted and fed to larvae. *Artemia* were refrigerated between feeds.

Barramundi larvae at 14 DAH were used in this experiment. Larvae were reared according to standard rearing protocols (Appendix 1) until the start of the experiment. Larvae were stocked into 20 L aquaria at a density of 15 L^{-1}. Mean (± S.E., n=30) total length (TL) and dry weight (DW) at this time were 5.17 ± 0.22 mm and 0.57 ± 0.05 mg, respectively. Each aquarium was provided with a flow-through supply of 1-µm filtered seawater through a 4 mm diameter tube at a rate of 5 L.h^{-1} between the hours of 23:00 h and 07:00 h daily. Water exited through a 500 µm screened outlet. Water was lightly aerated and a photoperiod of 16L:8D was maintained for the duration of the trial. There were five diet treatments:

1) Algalmac 2000 enriched *Artemia*;

2) Super Selco enriched *Artemia*;

3) Super HUFA enriched *Artemia*;

4) *Artemia* enriched with the experimental enrichment preparation; and

5) Non-enriched *Artemia* (control).

Each treatment was assessed in triplicate and randomly assigned to each aquarium. Larvae were fed four times daily at 09:00 h, 11:30 h, 14:00 h and 16:30 h, at a rate of 1 nauplius.mL^{-1} per feed.

The growth trial ran for 14 days and was terminated when the larvae were 28 days after hatch. Larval growth was assessed, by measuring TL and DW as described above. The physiological condition of the larvae at the end of the experiment was evaluated by measuring their resistance to stress using a salinity-stress test (Dhert et al. 1990). Ten fish from each replicate were transferred into beakers and 65 ‰ water was added. Larval mortality was recorded at 10 minute intervals for 140 minutes.

1.1.1.3 Results

Experiment 1

There was no significant difference in mean percentage survival between the treatments. However, larvae fed micro-algae enriched rotifers and Super Selco enriched *Artemia* had the highest mean percentage survival (54%) (Fig. 1).

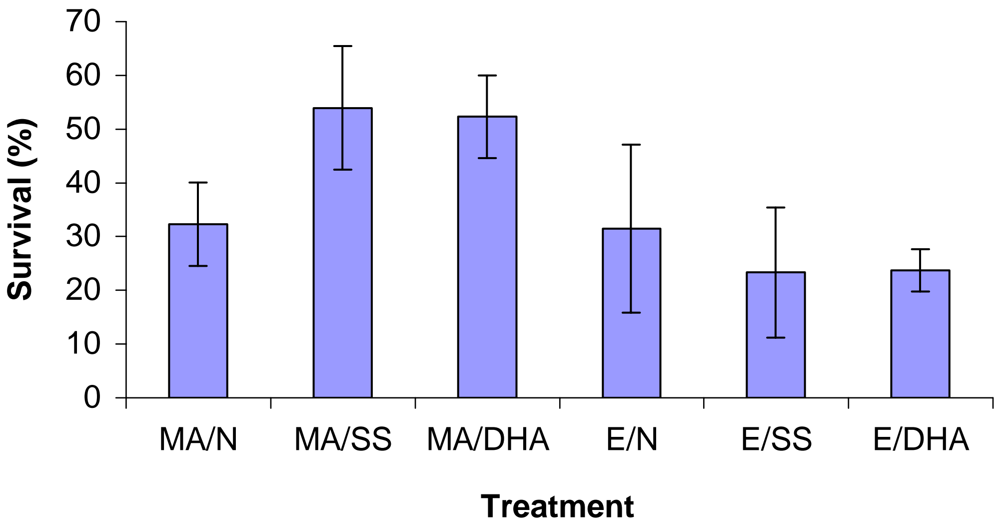

Figure 1. Mean (± S.E., n=3) survival of barramundi larvae fed various enrichment treatments, where MA = rotifers fed microalgae only, E = DHA protein enriched rotifers, N = non-enriched *Artemia*, SS = *Artemia* enriched with Super Selco, and DHA = *Artemia* enriched with DHA Selco. There were no significant differences between means ($P > 0.05$).

There was no significant difference in mean DW of larvae between treatments. Although larvae in the control treatment receiving micro-algae enriched rotifers and non-enriched *Artemia*, had a lower mean DW (1.38 mg) than larvae in other treatments (Fig. 2).

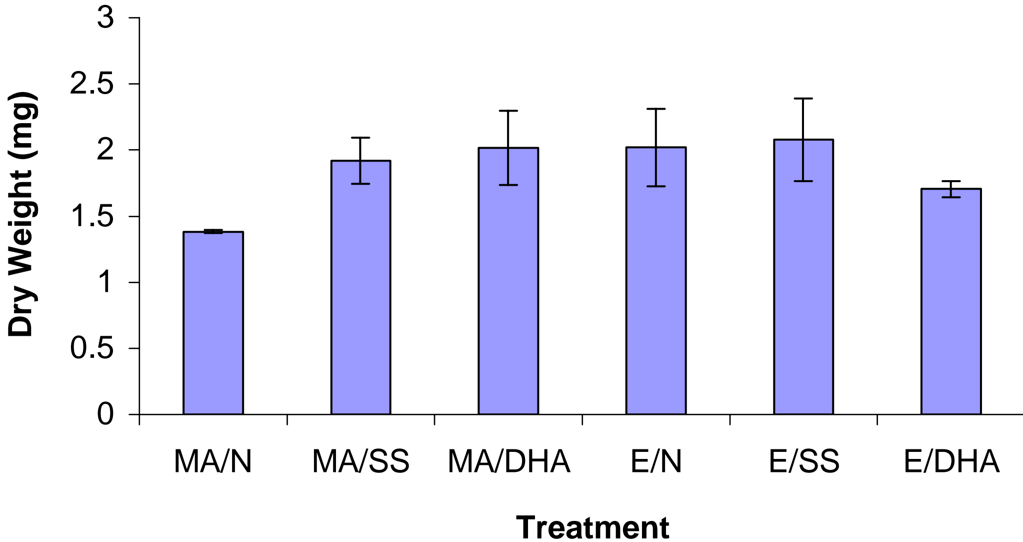

Figure 2. Mean (± S.E., n=3) dry weight of barramundi larvae fed enrichment treatments, where MA = rotifers fed microalgae only, E = DHA protein enriched rotifers, N = non-enriched *Artemia*, SS = *Artemia* enriched with Super Selco, and DHA = *Artemia* enriched with DHA Selco. There were no significant differences between means (P > 0.05).

There was no significant in mean TL between treatments (Fig. 3).

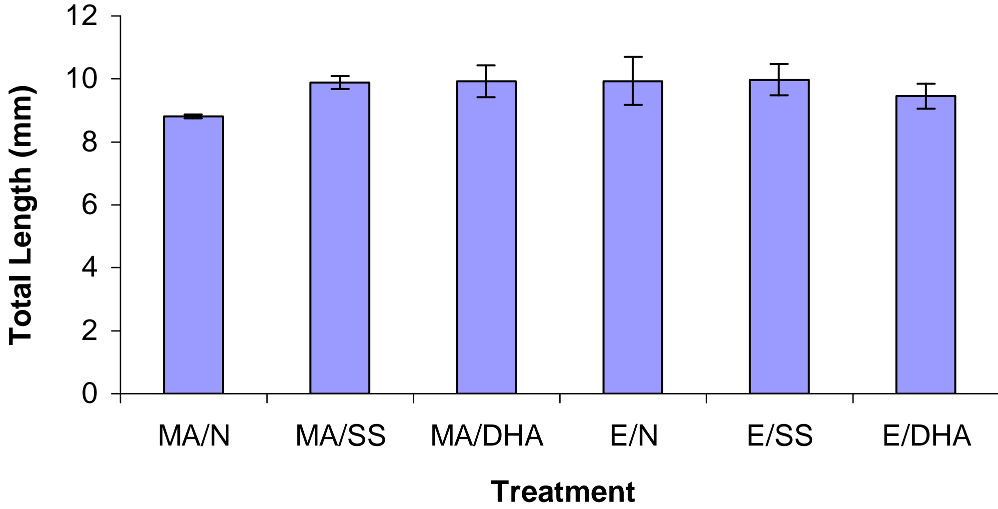

Figure 3. Mean (± S.E., n=3) total length of barramundi larvae fed various enrichment treatments (same abbreviations as above). There were no significant differences between means (P > 0.05).

Experiment 2

There was no significant difference in percentage survival of barramundi larvae between the different dietary treatments (Fig. 4).

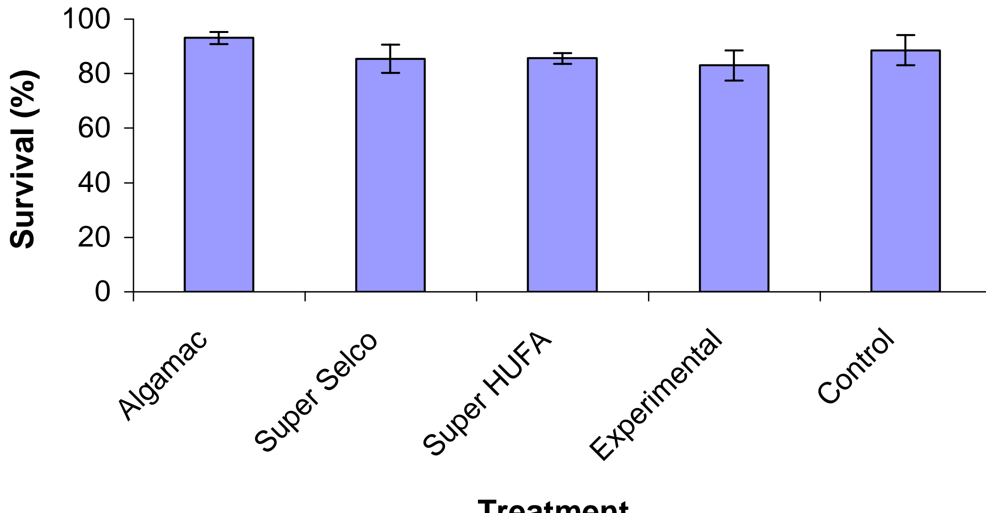

Figure 4. Mean (± S.E., n=3) survival of barramundi larvae fed enriched or unenriched *Artemia*. There were no significant differences between means (P > 0.05).

There was a significant difference in DW between treatments (Fig. 5). Larvae fed *Artemia* enriched with either Super-HUFA or the experimental enrichment diet had significantly greater DW than that of the larvae offered *Artemia* enriched with Algamac or unenriched *Artemia*, although not significantly different to larvae fed *Artemia* enriched with Super Selco.

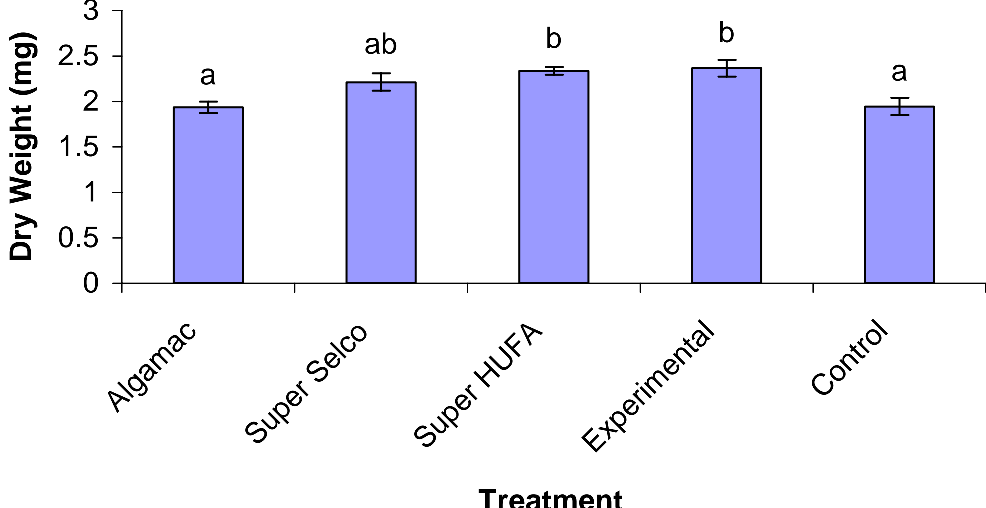

Figure 5. Mean (± S.E., n=3) dry weight of barramundi larvae fed enriched or unenriched *Artemia*. Means sharing the same superscript are not significantly different (P > 0.05).

There was no significant difference in mean TL of larvae between treatments although larvae fed *Artemia* enriched with the experimental enrichment diet showed the highest growth during the experiment period (Fig. 6).

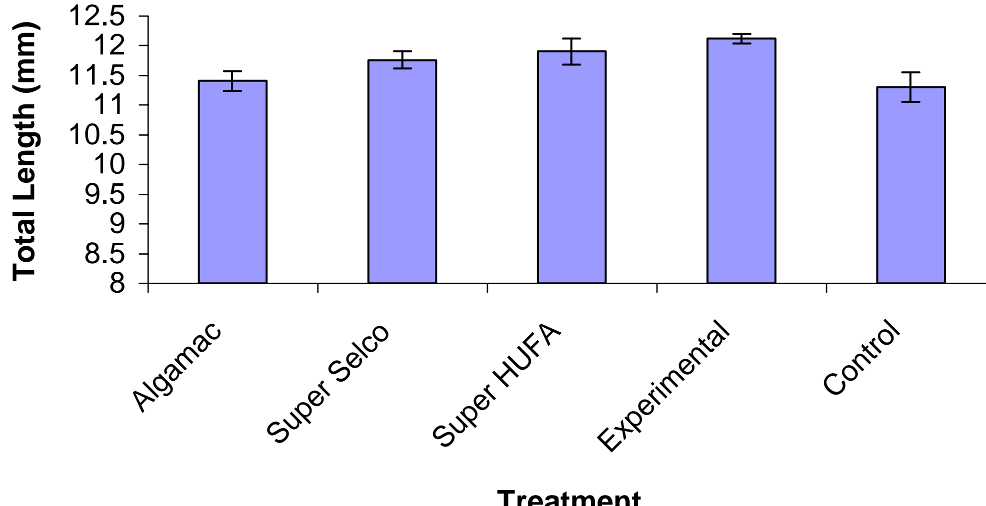

Figure 6. Mean (± S.E., n=3) total length of barramundi larvae fed enriched or unenriched *Artemia*. There were no significant differences between means (P > 0.05).

Behavioural differences between larvae fed *Artemia* enriched with the various preparations were observed. For example, larvae fed *Artemia* enriched with Super Selco, Super HUFA or the experimental enrichment preparation, appeared more silvery in colour, occupied the corners of aquaria in the bottom quarter of the water and moved with rapid, darting movements. Those fed Algamac-enriched *Artemia* or unenriched *Artemia* were darker in colour with the characteristic stripes; they stayed towards the water's surface and were placid and difficult to frighten.

Larvae fed *Artemia* enriched with Super Selco showed the lowest rate of mortality during exposure to high salinity and significantly lower mortality than the larvae in all other treatments up to 100 h (P < 0.05). Larvae fed unenriched *Artemia* showed the highest and most rapid rate of mortality under the same conditions. Mortality of larvae fed *Artemia* enriched with Algamac, Super HUFA and the experimental preparation did not differ significantly during the high salinity stress test (Fig. 7).

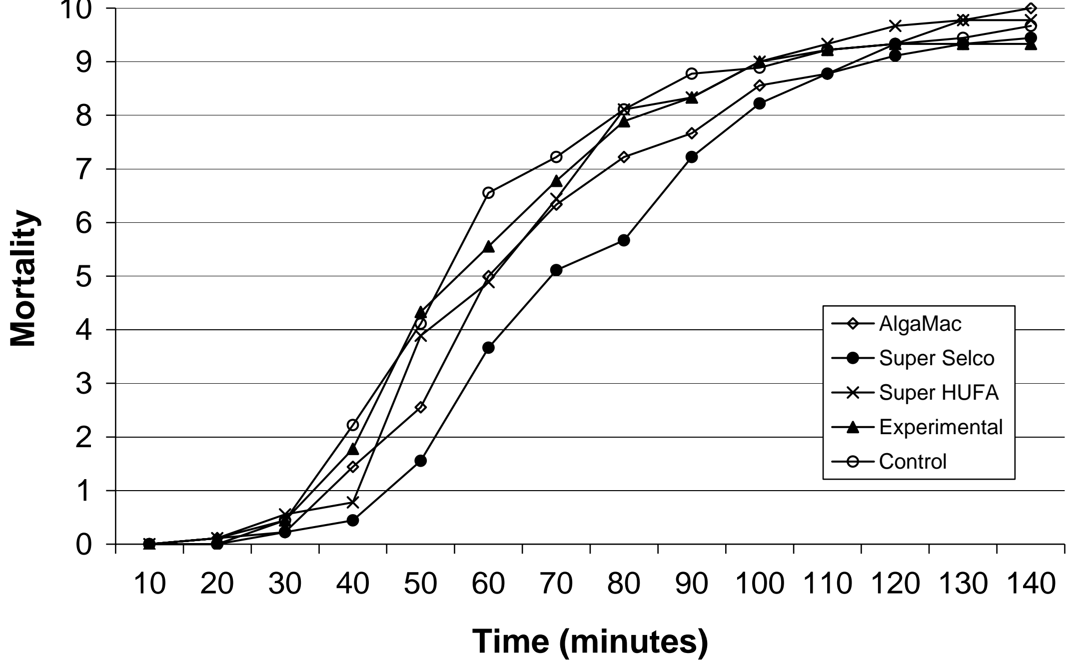

Figure 7. Mean mortality of barramundi larvae subject to high salinity (65‰) stress test for 140 minutes. Error bars omitted for clarity.

1.1.1.4 Discussion

The results of Experiment 1 showed that enrichment of rotifers (with anything other than micro-algae) prior to feeding to barramundi larvae provided no advantage in terms of growth and survival. Larvae in treatments receiving micro-algae enriched rotifers and *Artemia* enriched with either Super Selco or DHA Selco, had higher survival than those in any other treatment. The control treatment of micro-algae enriched rotifers and non-enriched *Artemia* resulted in larvae with the lowest mean TL and DW of all treatments. These results clearly indicate the benefit of providing barramundi larvae with enriched *Artemia*. This may reflect increasing requirement for dietary (*n*-3) HUFA during larval development as shown for red seabream larvae (Izquierdo et al. 1989a).

Although there were no significant differences between treatments for mean TL or survival in Experiment 2, larvae fed *Artemia* enriched with 'Super-HUFA' and the experimental enrichment preparation had significantly greater DW than larvae fed Algamac-enriched *Artemia* and those fed unenriched *Artemia*. It is well established that dietary (*n*-3) HUFA increase resistance of marine fish larvae to stress (Dhert et al. 1990). This study showed that larvae fed *Artemia* enriched with Super Selco had superior tolerance to stress when compared to those fed unenriched *Artemia* and *Artemia* enriched with Algamac, Super-HUFA and the experimental enrichment preparation.

Dietary lipid provides both energy and cell membrane components to developing fish larvae (Watanabe and Kiron, 1994). The levels of total lipid in larval diets are generally high in order to maximise growth and to meet energy and nutritional requirements of the developing larvae. Prior studies investigating dietary lipid requirements of marine fish larvae have reported that optimal growth was achieved with levels (in *Artemia*) of 29-37% for sea bream (Koven et al. 1992) and 25% *P. olivaceau* (Furuita et al. 1998). Optimum dietary lipid levels when presented in formulated feeds vary from 18-30% (Brinkmeyer and Holt, 1995; Salhi et al. 1999; Infante and Cahu, 1999). Similar studies using live prey have concluded that optimal level of dietary EPA and DHA for marine fish larvae are approximately 3% dry matter (Izquierdo et al. 1989; Watanabe and Kiron, 1994; Cahu and Infante, 2001). *n*-3 HUFA, particularly the essential EPA (C20: 5n-3) and DHA (C22: 6n-3), are found in large amounts within cell membranes (Cahu and Infante, 2001). Low levels of dietary (*n*-3) HUFA has been shown to result in poor growth and slow development of marine fish larvae, together with reduced survival and difficulties in metamorphosis (Bisbal and Bengston, 1991; Dhert et al. 1991; Koven et al. 1992; Watanabe, 1993; Watanabe and Kiron, 1994; Cahu and Infante, 2001). Clearly, commercially available fatty acid enrichment preparations must account of these requirements and Super Selco, Algamac and Super-HUFA contained 40%, 46.1% and 45% *n*-3 HUFA, respectively.

On the basis of these results, the preferred protocol for feeding live foods to barramundi larvae is to use rotifers fed with *Nannochloropsis* followed by enriched *Artemia*. Of the four fatty acid enrichment preparations assessed in Experiment 2, *Artemia* enriched with Super HUFA and the experimental enrichment preparation supported the greatest increase in larval dry weight, while those enriched with Super Selco supported greater stress tolerance in barramundi larvae. These findings have clear implications for commercial culture of barramundi larvae and provide the basis for optimising the larval rearing protocol used in subsequent research during this project.

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1.2 CO-FEEDINGS AND PROTOCOLS

1.2.1 Towards replacing *Artemia* with commercially available formulated feeds for barramundi (*Lates calcarifer*) larvae

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1.2.1.1 Introduction

There are a number of problems associated with the use of live foods for marine fish larvae including nutritional deficiency, nutritional inconsistency, reliability and, in particular, the cost of live food production (Southgate, 2003). As well as 'off-the-shelf' convenience, complete or partial replacement of live food during larval culture offers significant advantages relating to hatchery operating costs and more efficient hatchery production. However, numerous studies have shown that marine fish larvae reared exclusively on inert or formulated foods do not match the growth and survival performance of larvae fed live food organisms (Kanazawa et al. 1982; Tandler and Kolkovski, 1991; Southgate and Lee, 1993; Khemis et al. 2000). Some success has been reported, however, when inert foods are offered as a partial replacement for, or supplement to, live foods (Kolkovski and Tandler, 1995). In some cases, combined feeding of live and artificial diets (co-feeding) has supported superior growth and survival of early larvae that either live foods or inert foods alone (Walford et al. 1991; Jones et al. 1993; Rosenlund et al. 1997; Önal and Langdon, 2000).

The aim of these experiments was to investigate growth and survival of barramundi larvae fed commercially available and experimental formulated foods as a partial substitute for an *Artemia* ration.

1.2.1.2 Materials and methods

Experiment 1 – Replacement of 50%* Artemia *ration with commercial foods

In order to calculate the quantity of formulated diet needed to replace 50% of a standard *Artemia* ration, the mean dry weight of a single enriched *Artemia* nauplius was determined. *Artemia* were on grown for 24 h before being enriched with Super-HUFA (Aquasonics) for a further 24 h. *Artemia* were then harvested and rinsed in freshwater before being individually counted into five pre-weighed foil weighing-boats. *Artemia* were then oven-dried at 50°C to constant weight before the foils were re-weighed. The resultant dry weight per *Artemia* was determined as 4.13 µg. The total weight of *Artemia* based on a 50% ration size (0.5 nauplii.mL^{-1}) was then calculated to be 0.041 g. Treatment receiving 50% replacement of *Artemia* were fed a 50% *Artemia* ration along with 0.041 g of the designated formulated food. The commercial diets used in this experiment were:

1) Proton (source: Primo);

2) Aller Aqua (AA) (source: Ridley); and

3) Higashi Maru 'Kinko'(HM) (source: Higashi Maru, Japan).

Barramundi larvae at 12 and 16 days of age were used in this experiment. Day 16 larvae were offered particles in the size range of 300-500 µm. Day 12 larvae were offered 200-400 µm particles until day 16 and then larger 300-500 µm particles. Larvae were reared according to standard rearing protocols (Appendix 1). At 12 days of age, mean (± S.E., n=20) total length (TL) and dry weight (DW) of the larvae were 4.21 ± 0.11 mm and 0.23 ± 0.07 mg, respectively. At 16 days of age,

TL and DW of the larvae were 5.05 ± 0.06 mm and 0.31 ± 0.02 mg, respectively. Five dietary treatments were assessed in triplicate:

1) 50% *Artemia* ration and 50% Proton diet;

2) 50% *Artemia* ration and 50% Aller Aqua (AA) diet;

3) 50% *Artemia* ration and 50% Higashi Maru (HM) diet;

4) 50% *Artemia* ration only; and

5) 100% *Artemia* ration only.

Prior to the start of each growth trial, 300 larvae (15 larvae.L^{-1}) were randomly stocked into replicate 20 L flat-bottomed aquaria. Each was provided with a flow-through supply of 1 µm filtered seawater through a 4 mm diameter tube at a rate of 5 L.h^{-1} between 11:00 h and 07:00 h daily. Water exited aquaria through a 500 µm screened outlet, which prevented exit of the larvae. Water was gently aerated and a photoperiod of 16L:8D was maintained for the duration of the trial.

Treatments were randomly assigned to each aquarium. Larvae were fed 4 times daily, at 09:00 h, 11:30 h, 14:00 h and 16:30 h. Larvae fed the 100% *Artemia* ration were fed at a density of 1 nauplius.mL^{-1}. Unfed controls were included in the growth trials and larvae in these replicates were not fed for the duration of the trial. The bottom of each aquarium was cleaned (siphoned) daily.

Both growth trials (with both 12 DAH and 16 DAH larvae) ran for 14 days, after which larvae were removed by siphoning onto screens and then killed in ice slurry. Larval growth was determined as the increase in TL and DW. TL was measured from digital images taken with a JVC300 camera mounted on a Leica 12.5 dissecting microscope and measured using Leica IM50 measurement module. DW was determined by firstly washing the larvae with distilled water before oven drying at 50°C to constant weight. Larvae were then weighed on a Cahn 33 microbalance to 0.1 mg. Data were analysed using a one-way ANOVA and Tukey's HSD test. TL data for 12 day old larvae was log transformed to correct for heterogeneity of variance. Percentage survival data for 16 day old larvae was first converted to proportions and then arcsine transformed prior to analysis.

Experiment 2 – Replacement of 30% Artemia *ration with commercial foods*

The results of Experiment 1 showed that partial replacement of *Artemia* with commercial foods was feasible; however, growth of barramundi larvae fed 50% replacement was poor compared to those fed 100% *Artemia*. The results also showed that larvae weaned at an early age of 12 DAH showed significant less growth than those weaned at 16 DAH. This experiment was conducted to investigate growth and survival of 14 DAH barramundi larvae fed commercially available foods in the place of 30% of the *Artemia* ration.

Mean dry weight of *Artemia* was determined as described in Experiment 1. Based on a mean dry weight of 4.13 µg per nauplius, the total weight of a 30% ration size (0.3 nauplius.ml^{-1}) of *Artemia* was calculated to be 0.08 g. Therefore, treatment receiving partial replacement of *Artemia* at a 30% level, received a 70% *Artemia* ration along with 0.08 g of the designated commercial diet. The commercial diets used in this experiment were:

1) Proton (source: Primo);

2) Aller Aqua (AA) (source: Ridley);

3) Higashi Maru 'Kinko' (HM) (source: Higashi Maru, Japan); and

4) Skretting diet (source: Skretting).

All diets were used in a particles size range of 300–500 µm.

Fourteen-day old barramundi larvae were used in this experiment. Larvae were reared according to standard rearing protocols (Appendix 1). Six dietary treatments were used in this experiment:

1) 100% *Artemia* ration;

2) 70% *Artemia* ration only;

3) 70% *Artemia* ration and 30% Higashi Maru diet;

4) 70% *Artemia* ration and 30% Proton diet;

5) 70% *Artemia* ration and 30% Aller Aqua diet; and

6) 70% *Artemia* ration and 30% Skretting diet.

Larvae were stocked into 16 L conical fibreglass tanks at a density of 15 larvae.L^{-1}. Each tank was provided with a flow-through supply of 1-µm filtered seawater at a rate of 1 L min^{-1} (equivalent to 3 volume exchanges per hour). Water to each tank entered through the base following degassing, and exited through a 500 µm screened outlet, at the water surface (Fig. 1). Aeration was supplied in the degassing column, but not directly to the tank and a photoperiod of 16L:8D was maintained.

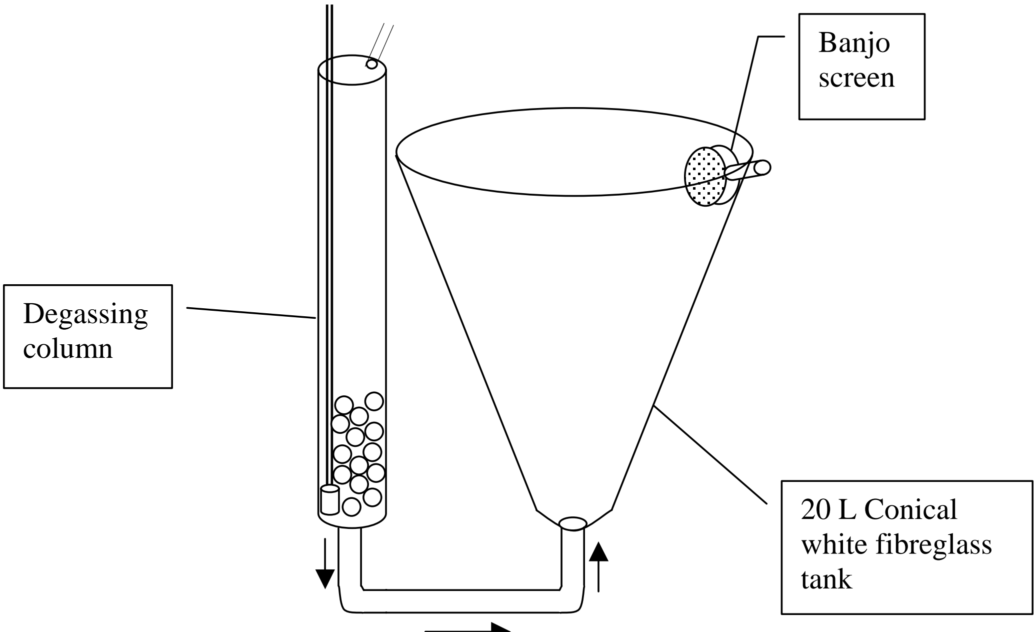

Figure 1. General tank design used in Experiment 2.

Larvae were fed 4 times daily, at 09:00 h, 11:30 h, 14:00 h and 16:30 h. Larvae fed the 100% *Artemia* ration received 1 nauplius.mL^{-1} per feed. Unfed controls were included in the growth trial. The bottom of each tank was cleaned (siphoned) daily. The growth trial ran for 14 days, after which larvae were removed by siphoning onto screens and then killed in an ice slurry. Larval growth was assessed as increases in TL and DW which were determined as described for Experiment 1. Data were analysed as described for Experiment 1.

Experiment 3 – Partial replacement of Artemia *with Higashi Maru*

The results of Experiment 2 showed that the commercial diet 'Higashi Maru' was the superior diet when used to replace 30% of dietary *Artemia* for barramundi larvae. Experiment 2 used 14 day-old larvae and the highest survival was under 50%. This follow-up experiment was designed to determine the optimum weaning age for barramundi larvae when 30% of the *Artemia* ration is replaced with Higashi Maru. This experiment was conducted with barramundi of 12, 14, 16 and 18 days after hatch.

Larvae in all treatment received a 70% *Artemia* ration along with 0.08 g of the Higashi Maru diet (particles size range 300–500 µm) as detailed for Experiment 2. Barramundi larvae were reared according to standard protocols (Appendix 1) and used in the experiment at 12, 14, 16 and 18 DAH. Six treatments were assessed in triplicate:

1) 100% *Artemia* ration only;

2) 70% *Artemia* ration only;

3) 12 DAH 30% replacement Higashi Maru diet and 70% *Artemia* ration;

4) 14 DAH 30% replacement Higashi Maru diet and 70% *Artemia* ration;

5) 16 DAH 30% replacement Higashi Maru diet and 70% *Artemia* ration; and

6) 18 DAH 30% replacement Higashi Maru diet and 70% *Artemia* ration.

Experimental design, feeding protocol and tank maintenance followed the protocol described for Experiment 2. The growth trial ran for 21 days when larvae were processed and their TL and DW determined as described for Experiment 2. Percentage survival and DW data were analysed using a one-way ANOVA and Tukey's HSD. Data for TL was analysed using Kruskal-Wallis test.

Experiment 4 – Partial replacement of **Artemia** *by Higashi Maru and an MBD*

Previous experiments identified the commercially available Japanese diet Higashi Maru as a high quality partial replacement for *Artemia* fed to barramundi larvae. However, this diet is not specifically formulated for barramundi. The major aim of this experiment was to determine the nutritional value of a prototype microbound diet (P-MBD) formulated for barramundi and to assess its performance against Higashi Maru (HM). A second aim of Experiment 4 was to identify the optimum level for *Artemia* ration replacement for both P-MBD and HM.

Based on the calculated mean dry weight of enriched *Artemia* of 4.67 µg (Experiment 2), the total weight of *Artemia* based on a 100%, 75%, 50% and 25% ration size was calculated as 0.3, 0.22, 0.15 and 0.07 g, respectively. The P-MBD used in this experiment was developed for barramundi and shown to be of high nutritional value for barramundi larvae in previous research at James Cook University (Partridge, 1996)(Appendix 2).

Fourteen day-old barramundi larvae were used in this experiment. Larvae were reared according to standard rearing protocols (Appendix 1) and, at 14 days of age had mean (±S.E., n=20) TL and DW of 4.052 ± 0.16 mm and 0.27 ± 0.014 mg, respectively. Nine treatments were assessed in triplicate in this experiment:

1) 100% *Artemia* ration only;

2) 100% P-MBD;

3) 75% replacement experimental diet + 25% *Artemia* ration;

4) 50% P-MBD + 50% *Artemia* ration;

5) 25% P-MBD + 75% *Artemia* ration;

6) 100% Higashi Maru;

7) 75% Higashi Maru + *Artemia* ration;

8) 50% Higashi Maru + 50% *Artemia* ration; and

9) 25% Higashi Maru + 75% *Artemia* ration.

Experimental design, feeding protocol and tank maintenance followed the protocol described for Experiment 2. The growth trial ran for 21 days when larvae were processed and their TL and DW determined as described in Experiment 2. Percentage survival and DW data were analysed using a one-way ANOVA and Tukey's HSD. Data for TL was analysed using Kruskal-Wallis test.

Experiment 5 – Optimising weaning protocol using HM and P-MBD

The aim of this experiment was to determine an appropriate weaning protocol for barramundi based on the P-MBD and HM diets. Fourteen day-old barramundi larvae were used in this experiment with a mean (±S.E, n=20) TL and DW of 4.84 ± 0.03 mm and 0.17 mg, respectively. Nine weaning protocols were assessed in triplicate as outlined in Table 1.

Table 1. Nine weaning protocols assessed in Experiment 5. Each protocol varied in the percentage (figure shown in Table) of either P-MBD or HM fed to barramundi larvae from 14 days after hatch (DAH) until 28 DAH. The remainder of the diet in each protocol is made up by *Artemia*.

Age	Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5	Protocol 6	Protocol 7	Protocol 8	Protocol 9
(DAH)	Control*	P-MBD	HM	P-MBD	HM	P-MBD	HM	P-MBD	HM
14	0	10	10	10	10	50	50	**100**	**100**
15	0	15	15	20	20	60	60	**100**	**100**
16	0	20	20	30	30	70	70	**100**	**100**
17	0	25	25	40	40	80	80	**100**	**100**
18	0	30	30	50	50	90	90	**100**	**100**
19	0	35	35	60	60	**100**	**100**	**100**	**100**
20	0	40	40	70	70	**100**	**100**	**100**	**100**
21	0	45	45	80	80	**100**	**100**	**100**	**100**
22	0	50	50	90	90	**100**	**100**	**100**	**100**
23	0	55	55	**100**	**100**	**100**	**100**	**100**	**100**
24	0	60	60	**100**	**100**	**100**	**100**	**100**	**100**
25	0	70	70	**100**	**100**	**100**	**100**	**100**	**100**
26	0	80	80	**100**	**100**	**100**	**100**	**100**	**100**
27	0	90	90	**100**	**100**	**100**	**100**	**100**	**100**
28	0	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

*100% *Artemia*

Experimental design, feeding protocol and tank maintenance followed the protocol described for Experiment 2. The growth trial ran for 14 days when larvae were processed and their TL and DW determined as described for Experiment 2. Data were analysed using a one-way ANOVA and Tukey's HSD. DW data were log transformed to correct for heterogeneity of variance.

1.2.1.3 Results

Experiment 1 – Replacement of 50% Artemia *ration with commercial foods*

Larvae in the unfed treatment died by day 9 of the experiment. There were no significant differences between treatments in overall survival of the larvae (Fig. 2).

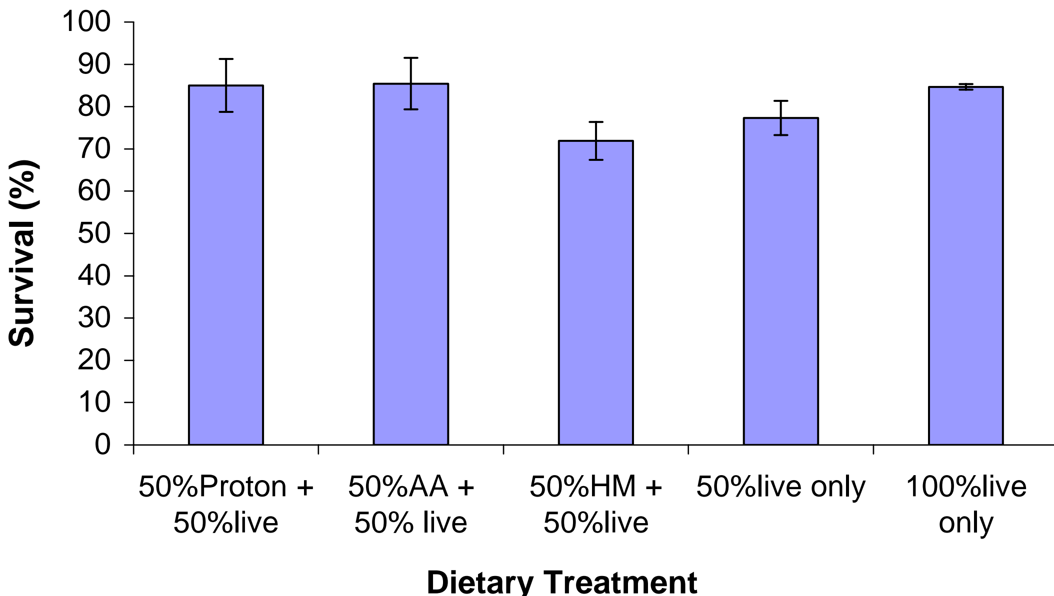

Figure 2. Mean (± S.E., n=3) survival of barramundi larvae from day 12 when fed commercial diets at a rate of 50% replacement for live *Artemia* ration. AA, Aller Aqua; HM, Higashi Maru. There were no significant differences between means (P > 0.05).

Mean DW was significantly greater for larvae fed the 100% *Artemia* diet. Larvae fed 50% *Artemia*/50% Proton, 50% *Artemia*/50% Aller Aqua or a 50% *Artemia* ration recorded the lowest mean DW. Larvae receiving 50% *Artemia*/50% Higashi Maru weighed significantly more than larvae fed either of the other commercial diets, but did not differ significantly in DW from larvae fed 50% *Artemia* only (P > 0.05) (Fig. 3).

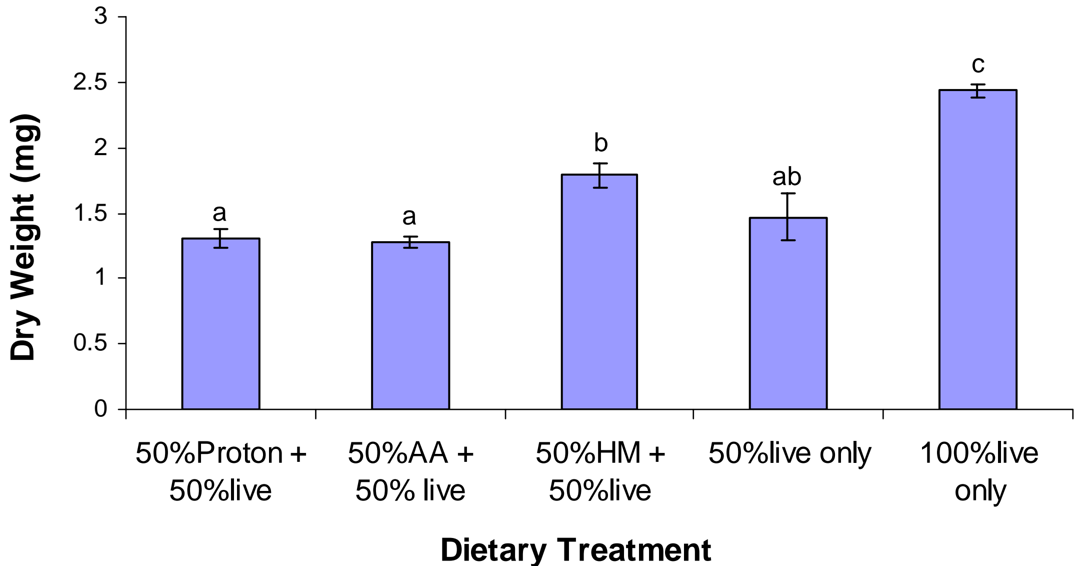

Figure 3. Mean (± S.E., n=3) dry weight of barramundi larvae from day 12 when fed commercial diets at a rate of 50% replacement for live *Artemia* ration. Means sharing the same superscript are not significantly different (P > 0.05). AA, Aller Aqua; HM, Higashi Maru.

Larvae fed 100% *Artemia* grew to the greatest length although mean TL did not differ significantly from that of larvae fed 50% *Artemia*/50% Higashi Maru. Larvae fed 50% *Artemia*, 50% *Artemia*/50% Proton and 50% *Artemia*/50% Aller Aqua were significantly shorter than those fed 100% *Artemia* ($P < 0.05$), but did not differ significantly in TL from larvae fed 50% *Artemia*/50% Higashi Maru (Fig. 4).

When barramundi larvae were fed 50% rations of Proton, Aller Aqua and Higashi Maru from 16 DAH, survival did not differ significantly between treatments. Larvae fed 50% *Artemia*/50% Higashi Maru showed the highest mean survival, although this did not differ significantly from that of larvae in other treatments (Fig. 5). All unfed larvae had died by day 7 of the experiment.

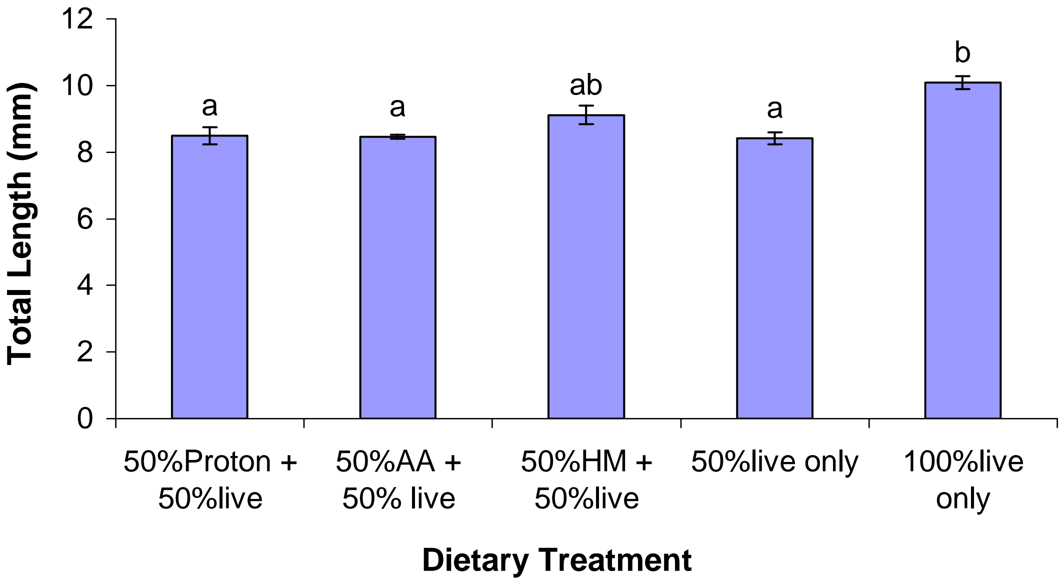

Figure 4. Mean (± S.E., n=3) total length of barramundi larvae from day 12 when fed commercial diets at a rate of 50% replacement for live *Artemia* ration. Means sharing the same superscript are not significantly different ($P > 0.05$). AA, Aller Aqua; HM, Higashi Maru.

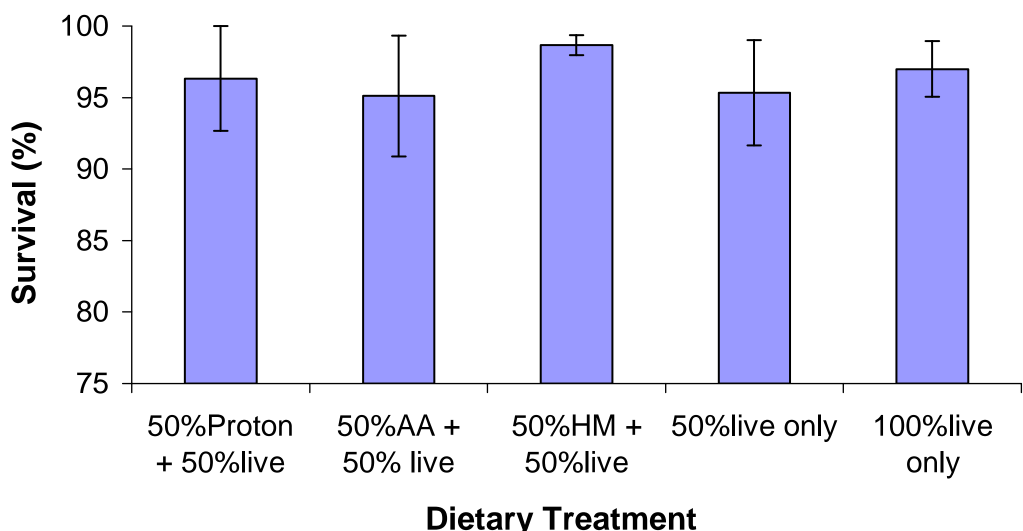

Figure 5. Mean (± S.E., n=3) survival of barramundi larvae from day 16 when fed commercial diets at a rate of 50% replacement for live Artemia ration. AA, Aller Aqua; HM, Higashi Maru. There were no significant differences between means ($P > 0.05$).

Final mean DW was significantly greater for larvae fed 100% *Artemia*. Larvae offered 50% *Artemia*/50% Higashi Maru had significantly greater mean DW than larvae fed either 50% *Artemia*/50% Proton, *Artemia*/50% Aller Aqua or 50% *Artemia* only (Fig. 6).

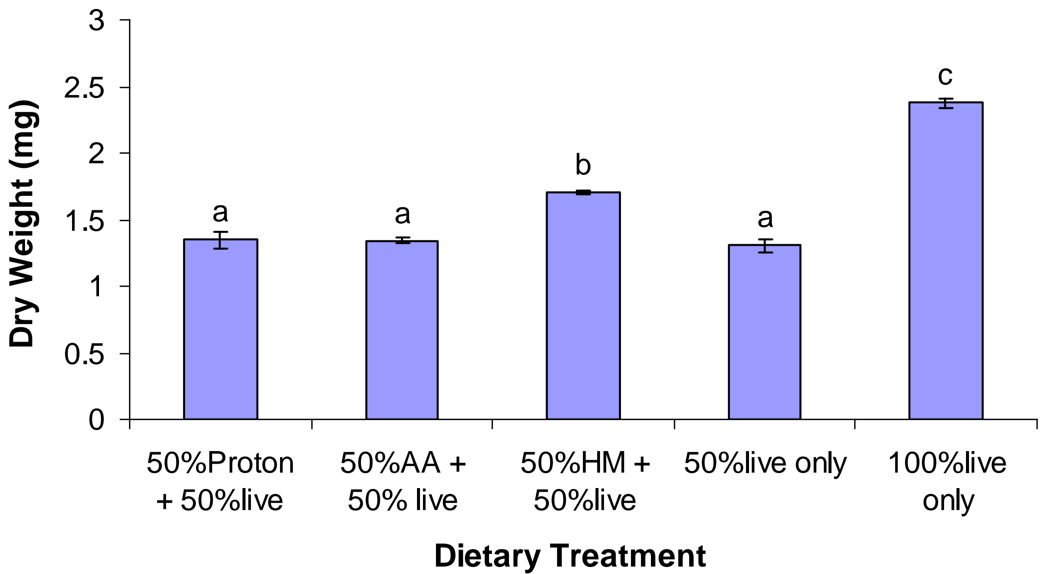

Figure 6. Mean (± S.E., n=3) dry weight of barramundi larvae from day 16 when fed commercial diets at a rate of 50% replacement for live *Artemia* ration. Means sharing the same superscript are not significantly different (P > 0.05). AA, Aller Aqua; HM, Higashi Maru.

Larvae fed 100% *Artemia* had a significantly greater TL than larvae in all other treatments (P < 0.05). Larvae fed 50% *Artemia* were the shortest, although their mean TL did not differ significantly from that of larvae fed 50% *Artemia*/50% Proton or 50% *Artemia*/50% Aller Aqua. Larvae offered 50% *Artemia*/50% Higashi Maru had a significantly greater mean TL than those fed 50% *Artemia* only but were not significantly longer than larvae fed 50% *Artemia* in combination with either Proton or Aller Aqua (Fig. 7).

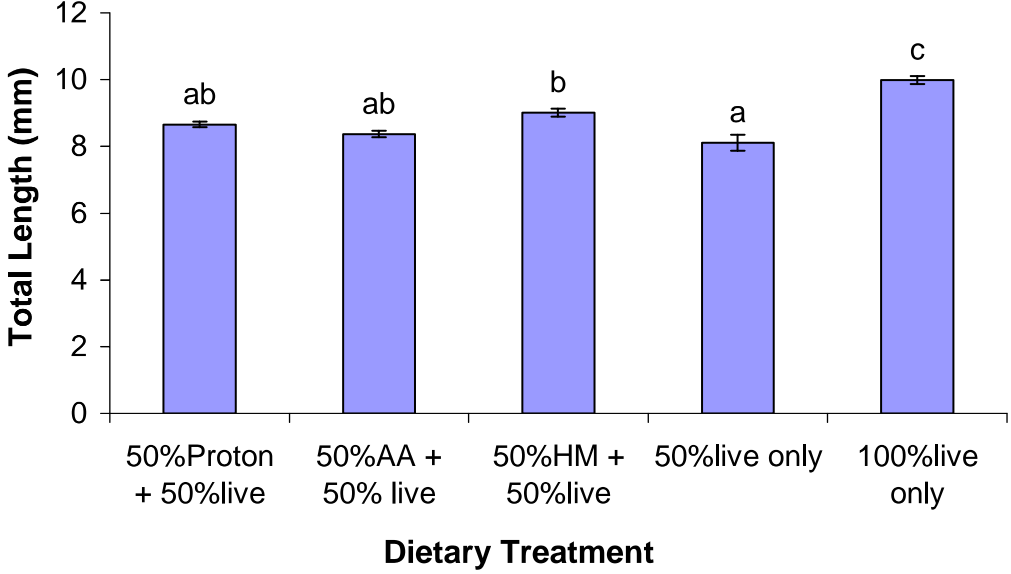

Figure 7. Mean (± S.E., n=3) total length of barramundi larvae from day 16 when fed commercial diets at a rate of 50% replacement for live *Artemia* ration. Means sharing the same superscript are not significantly different (P > 0.05). AA, Aller Aqua; HM, Higashi Maru.

Experiment 2 – Replacement of 30% **Artemia** *ration with commercial foods*

Although larvae fed 70% *Artemia* ration and 30% Higashi Maru diet had a higher mean survival then any of the other treatments, there were no significant differences in survival between treatments (Fig. 8).

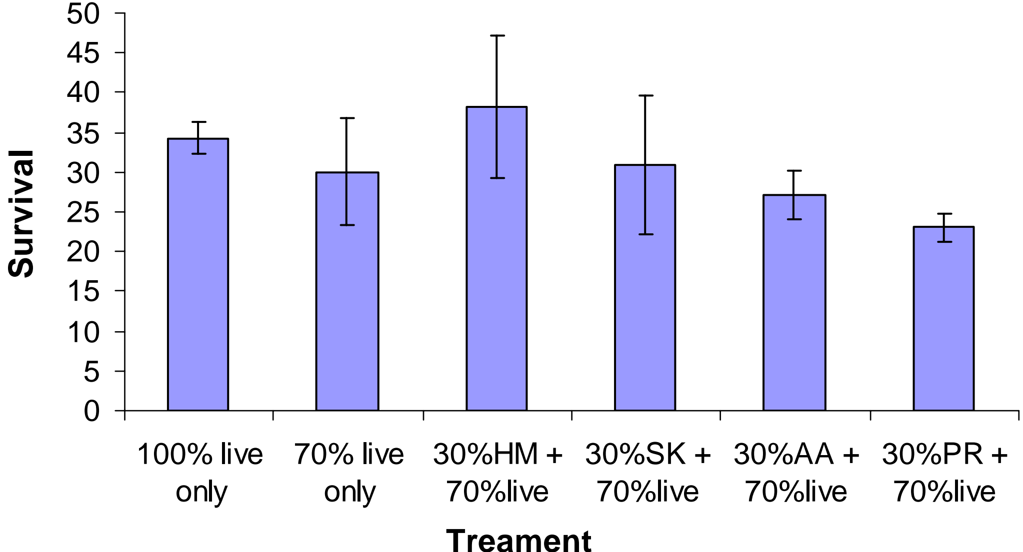

Figure 8. Mean (± S.E., n=3) survival of barramundi larvae from day 14 when fed commercial diets at a rate of 30% replacement for live *Artemia* ration. HM, Higashi Maru; AA, Aller Aqua; SK, Skretting diet; PR, Proton. There were no significant differences between means (P > 0.05).

There was a significant difference in mean DW between larvae fed 100% *Artemia* and those fed the 70% *Artemia* only treatment. However, there was no significant difference between the mean DW of larvae in these two treatments and those fed a ration of 70% *Artemia* /30% commercial diet (Fig. 9).

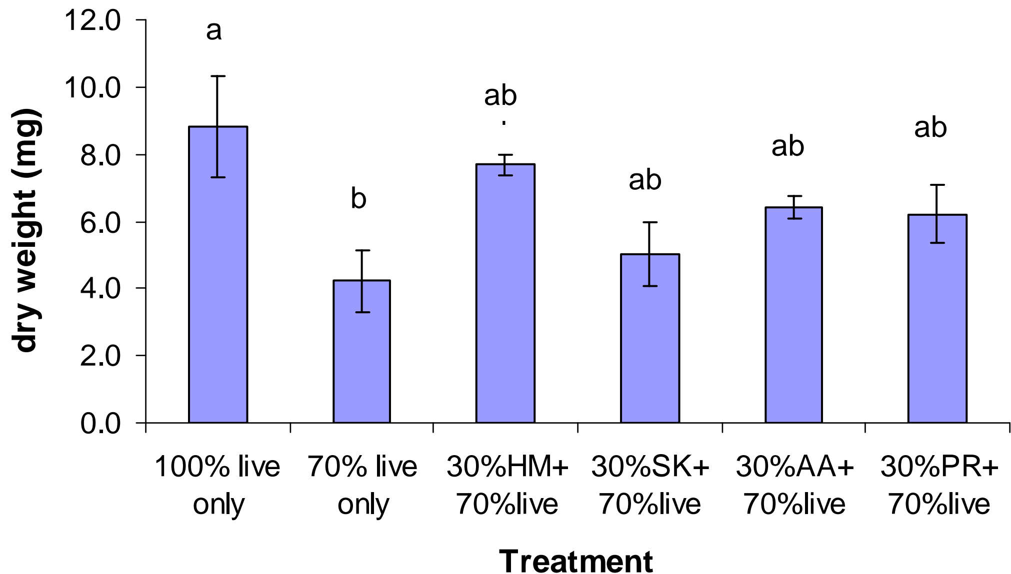

Figure 9. Mean (±S.E., n=3) dry weight of barramundi larvae from day 14 when fed commercial diets at a rate of 30% replacement for live *Artemia* ration. HM, Higashi Maru; AA, Aller Aqua; SK, Skretting diet; PR, Proton. Means sharing the same superscript are not significantly different (P > 0.05).

Although larvae fed the 100% *Artemia* and 30% *Artemia* rations replaced with Higashi Maru showed slightly higher mean TL than the larvae in other treatments, there were no significant differences in mean TL between treatments (Fig. 10).

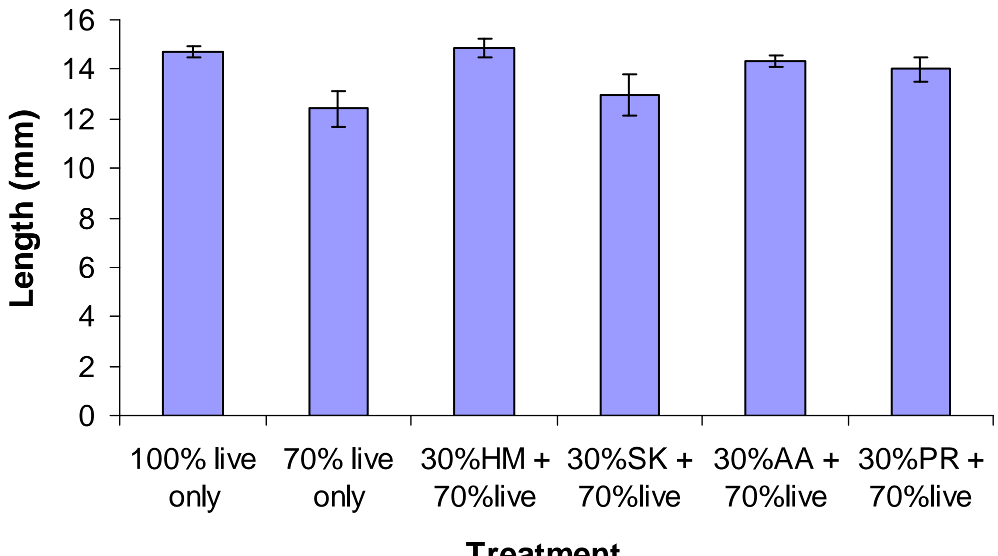

Figure 10. Mean (± S.E., n=3) total length of barramundi larvae from day 14 when fed commercial diets at a rate of 30% replacement for live *Artemia* ration. HM, Higashi Maru; AA, Aller Aqua; SK, Skretting diet; PR, Proton. There were no significant differences between means (P > 0.05).

Experiment 3 – Partial replacement of **Artemia** *with Higashi Maru*

Larvae fed 70% *Artemia*/30% HM from 16 DAH showed significantly greater survival (P < 0.05) than larvae fed 70% *Artemia* only. However, there were no other significant differences for survival between other treatments (Fig. 11).

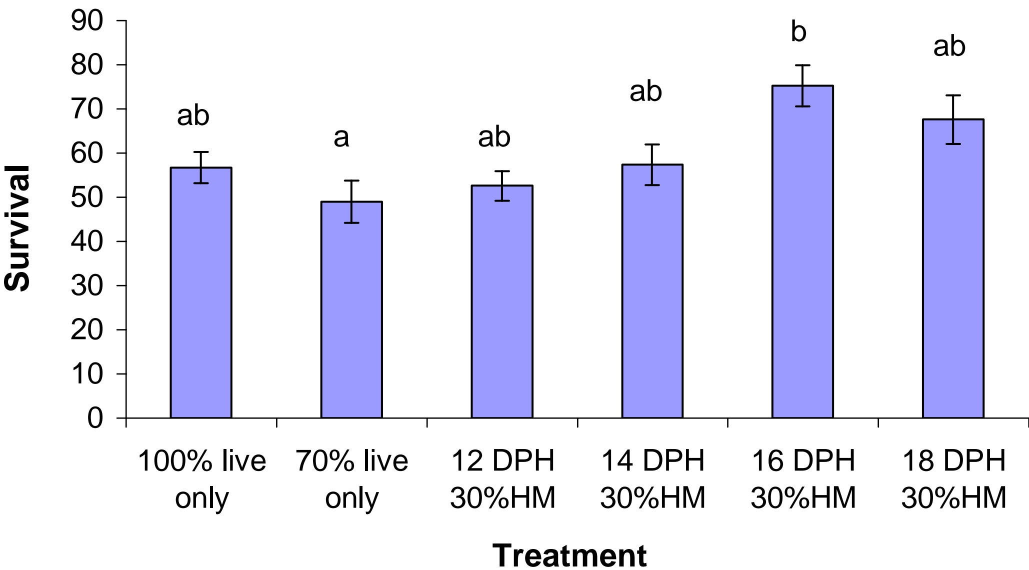

Figure 11. Mean (± S.E., n=3) survival of barramundi larvae fed Higashi Maru (HM) at a 30% replacement level for *Artemia* at different ages (days after hatch, DAH). Means sharing the same superscript are not significantly different (P > 0.05).

Similarly, there was a significant difference in mean DW between larvae fed 70% *Artemia*/30% HM from 16 DAH and those fed 70% *Artemia* only. Interestingly, larvae fed 70% *Artemia*/30% HM from 12, 14 and 16 DAH had significantly higher mean DW than larvae fed the 100% or 70% *Artemia* ration (Fig. 12). There were, however, no significant differences in mean DW between larvae of different weaning ages.

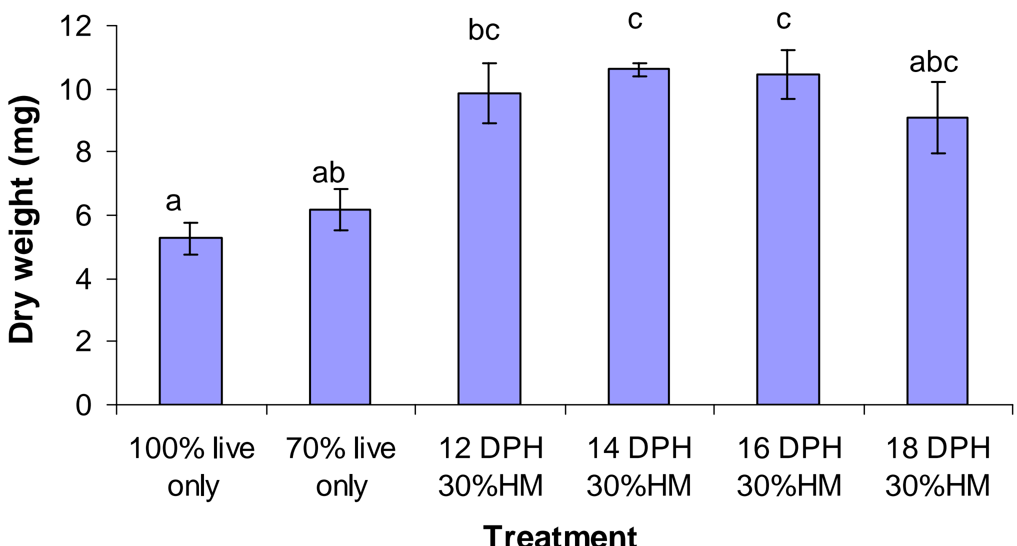

Figure 12. Mean (± S.E., n=3) dry weight of barramundi larvae fed Higashi Maru (HM) at a 30% replacement level for *Artemia* at different ages (days after hatch, DAH). Means sharing the same superscript are not significantly different (P > 0.05).

Larvae fed 70% *Artemia*/30% HM from 14 DAH attained significantly greater mean TL then larvae fed 100% *Artemia*. This was the only significant difference for TL between treatments (Fig. 13).

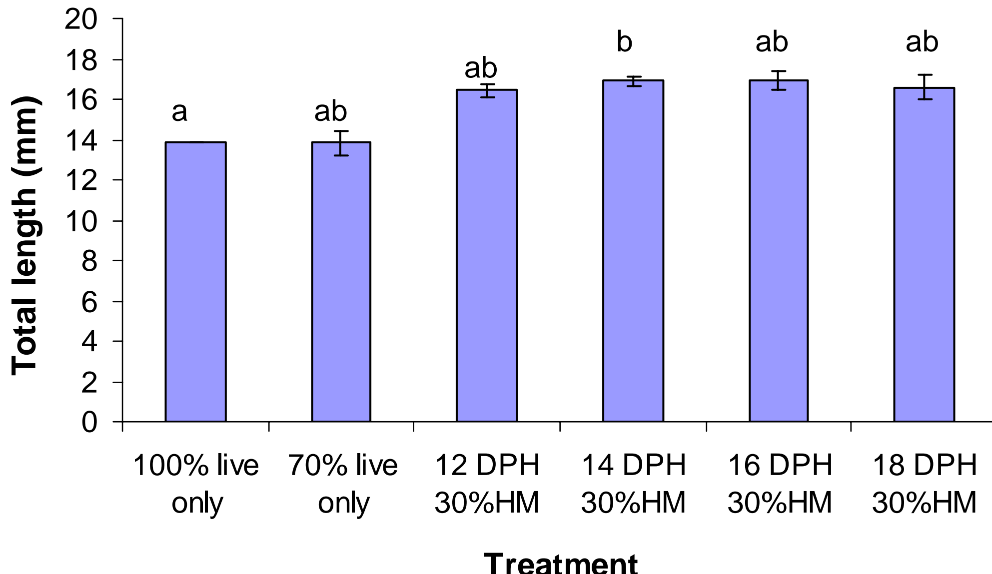

Figure 13. Mean (± S.E., n=3) total length of barramundi larvae fed Higashi Maru (HM) at a 30% replacement level for *Artemia* at different ages (days after hatch, DAH). Means sharing the same superscript are not significantly different (P > 0.05).

Experiment 4 – Partial replacement of* Artemia *by Higashi Maru and an MBD

Survival was variable across the nine treatments, with larvae fed 100% *Artemia*, 50% *Artemia*/50% HM, 50% *Artemia*/50% P-MBD and 75% *Artemia*/25% P-MBD achieving significantly higher survival than larvae fed either 100% HM or P-MBD, or 75% HM or P-MBD with 25% *Artemia* (Fig. 14).

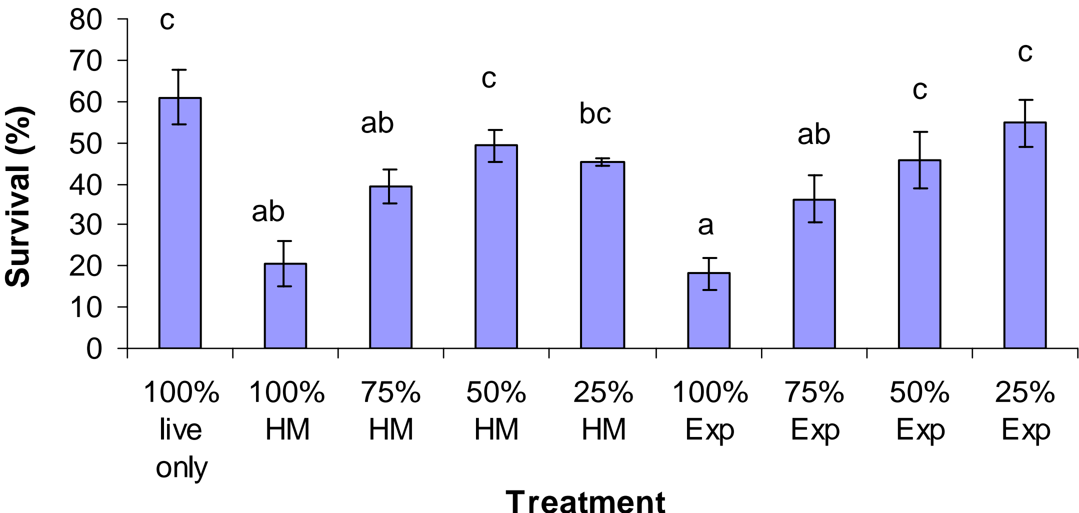

Figure 14. Mean (± S.E., n=3) survival of barramundi larvae fed Higashi Maru (HM) or the experimental P-MBD (Exp) from day 14 at different levels of replacement (%) for live *Artemia*. Means sharing the same superscript are not significantly different (P > 0.05).

Larvae fed 50% and 25% HM had the highest mean DW which did not differ significantly from the mean DW of larvae fed 100% *Artemia* or 25% P-MBD. HM and P-MBD could replace 50% and 25% of the *Artemia* ration, respectively, before mean larval dry weight was significantly reduced (Fig. 15). The lowest mean DW was shown by larvae fed 100% HM or 100% P-MBD and these values did not differ significantly.

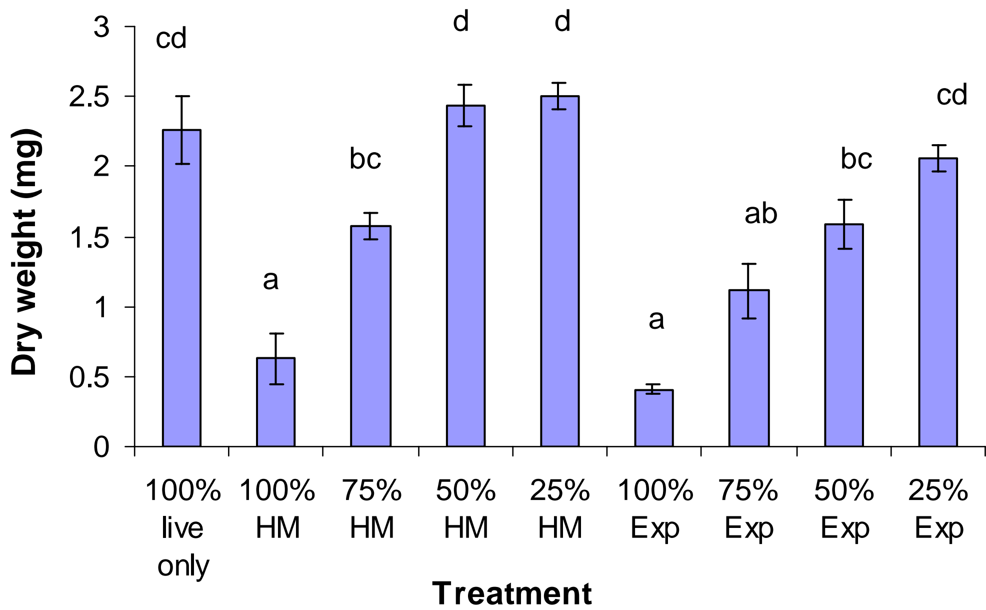

Figure 15. Mean (± S.E., n=3) dry weight of barramundi larvae fed Higashi Maru (HM) or the experimental P-MBD (Exp) from day 14 at different levels of replacement (%) for live *Artemia*. Means sharing the same superscript are not significantly different (P > 0.05).

The 100% HM and 100% P-MBD treatments resulted in significantly lower larval TL than all other diets except 75% P-MBD. Larvae fed 75%, 50% and 25% levels of HM and P-MBD did not differ significantly in mean TL from those fed 100% *Artemia* (Fig. 16).

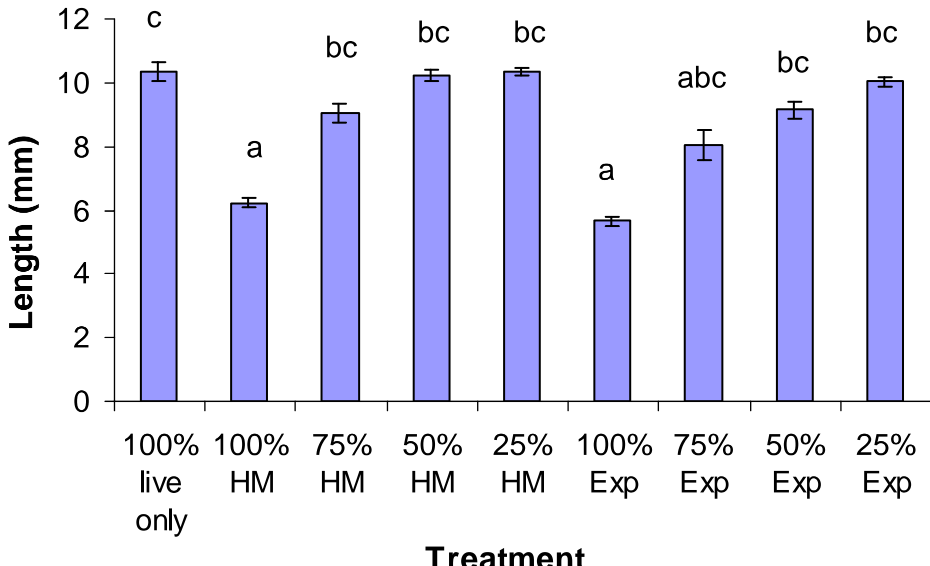

Figure 16. Mean (± S.E., n=3) total length of barramundi larvae fed Higashi Maru (HM) or the experimental P-MBD (Exp) from day 14 at different levels of replacement (%) for live *Artemia*. Means sharing the same superscript are not significantly different ($P > 0.05$).

It is notable that the HM diet particles floated on the water's surface in experimental aquaria for far longer than P-MBD diet particles, which sank soon after feeding. This difference in behaviour between the particle types has major implications for their suitability as a larval feed. This aspect is the subject of research and discussed in greater detail in section 6.3.2 of this report.

Experiment 5 – Optimising weaning protocol using HM and P-MBD

Survival of larvae amongst the different feeding protocols ranged from 85% to 25% and was positively correlated to increasing provision of *Artemia*. Larvae fed 100% *Artemia* throughout the experiment (Protocol 1) showed the highest mean survival while those weaned onto HM or P-MBD from 14 DAH (Protocols 8 and 9) showed the lowest mean survival (Fig 17). Survival of larvae weaned according to Protocols 2 and 3, which were fully weaned to P-MBD and HM by 28 DAH (respectively), did not differ significantly to that of larvae fed 100% *Artemia* throughout the experiment. There was no significant difference in mean survival between larvae fed the same levels of P-MBD and HM (i.e. between protocols 2 and 3, protocols 4 and 5, protocols 6 and 7 and protocols 8 and 9)(Fig. 17).

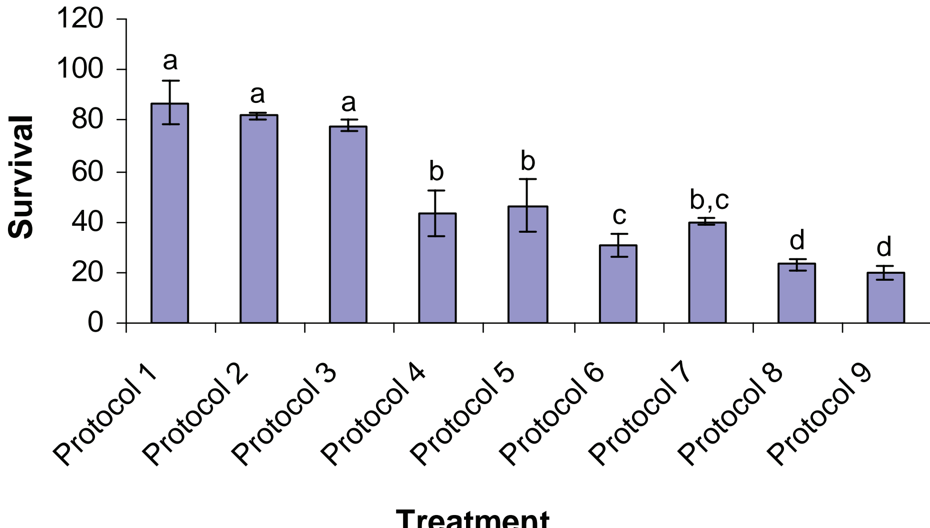

Figure 17. Mean (± S.E., n=3) survival of barramundi larvae cultured according to nine weaning protocols from 14 DAH. Means sharing the same superscript are not significantly different (P > 0.05).

The mean DW of larvae fed the nine different weaning protocols is shown in Fig. 18. Generally again, mean DW was positively correlated to increasing provision of *Artemia* with the highest mean DW recorded for larvae in Protocol 1 and the lowest recorded for larvae in Protocols 8 and 9. However, there were no significant differences for mean DW between Protocols 1 to 5. The DW of larvae weaned to inert feeds by 28 DAH (Protocols 2 and 3) did not differ significantly to that of larvae weaned to inert feeds by 23 DAH (Protocols 4 and 5). However, there were significant differences in the mean DW between larvae weaned to inert feeds by 23 DAH (Protocol 4) and those weaned to the same diets by 19 DAH. Similarly, there were significant differences in the mean DW between larvae weaned to inert feeds by 23 DAH (Protocols 4 and 5) and those weaned to the same diets from 14 DAH. (Fig. 18). Again, there were no significant differences in mean DW between larvae fed the same levels of P-MBD and HM (i.e. between protocols 2 and 3, protocols 4 and 5, protocols 6 and 7 and protocols 8 and 9) (Fig. 18).

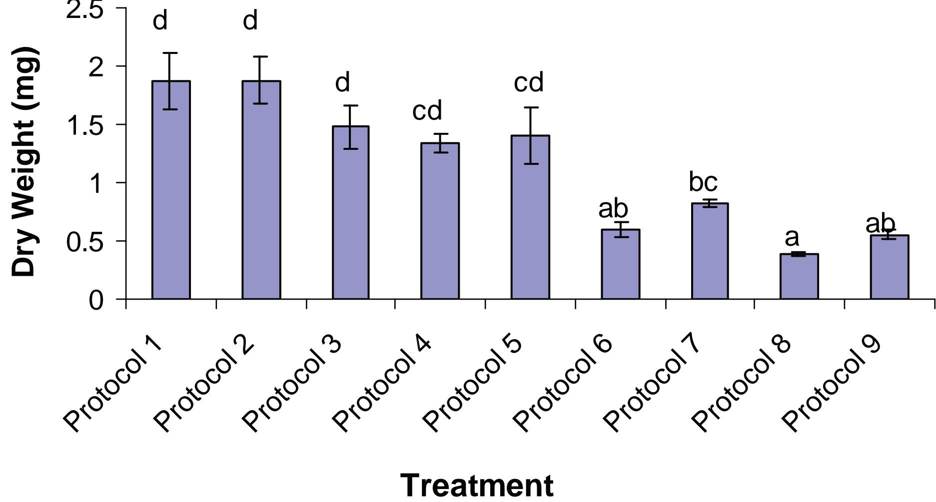

Figure 18. Mean (± S.E., n=3) dry weight of barramundi larvae cultured according to nine weaning protocols from 14 DAH. Means sharing the same superscript are not significantly different (P > 0.05).

The mean TL of larvae subject to the nine different weaning protocols are shown in Fig. 19. Again higher means were associated with higher levels of *Artemia* provision. The highest mean TL was recorded for larvae in Protocol 1 and the lowest for larvae in Protocols 8 and 9. However, there were no significant differences for mean TL between Protocols 1 to 5. Again, there were no significant differences in mean TL between larvae fed the same levels of P-MBD and HM (i.e. between protocols 2 and 3, protocols 4 and 5, protocols 6 and 7 and protocols 8 and 9)(Fig. 19).

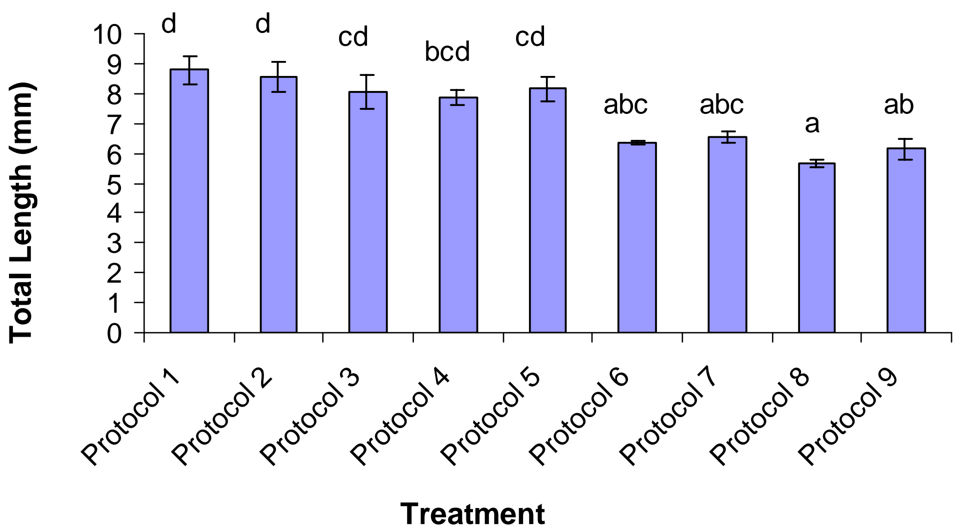

Figure 19. Mean (± S.E., n=3) total length of barramundi larvae cultured according to nine weaning protocols from 14 DAH. Means sharing the same superscript are not significantly different (P > 0.05).

1.2.1.4 Discussion

Co-feeding of live foods with inert foods can result in superior growth and survival of early staged marine fish larvae to that achieved with either live or inert foods alone Ehrlich et al. 1989; Holt, 1991; Marte et al. 1991). Corneillie et al. (1989), for example, reported that when 48 day old *S. aurata* were fed an inert food at a 50% inclusion level, growth rate (weight and length) was significantly improved compared to larvae fed live food only. Similarly, Canavate and Fernandez-Diaz (1999) reported that *Solea senegalensis* larvae were able to complete metamorphosis when fed a 50% combination of live foods and inert food and achieved similar growth and survival as larvae fed live feeds alone. However, similar to the findings in this study, much of research in this field has reported that significant replacement of live foods with inert food particles results in reduced growth rates of marine fish larvae and poorer survival. The following are major outcomes of these experiments:

- Replacement of 50% of an *Artemia* ration with commercially available inert foods significantly affected the growth rate of larvae at 12 DAH when compared to those fed a full ration of enriched *Artemia*. Larvae fed 50%*Artemia*/50% inert food showed no improvement in growth than larvae fed 50% *Artemia* alone.
- Replacement of 30% of the *Artemia* ration with inert foods for 14 DAH larvae improved their growth rates compared to those fed a full *Artemia* ration. There were no significant differences in either mean DW or TL between larvae that were co-fed with *Artemia* and inert foods and those fed *Artemia* alone. Larvae fed 70% *Artemia* and 30% inert food had greater mean DW than larvae fed 70% *Artemia* alone.
- Of the four commercially available foods used in co-feeding experiments with barramundi

larvae, the Japanese made 'Higashi Maru' supported greater larval growth and was used as the benchmark in subsequent experiments.

- When 'Higashi Maru' was used to replace 30% of the *Artemia* ration for larvae of 12, 14, 16 and 18 DAH, mean larval DW was significantly greater than that of larvae receiving 100% *Artemia* and mean TL did not differ significantly from that of larvae fed 100% *Artemia*.
- There were no significant differences in mean DW or TL between larvae fed 70%*Artemia*/30% Higashi Maru beginning from 12 to 18 DAH.
- An experimental MBD (P-MBD) used to feed barramundi larvae at various levels of *Artemia* replacement from 14 DAH, supported similar growth and development rates to Higashi Maru used at the same levels of replacement.
- Fourteen DAH barramundi larvae fed rations where 50% and 25% of *Artemia* were replaced by Higashi Maru and 25% *Artemia* were replaced by P-MBD did not differ significantly in mean DW to larvae fed a full *Artemia* ration. Similarly, larvae fed rations where 25%, 50% and 75% of *Artemia* were replaced by Higashi Maru or P-MBD did not differ significantly in mean TL from larvae fed a full *Artemia* ration.
- Generally, the more rapidly barramundi larvae are weaned onto inert foods from 14 DAH then the lower is their growth rate and survival to 28 DAH.
- Fourteen DAH barramundi larvae weaned gradually by 23 DAH did not differ significantly in mean DW or TL from larvae fed *Artemia* alone. However, a more rapid weaning protocol in which larvae were fully weaned by 19 DAH resulted in a significant decrease in mean DW and TL.
- There were no significant differences in mean survival, DW or TL between larvae fed the same levels of P-MBD and HM in any of the weaning protocols assessed.

The results of these experiments indicate, not surprisingly, that the nutritional value of available commercial feeds varies when used as a partial replacement for *Artemia* fed to barramundi larvae. The results also show that the effectiveness of inert food is greatly influenced by the age of the larvae they are fed to and the degree to which they are used a replacement for *Artemia*. Barramundi larvae, from 14 DAH, showed no significant decrease in mean survival, total length or dry weight, compared to larvae fed a full *Artemia* ration, when fed a ration composed of up to 50% inert food (Higashi Maru or P-MBD). Both Higashi Maru and the experimental P-MBD were of high nutritional value for barramundi larvae and performed similarly in weaning trials. Barramundi larvae weaned to Higashi Maru and the P-MBD by 23 DAH showed no significant difference in mean DW or TL to larvae fed *Artemia* alone for the same period. This indicates that barramundi larvae can be weaned more rapidly than in some 'in-house' weaning protocols used by commercial barramundi hatcheries, where the weaning process generally begins at around 20 DAH and is not completed until ≥ 25 DAH. The successful weaning protocols reported here are also more rapid than some experimental weaning protocols recently examined for barramundi larvae in which larvae were weaned over an 8 day period from 16 DAH (Curnow et al. 2004). Unfortunately no growth or survival data were presented by Curnow et al. (2004) to allow comparison with the results of this study.

The performance of the experimental P-MBD in this study was encouraging as it promoted similar rates of growth and survival of barramundi larvae to the best of the commercial foods tested. A logical next step in assessing the potential of the P-MBD is to test its performance under large-scale commercial conditions. Unfortunately, this was not possible during this project. However, Bosmans

et al. (2004) recently reported development of a commercial weaning protocol for barramundi which allows hatchery production of barramundi without use of *Artemia*. In this protocol, rotifers are fed to larvae up to 15 DAH and inert food particles are provided to larvae from 8 DAH. Of these, 'Gemma Micro' (Skretting) is provided from 8 DAH until harvest (23 DAH) and Proton is provided from 16 DAH until harvest. This feeding protocol allows a 20% cost saving compared to a standard feeding protocol (Bosmans et al. 2004).

1.2.1.5 References

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1.2.1.6 Appendix 1 – Standard larval rearing protocol

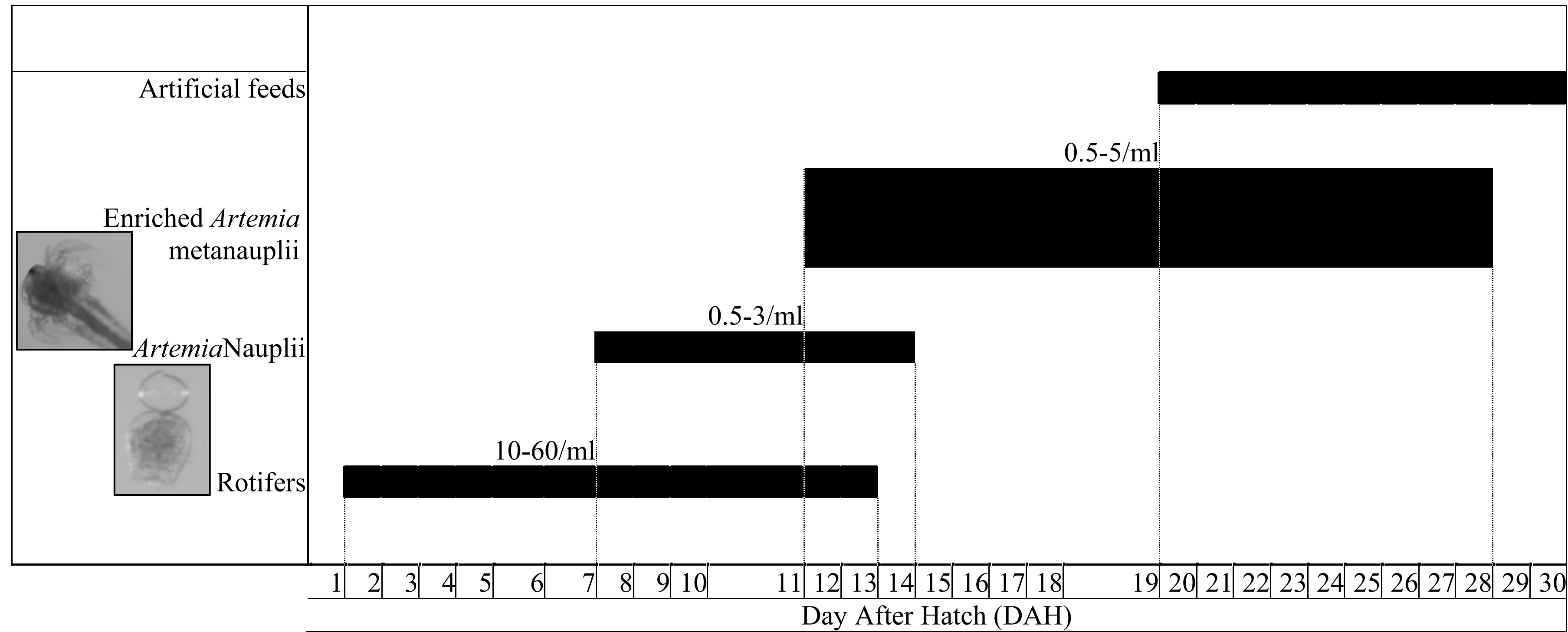
Larvae at one-day after hatch were obtained from either the Department of Primary Industries Northern Fisheries Centre in Cairns or from a commercial hatchery (Bluewater Barramundi, Innisfail). Larvae were reared in 2000 L round tanks, in a temperature controlled room until needed for specific experiments. Larvae were reared under green-water conditions, with *Nannochloropsis oculata* being added daily to maintain a density of 500,000 cells/mL in the tanks until the larvae were ten days after hatch. Microalgae were gradually dripped into the tanks. When larvae reached eight days after hatch, the tanks received a partial water exchange over night with 1-µm filtered seawater introduced via a degassing chamber at a rate of 1L/min. Heaters were placed in the tanks to maintain the temperature within the range of 27-29°C. The mean water quality parameters of the larval rearing tanks is shown in Table 1.

Table 1. Mean water quality parameters for larval rearing.

Salinity (‰)	pH	Phosphate (mg/L)	Nitrite (mg/L)	Nitrate (mg/L)	Temp. Min. (°C)	Temp. Max. (°C)
32 ± 0.26	8 ± 0.02	0.65 ± 0.1	0.02 ± 0.003	10.88 ± 0.50	27.8 ± 0.09	28.8 ± 0.1

Larvae were fed rotifers, *Artemia* nauplii and enriched *Artemia* at different stages of development. Table 2 outlines the standard feeding protocol used to rear larvae until they reached the desired age for experiments. Rotifers were initially stocked at a rate of 20/mL and maintained at this level.

Table 2. Standard feeding protocol for larval rearing tank.



1.2.1.7 Appendix 2 – Composition of experimental MBD

Ingredient	% inclusion	g/1000g	Protein (% of diet)	Energy (MJ/kg)	g/30g
[1]Fish meal[1]	54.059601	540.59601	35.2063146	10.31457171	16.21788005
[2]Squid powder[2]	20.702290	207.0229	17.6031573	4.815352685	6.21068704
Gelatin	3	30	2.19	0.5616	0.9
Squid oil	13.576446	135.76446		5.308475606	4.072933982
Lecithin	1	10			0.3
Choline chloride	0.5	5			0.15
Pancreatin[3]	0.05	0.5			0.015
Mineral mix[4]	0.5	5			0.15
Vitamin Mix[5]	0.5	5			0.15
Vitamin C[6]	0.5	5			0.15
Cellulose	5.611663	56.11663			1.698498932
Total	**100**	**1000**	**54.9994719**	**21**	**30**

[1] Skretting Australia

[2] Ribber and Son, Norway

[3] Porcine pancrease extract

[4,5] Standard mineral amd vitamin mix, Skretting Australia

[6] StayC, Hoffman La Rosh

Method of preparation

1. First combine Fish meal, squid powder and cellulose, thoroughly mix.
2. Oil and lecithin are added next, mixed thoroughly with above ingredients.
3. Add remaining ingredients and thoroughly mix. Add the gelatin last.
4. Add sufficient amount of hot water to dissolve ingredients.
5. All ingredients are then mixed thoroughly (using a hand held blender) to ensure homogeneity of diet.
6. Diet mixture is then spread thinly and evenly in plastic trays.
7. Place trays in oven at 50^{0}C for 24 - 48 hours.
8. Once cooled, grind flat diet up using mortar and pestle.
9. Sieve crumble to required particle size. Less than 425 µm.

1.3 MICRODIET DEVELOPMENT

1.3.1 Protein and energy nutrition of barramundi (*Lates calcarifer*) larvae

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1.3.1.1 Introduction

Development of successful formulated feeds for marine fish larvae requires (1) preparation of diet particles with appropriate behavioural and physical characteristics to allow ingestion and digestion and (2) detailed knowledge of the nutritional requirements of the target species. Little is known of the nutritional requirements of marine fish larvae, particularly those of tropical species. Protein is the largest and most expensive component of formulated aquaculture foods and, on this basis has primary importance in terms of diet formulation. The experiments reported below examine the protein and energy nutrition of barramundi (*Lates calcarifer*) larvae as a major step towards formulation of a species-specific diet for this species.

The relatively high growth rate of marine fish larvae would logically prescribe a high amino acid requirement (Rønnestad et al. 2003). However, few studies have quantitatively manipulated the protein component of diets for larval fish (Péres et al. 1996). Furthermore, although the interaction of dietary energy and protein has been very well documented in juvenile fish (Cowey and Sargent, 1972) no previous studies have accounted for the influence of dietary energy on the protein requirement of larvae. As amino acids and lipids are the major energy substrates in larval fish nutrition (Fyhn, 1989; Watanabe and Kiron, 1994), lipid-derived energy may influence their quantitative protein requirement.

When considering dietary protein requirements we must also consider the qualitative aspects of protein delivery. As larger fish eat an easily quantifiable amount of food, their amino acid intake can be easily determined. The relatively large volume of the juvenile and adult food compared to its surface area also precludes amino acid leaching to some extent, allowing the use of crystalline amino acids to manipulate formulated foods in a dose-response manner in order to determine amino acid requirements (Murillo-Gurrea et al. 2001; Alam et al. 2002). Due to the small size of larvae and their foods, and the consequent influence on leaching (Baskerville-Bridges and Kling, 2000) and quantification of ingestion, these traditional amino acid manipulation experiments are not applicable to fish larvae. High leaching rates of amino acids in microbound diets also necessitates the use of native protein sources or semi-purified diets rather than water-soluble crystalline amino acids. To determine the potential of such native and semi-purified protein sources in the formulation of larval foods, representative EAA profiles have been identified, assuming that the optimal EAA profile for larval foods corresponds to the profile of larvae tissue (Conceicao et al. 1998), their eggs (Ketola, 1982), or their natural food (Dabrowski and Rusiecki, 1983). While such representative amino acid profiles have their application in the selection of potential protein sources, it is apparent that feeding trials are a more decisive measure of the application of protein sources to microparticulate foods for fish larvae (Eid and Matty, 1989).

Growth in fish has been shown to be regulated by hormones, including thyroid hormones (L-Thyroxine, T4 and Triiodothyronine, T3), growth hormone (GH), insulin-like growth factors (IGF's), sex steroids (mostly androgens such as 17 β methyl-testosterone) and cortisol (Eales, 1985; Gray and Kelley, 1991; MacKenzie et al. 1998). These hormones mediate the effects of extrinsic processes such as nutrition, on growth. While there is a strong association between thyroid

hormones and growth (Hey et al. 1996), survival (Ayson and Lam, 1993) development (Kim and Brown, 1997) and metamorphic success (Soffientino and Specker, 2001; Gavlik et al. 2002) in fish larvae, data are lacking on the link between larval nutrition and these hormonal processes. By applying an endocrinal approach to the nutritional control of performance indices such as growth and survival, we can achieve a better understanding of the underlying processes governing the physiological status of fish. This approach has been taken by several authors working with juvenile fish, particularly salmonids (eg. Farbridge et al. 1992; Gelineau et al. 1996; Dosanjh et al. 1998). However there is little information relating to the nutrition-growth-thyroid interrelationship for tropical species (Nankervis et al. 2000).

These experiments aimed to further develop our understanding of the processes involved in feeding artificial diets to altricial fish larvae. Dietary macronutrients were investigated in terms of their affect on growth and survival of barramundi, and essential amino acid profiles related to perceived requirements. The use of thyroid hormones as markers of nutritional status in fish larvae was also investigated.

1.3.1.2 Materials and methods

Barramundi larvae were spawned at the Queensland Department of Primary Industries (DPI) Northern Fisheries Centre, Cairns, Australia, and at Bluewater Barramundi, Mourylian, Australia. Larvae were obtained one day after hatching (DAH) at a yolk-sac larval stage, and were stocked at a density of 50 L^{-1} in a 2,000 L cylindrico-conical tank, where they were reared to 13 DAH using standard green-water rearing techniques.

Experiments were conducted in 50 L parabolic-based tanks, containing gently aerated flow-through (1.2 $L.min^{-1}$) seawater (33 ± 0.5 ‰ salinity), at 28 (± 1)°C. Photoperiod was maintained at 12hL:12hD. At 13 DAH barramundi larvae were counted individually into each experimental tank to a density of $6.L^{-1}$. The feeding trial began the following day. Larvae were fed *ad libitum*, four times daily.

Any individuals showing obvious cannibalistic behaviour were removed from their tanks and their removal recorded and accounted for in calculations of larval survival. The growth trial was conducted for 14 days, after which all fish were euthanased in chilled water.

Six experiments were conducted to refine our current understanding of the protein nutrition of barramundi larvae from 14–28 DAH, and the physiological responses of larvae to nutritional inputs. Experiment 1 was designed to analyse the influence of dietary protein and energy on larval growth and physiology. The strong caloric effect found in Experiment 1 lead to Experiment 2; refining the dietary energy requirements of barramundi larvae fed microbound diets. The qualitative protein requirements were then analysed, with Experiment 3 studying the effect of the dietary inclusion of different native protein meals (fish meal, squid powder, mussel meal, prawn meal and decapsulated *Artemia* cyst meal). A colour control (fish meal diet with an orange food dye) was also included to control for colour variation between diets. Three native protein meals were selected from this experiment (fish meal, squid powder and mussel meal) for Experiment 4; refining the qualitative protein requirements of barramundi larvae fed microbound diets. Fish meal and squid powder were selected from this experiment for further analysis in Experiment 5; a detailed analysis of the ability of fish meal and squid powder to meet the qualitative protein requirement for barramundi larvae fed microbound diets. As krill meal became available at an intermediate phase in this experimental regime, experiment 6 was performed to analyse the potential of krill meal to meet part of the qualitative protein requirements of barramundi larvae fed microbound diets.

Experimental microbound diets (MBD) were formulated according to Tables 1-5. Fishmeal was included as the protein source, while squid oil was used as the primary lipid source and non-protein

energy derivative. Other lipids were derived from soy lecithin and fishmeal. Cellulose was used as an inert filler to balance dietary formulations.

All fish were counted before being photographed using a stereo dissecting microscope. Total length of each fish was determined using Leica IM50 software (Leica Microsystems AG). A random sub-sample of fish (0.2 g wet weight) was taken from each tank at the end of the experiment for thyroid hormone extraction (NaOH/6-N-propylthiouracil made up in methanol) and analysis (RIA; ICN Diagnostics), while the remainder of the fish were dried at 50°C to constant weight. Dry larvae were weighed to the nearest 0.001 mg. Essential amino acid index (EAAI) and AA/aa ratio of protein sources was calculated according to Penaflorida (1989). Significant differences between treatments were determined by one-way and two-way analysis of variance (ANOVA), while Tukey's HSD was used post-hoc. Pearson's correlation was used to determine relationships between thyroid hormones and growth parameters.

Table 1. Formulation and proximate analysis of the six microparticulate diets used in Experiment 1; containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 $MJ.kg^{-1}$).

Ingredient (%)	Diet (Protein/Energy content)					
	45/18	45/21	50/18	50/21	55/18	55/21
Fishmeal[1*]	65.74	65.74	73.41	73.41	81.09	81.09
Gelatin[*]	3	3	3	3	3	3
Squid oil	12.52	20.19	8.78	16.45	5.03	12.7
Soybean lecithin	1	1	1	1	1	1
Choline Chloride[*]	0.5	0.5	0.5	0.5	0.5	0.5
Mineral Mix[2*]	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin Mix[3*]	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin C [4*]	0.6	0.6	0.6	0.6	0.6	0.6
Cellulose[*]	15.64	7.97	11.71	4.04	7.78	0.11
Analysis						
Crude Protein [a*]	45.34	45.13	50.31	49.38	55.51	56.2
Gross Energy [b*]	18.61	21.78	18.59	21.66	18.69	21.22

*% dry matter by mass.

[1]Skretting Australia.

[2]Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate, sodium selenate.

[3]Citric acid, ascorbic acid, alpha tocopherol, myo-inositol, ethoxyquin, nicotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol.

[4] Ascorbic acid.

[a] Kjeldahl nitrogen (N X 6.25).

[b] $MJ.kg^{-1}$ bomb calorimetry, corrected for cellulose inclusion.

Table 2. Formulation and proximate analysis of the five microparticulate diets used in Experiment 2; containing different utilisable energy levels (18-22 MJ/kg).

	Formulated Dietary Energy Level (MJ/kg)				
Ingredient:	**18**	**19**	**20**	**21**	**22**
Fishmeal[1*]	73.41	73.41	73.41	73.41	73.41
Gelatin[*]	3	3	3	3	3
Squid oil	8.78	11.33	13.89	16.45	19.01
Soybean lecithin	1	1	1	1	1
Choline Chloride[*]	0.5	0.5	0.5	0.5	0.5
Mineral Mix[2*]	0.5	0.5	0.5	0.5	0.5
Vitamin Mix[3*]	0.5	0.5	0.5	0.5	0.5
Vitamin C [4*]	0.6	0.6	0.6	0.6	0.6
Cellulose[*]	11.71	9.15	6.60	4.04	1.48
Analysis					
Crude Protein [a*]	49.97	50.16	50.03	50.06	50.11
GrossEnergy [b*]	17.96	19.08	20.12	20.92	22.14

*% dry matter by mass.

[1]Skretting Australia.

[2]Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate, sodium selenate.

[3]Citric acid, ascorbic acid, alpha tocopherol, myo-inositol, ethoxyquin, nicotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol.

[4] Ascorbic acid.

[a] Kjeldahl nitrogen (N X 6.25).

[b] $MJ.kg^{-1}$ bomb calorimetry, corrected for cellulose inclusion.

Table 3. Formulation and proximate composition of experimental diets used in Experiment 3; formulated to 48% crude protein and 20MJ/kg gross energy levels, with different native animal meals as dietary protein sources.

	Diet					
Ingredient	**Fish Meal**	**Colour Control**	***Artemia* Meal**	**Squid Powder**	**Prawn Meal**	**Mussel Meal**
Fishmeal[1]	70.34	70.34	0	0	0	0
Artemia Meal[2]	0	0	81.84	0	0	0
Squid Powder[3]	0	0	0	53.88	0	0
Prawn Meal[2]	0	0	0	0	55.55	0
Mussel Meal[2]	0	0	0	0	0	86
Gelatin	3	3	3	3	3	3
Squid oil	14.88	14.88	1.40	17.15	18.87	7.77
Soybean Lecithin	1	1	1	1	1	1
Choline Chloride	0.5	0.5	0.5	0.5	0.5	0.5
Mineral Mix[4]	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin Mix[5]	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin C[6]	0.6	0.6	0.6	0.6	0.6	0.6
Cellulose	8.68	8.28	10.66	22.87	19.48	0.14
Food Dye	0	0.5	0	0	0	0
Analysis						
Crude Protein [a*]	48.21	48.14	48.28	48.14	48.18	48.04
Gross Energy [b*]	19.89	19.94	19.72	19.86	19.95	19.77

*% dry matter by mass

[1]Skretting Australia

[2] Homogenised/freeze-dried

[3] Rieber & Son, Bergen, Norway

[4]Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate sodium selenate

[5]Citric acid, ascorbic acid, alpha tocopherol, myo-inositol, ethoxyquin, nicotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol,

[6] Ascorbic acid

[a] Kjeldahl nitrogen (N X 6.25)

[b] $MJ.kg^{-1}$ bomb calorimetry, corrected for cellulose inclusion

Table 4. Ingredient and proximate composition of experimental diets used in Experiment 4; formulated to 48% crude protein and 20 J/kg gross energy levels, with different native animal meals as dietary protein sources (fish meal, squid powder and mussel meal).

	Diet									
	100S	66S 33F	66S 33M	100M	66M 33F	66M 33S	100F	66F 33M	66F 33S	33F 33M 33S
Formulated to contain (% dietary protein):										
Fishmeal	0	33.33	0	0	33.33	0	100	66.67	66.67	33.33
Squid Powder	100	66.67	66.67	0	0	33.33	0	0	33.33	33.33
Mussel Meal	0	0	33.33	100	66.67	66.67	0	33.33	0	33.33
Ingredient *:										
Fishmeal[1]	0	23.45	0	0	23.45	0	70.34	46.89	46.89	23.45
Squid Powder[2]	53.88	35.92	35.92	0	0	17.96	0	0	17.96	17.96
Mussel Meal[3]	0	0	28.67	86.00	57.33	57.33	0	28.665	0	28.67
Gelatin	3	3	3	3	3	3	3	3	3	3
Squid oil	17.15	16.40	14.02	7.77	10.14	10.90	14.88	12.51	15.64	13.27
Lecithin	1	1	1	1	1	1	1	1	1	1
Choline Chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral Mix[4]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin Mix[5]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin C[6]	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Cellulose	22.87	18.14	15.29	0.14	2.99	7.72	8.68	5.83	13.41	10.56
Proximate Composition:										
Crude Protein[a]	47.9	48.31	48.43	48.11	48.46	48.28	48.06	48.35	48.22	47.81
Gross Energy[b]	19.77	19.8	19.82	19.75	19.84	19.74	19.82	19.88	19.74	19.92

*% dry matter by mass

[1]Skretting Australia

[2] Rieber & Son, Bergen, Norway

[3] Homogenised/freeze-dried

[4]Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate sodium selenate

[5]Citric acid, ascorbic acid, alpha tocopherol, myo-inositol, ethoxyquin, nicotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol,

[6] Ascorbic acid

[a] Kjeldahl nitrogen (N X 6.25)

[b] $MJ.kg^{-1}$ bomb calorimetry, corrected for cellulose inclusion

Table 5. Ingredient and proximate composition of experimental diets used in Experiment 5; formulated to 50% crude protein and 21MJ/kg gross energy levels, with different native animal meals as dietary protein sources (fish meal, squid powder and krill meal).

	Diet									
	100F	90F/10S	80F/20S	70F/30S	60F/40S	50F/50S	40F/60S	30F/70S	20F/80S	10F/90S
Formulated to contain (% dietary protein):										
Fishmeal	100	90	80	70	60	50	40	30	20	10
Squid Meal	0	10	20	30	40	50	60	70	80	90
Ingredient *:										
Fishmeal[1]	73.41	66.07	58.73	51.39	44.05	36.71	29.37	22.02	14.68	7.34
Squid Meal[2]	0.00	5.62	11.25	16.87	22.49	28.11	33.74	39.36	44.98	50.60
Gelatin	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Squid oil	16.45	16.69	16.92	17.16	17.40	17.64	17.87	18.11	18.35	18.59
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline Chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral Mix[3]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin Mix[4]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin C[5]	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Cellulose	4.04	5.52	7.00	8.48	9.96	11.44	12.93	14.41	15.89	17.37
Proximate composition:										
Crude Protein[a]	50.13	50.07	50.11	50.06	50.08	50.16	50.02	50.09	50.12	49.96
Gross Energy[b]	21.31	21.06	21.26	21.17	21.07	21.14	21.07	21.18	21.13	21.21

*% dry matter by mass

[1, 3] Skretting Australia

[3]Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate sodium selenate

[4]Citric acid, ascorbic acid, alpha tocopherol, myo-inositol, ethoxyquin, nicotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol,

[5]Ascorbic acid

[a] Kjeldahl nitrogen (N X 6.25)

[b] $MJ.kg^{-1}$ bomb calorimetry, corrected for cellulose inclusion

Table 6. Ingredient and proximate composition of experimental diets used in experiment 6; formulated to 53% crude protein and 22 MJ/kg gross energy levels, with different native animal meals as dietary protein sources (fish meal, squid powder and krill meal).

	Diet									
	90F/10S	80F/10S/ 10K	70F/10S/ 20K	60F/10S/ 30K	50F/10S/ 40K	40F/10S/ 50K	30F/10S/60K	20F/10S/70K	10F/10S/80K	10S/90K
Formulated to contain (% dietary protein):										
Fishmeal	90	80	70	60	50	40	30	20	10	0
Squid Meal	10	10	10	10	10	10	10	10	10	10
Krill Meal	0	10	20	30	40	50	60	70	80	90
Ingredient *:										
Fishmeal[1]	70.22	62.42	54.61	46.81	39.01	31.21	23.41	15.60	7.80	0.00
Squid Meal[2]	5.98	5.98	5.98	5.98	5.98	5.98	5.98	5.98	5.98	5.98
Krill Meal[3]	0.00	7.03	14.05	21.08	28.11	35.13	42.16	49.19	56.21	63.24
Gelatin	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Squid oil	17.01	17.42	17.83	18.25	18.66	19.07	19.48	19.90	20.31	20.72
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline Chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral Mix[4]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin Mix[5]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin C[6]	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Cellulose	0.70	1.06	1.42	1.79	2.15	2.51	2.87	3.24	3.60	3.96
Proximate composition:										
Crude Protein[a]	53.92	53.33	53.68	53.30	53.23	53.30	53.22	53.51	53.10	53.87
Gross Energy[b]	22.31	22.17	22.42	22.40	22.62	22.06	22.55	22.13	22.37	22.20

*% dry matter by mass

[1, 3] Skretting Australia

[2] Rieber & Son, Bergen, Norway

[4] Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate sodium selenate

[5] Citric acid, ascorbic acid, alpha tocopherol, myo-inositol, ethoxyquin, nicotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol,

[6] Ascorbic acid

[a] Kjeldahl nitrogen (N X 6.25)

[b] $MJ.kg^{-1}$ bomb calorimetry, corrected for cellulose inclusion

1.3.1.3 Results

Experiment 1. The influence of dietary protein and energy on growth, survival and thyroid hormone levels in barramundi larvae fed microbound diets from 14 to 28 DAH.

Increasing dietary energy levels from 18 to 21 $MJ.kg^{-1}$ led to increased growth performance in terms of final dry weight and total length ($P < 0.05$, Figs. 1 and 2, Tables 6 and 7). Dietary protein level had no significant effect on either growth index. Survival ranged from 13% to 33.67% (Fig. 3). There was no significant influence of diet on survival, though larvae fed the 50% protein, 21 $MJ.kg^{-1}$ diet had a slightly higher mean survival (28 ± 1.67%) than larvae from all other treatments.

The whole carcass T4 level of larvae fed the 45% protein, 18 $MJ.kg^{-1}$ diet (2.81 ± 0.33 $ng.g^{-1}$ body weight), was significantly lower than in those fed the 45% protein, 21 $MJ.kg^{-1}$ (4.04 ± 0.15 $ng.g^{-1}$), 50% protein, 21 $MJ.kg^{-1}$ (4.3 ± 0.16 $ng.g^{-1}$), 55% protein, 18 $MJ.kg^{-1}$ (4.02 ± 0.34 $ng.g^{-1}$) and 55% protein, 21 $MJ.kg^{-1}$ diets (4.3 ± 0.33 $ng.g^{-1}$; Fig. 4). Larvae fed the 50% protein, 18 $MJ.kg^{-1}$ diet had an intermediate value for carcass T4 concentration (3.81 ± 0.18 $ng.g^{-1}$). Mean whole carcass T3 levels ranged from 2.67 to 3.34 $ng.g^{-1}$ fish body weight (Fig. 5), with no significant differences between dietary treatments.

There was a significant correlation between larval total length and T4 level ($P < 0.01$, $r = 0.595$), and total length was significantly related to dry weight ($P < 0.01$, $r = 0.858$). There were no significant correlations between dry weight and T4 level or between T3 and either dry weight or total length ($P > 0.05$).

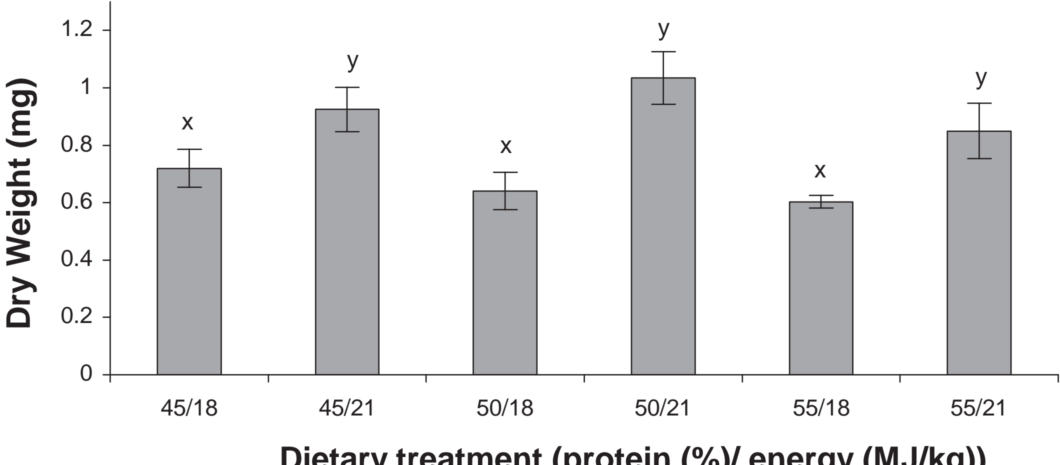

Figure 1. Final mean (±S.E. n = 4) dry weight (g) of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 $MJ.kg^{-1}$). Means with different superscripts (x-y, two-way ANOVA) are significantly different ($P < 0.05$).

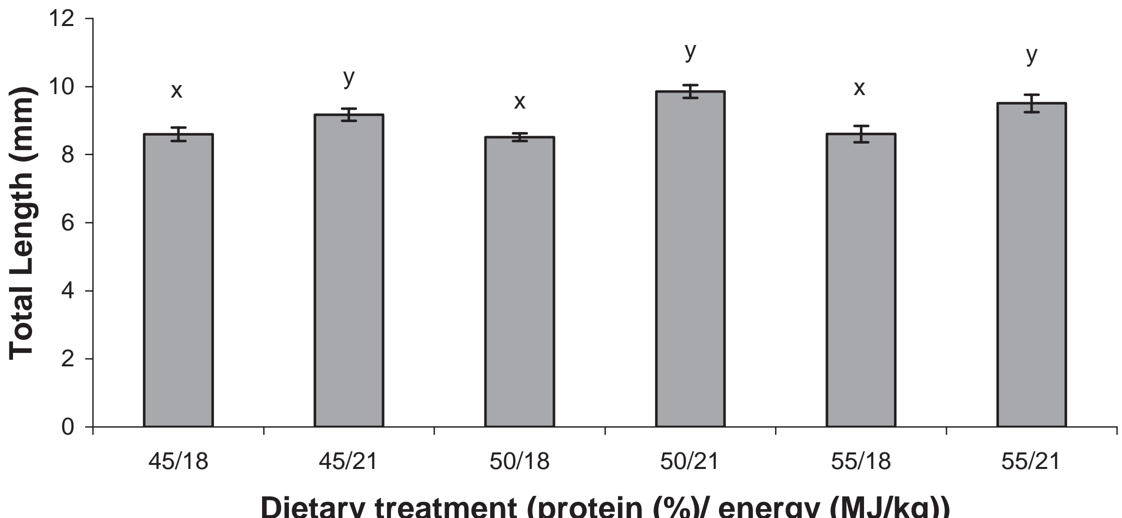

Figure 2. Final mean (±S.E. n = 4) total length of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 $MJ.kg^{-1}$). Means with a different superscript are significantly different (x-y, two-way ANOVA; $P < 0.05$; ±S.E. n = 4).

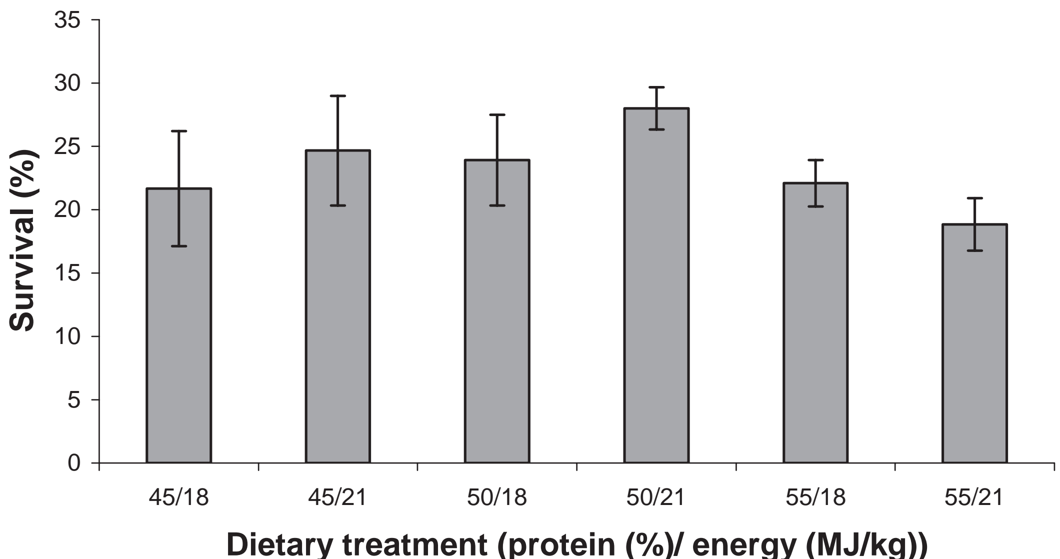

Figure 3. Survival of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 $MJ.kg^{-1}$). There were no significant differences between means (one-way ANOVA; $P > 0.05$; ±S.E. n = 4)

Table 6. Two-way ANOVA calculated on final dry weight of *L. calcarifer* larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ kg^{-1}).

Source	SS	DF	MS	F	Sig.
Protein	0.039	2	0.02	0.986	0.395
Energy	0.514	1	0.514	25.933	0
Interaction	0.069	2	0.035	1.748	0.206
Error	0.317	16	0.0198		

Table 7. Two-way ANOVA calculated on final total length of barramundi larvae fed artificial diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJkg^{-1}).

Source	SS	DF	MS	F	Sig.
Protein	0.32	2	0.16	0.989	0.394
Energy	4.76	1	4.76	29.38	0
Interaction	0.51	2	0.255	1.574	0.238
Error	2.592	16	0.162		

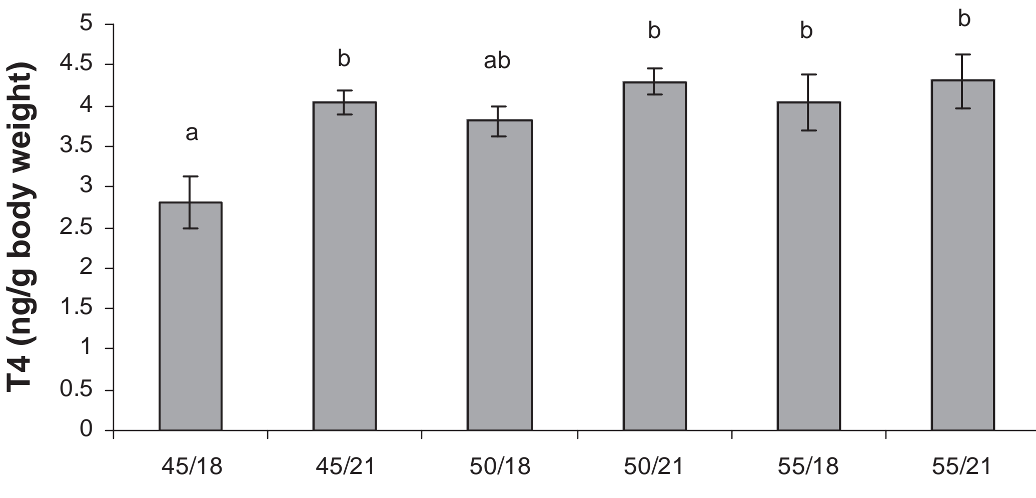

Figure 4. Whole carcass L-thyroxine levels of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ.kg^{-1}). Treatments with a different superscript (a-b) are significantly different (1-way ANOVA; $P < 0.05$; ±S.E. n = 3).

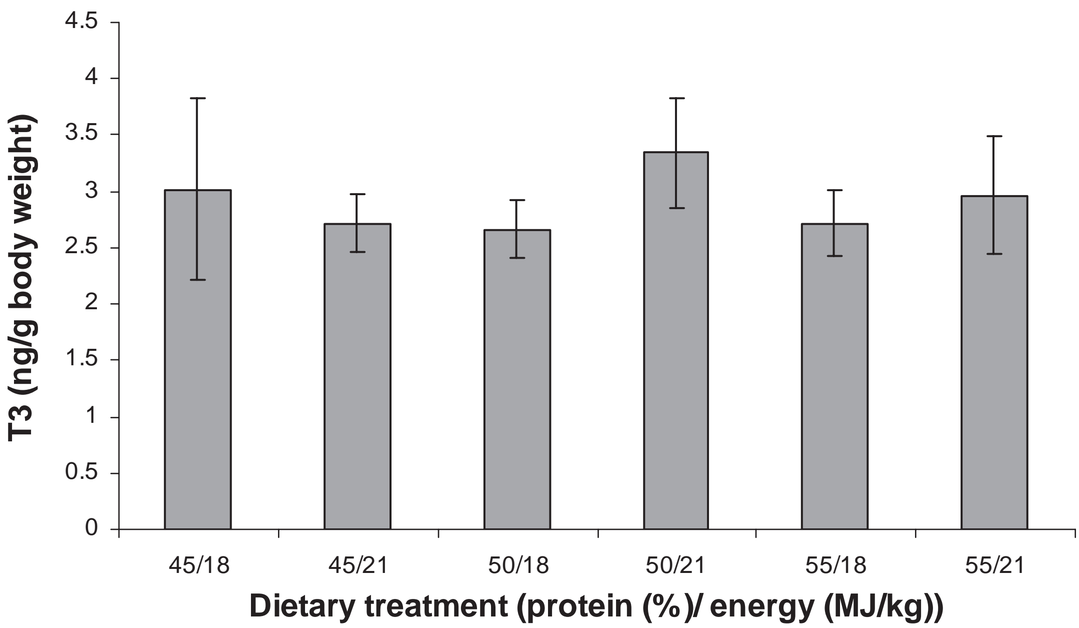

Figure 5. Whole carcass triiodothyronine concentration of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ.kg^{-1}). There were no significant differences between means. ($P < 0.05$; ±S.E. n = 3)

Experiment 2. The effect of dietary energy on growth, survival and thyroid hormone levels in* L. calcarifer *larvae fed microbound diets from 14 to 28 DAH.

The final dry weight and total length for larvae fed diets containing 21 and 22 MJ.kg^{-1} dietary utilisable energy was significantly higher than those fed the 18 and 19 MJ.kg^{-1}, while those fed the diet containing 20MJ.kg^{-1} had intermediate values for both parameters (Figs. 6 and 7). Survival of fish larvae throughout this experiment ranged from 24.6 – 55.2%, with no significant differences between dietary treatments (Fig. 8).

Carcass T4 and T3 levels ranged from 2.65 to 4.55 ng.g^{-1} and 2.53 to 4.85 ng.g^{-1} body weight respectively, with no significant differences between treatments (Figs. 9 and 10). There was however a significant regression, relating carcass T4 level to dietary energy inclusion ($r^2 = 0.203$, $P < 0.05$). Carcass T4 level was also correlated to the total length of larvae at the end of the experiment ($r^2 = 0.288$, $P < 0.05$), but not to dry weight ($P > 0.05$).

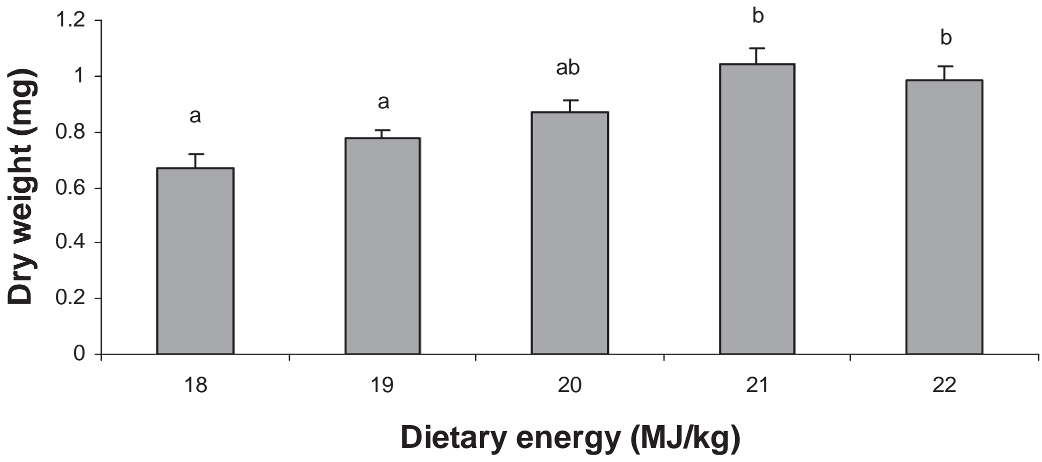

Figure 6. Final dry weight (g) of barramundi larvae fed microparticulate diets containing five different gross energy levels (18 – 22 MJ.kg^{-1}). Means with different superscripts (a-b) are significantly different (1-way ANOVA; P < 0.05; ±S.E. n = 3).

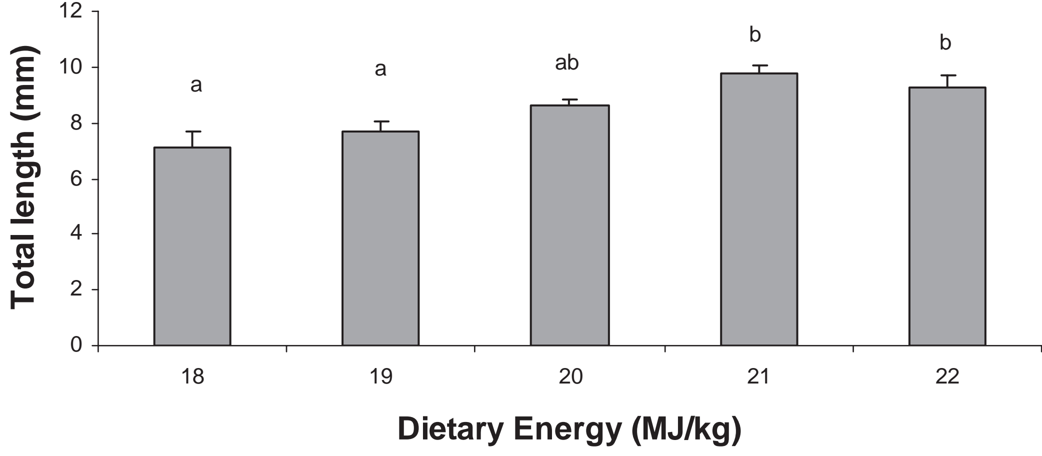

Figure 7. Final total length (mm) of barramundi larvae fed microparticulate diets containing five different gross energy levels (18 – 22 MJ.kg^{-1}). Means with different superscripts (a-b) are significantly different (1-way ANOVA; P < 0.05; ±S.E. n = 3).

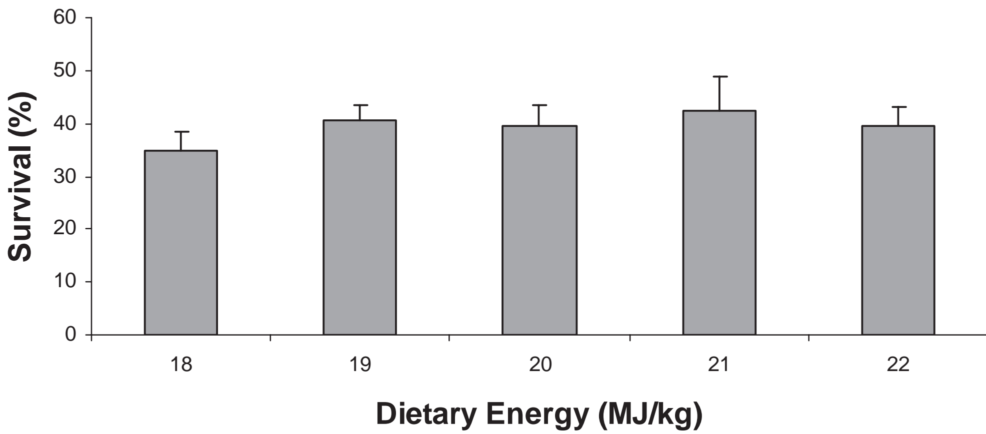

Figure 8. Survival of barramundi larvae fed microparticulate diets containing five different gross energy levels (18 – 22 MJ.kg^{-1}). There were no significant differences between means (P > 0.05; ±S.E. n = 3).

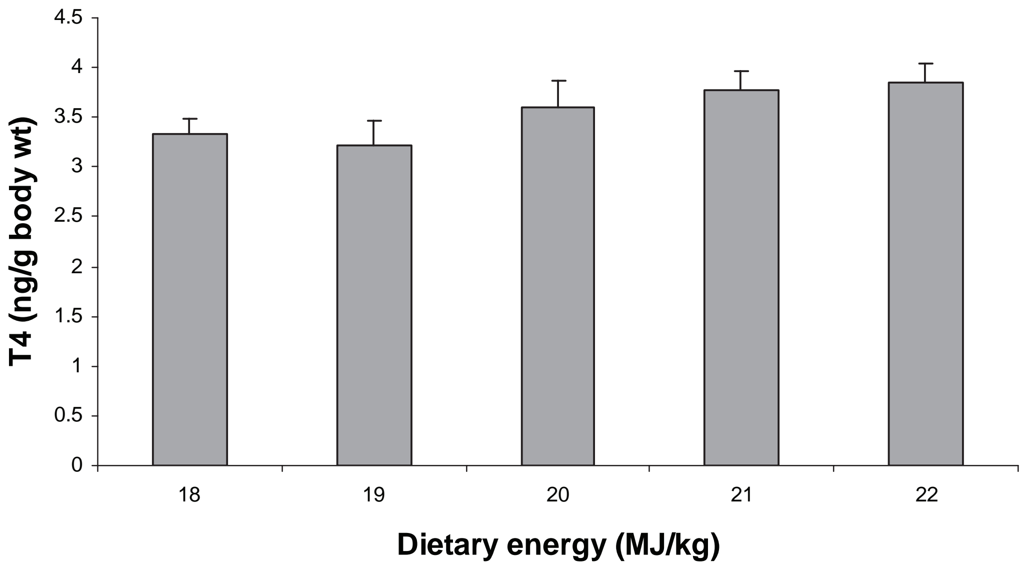

Figure 9. Carcass L-Thyroxine (T4) level of barramundi larvae fed microparticulate diets containing five different gross energy levels (18 – 22 MJ.kg^{-1}). There were no significant differences between means (P > 0.05; ±S.E. n = 3).

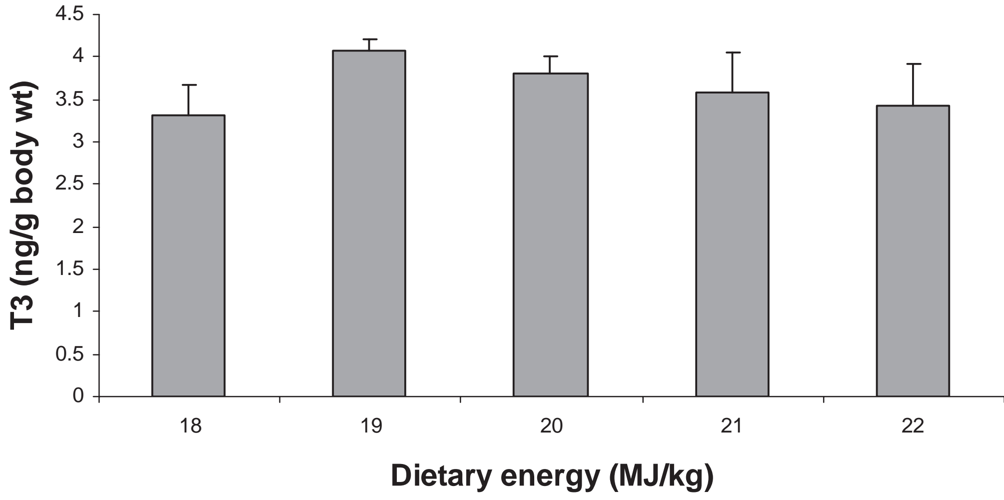

Figure 10. Carcass triiodothyronine (T3) level of barramundi larvae fed microparticulate diets containing five different gross energy levels (18 – 22 MJ.kg^{-1}). There were no significant differences between means (P > 0.05; ±S.E. n = 3).

Experiment 3. The use of native protein meals (fish meal, squid powder, mussel meal, prawn meal, and decapsulated* Artemia *cyst meal) to supply the dietary protein requirements of barramundi larvae fed microbound diets from 14 to 28 DAH.

Fish fed diets with *Artemia* cyst meal as the main protein source had the greatest total length (Fig. 11) and dry weight (Fig. 12). However the total length of fish fed diets based on *Artemia* cyst meal was not significantly different to the total length of fish fed fish meal or mussel meal based diets ($P > 0.05$). Likewise the dry weight of fish fed *Artemia* based diets was not significantly different to those fed fishmeal based diets (Fig. 12). The dry weight of fish fed the mussel meal based diet was significantly higher than the dyed fishmeal diet.

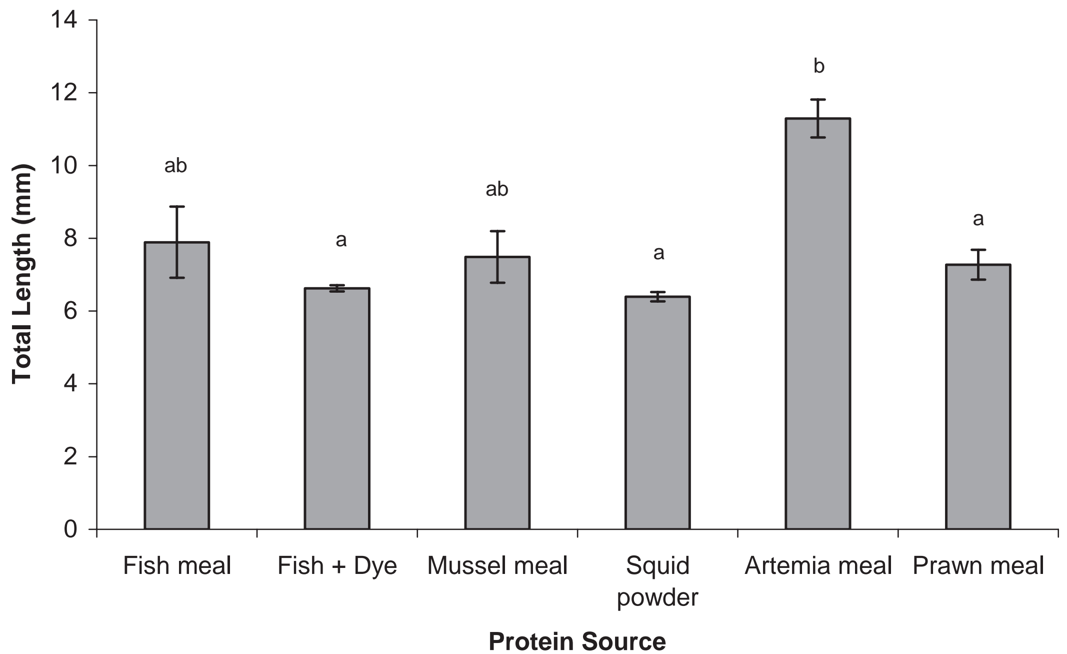

Figure 11. Total length of barramundi (*Lates calcarifer*) larvae, fed for 14 days on six isocaloric (20 MJ/kg), isonitrogenous (48% protein) artificial diets, varying in protein source. Different superscripts [(a-c)] denote statistically significant differences ($P < 0.05$; ±S.E. n = 3).

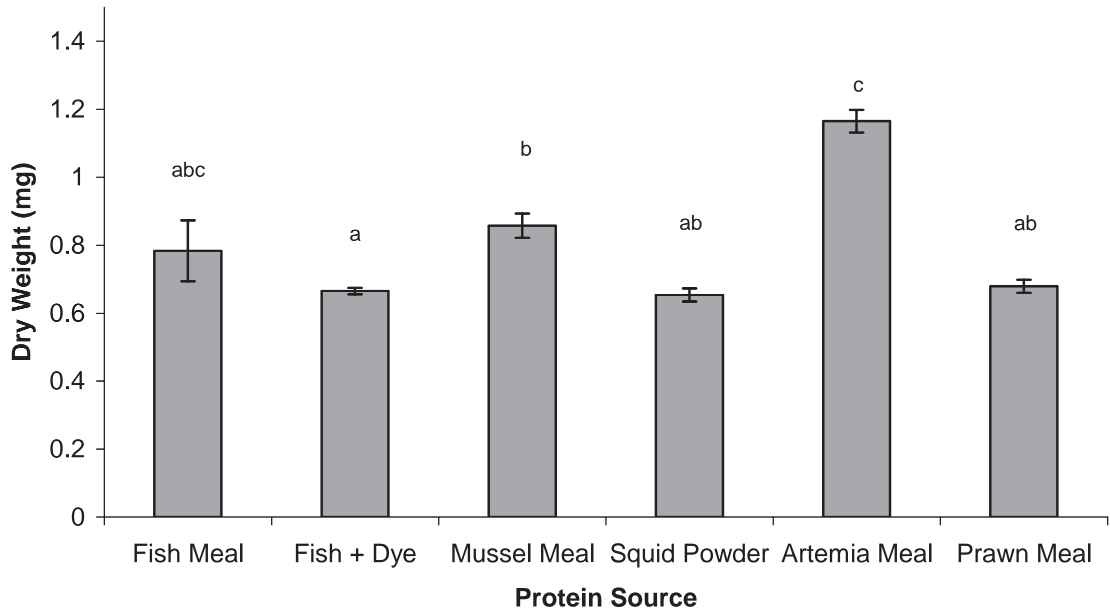

Figure 12. Final dry weight of barramundi (*Lates calcarifer*) larvae, fed for 14 days on six isocaloric (20 MJ/kg), isonitrogenous (48% protein) artificial diets, varying in protein source. Different superscripts [(a-c)] denote statistically significant differences ($P < 0.05$; ±S.E. n = 3).

Survival was significantly lower (P < 0.05) for fish fed the prawn-based diet (0.5%) to those fed the fish meal (11.8%) and dyed fish meal (8.4%) diets (Fig.13). All other treatments had intermediate values, though the mean survival value for the squid meal based diet (7.2%) was more than two-fold higher than the mussel meal based diet (2.7%) and four times the survival of the *Artemia* cyst based diet (1.75%).

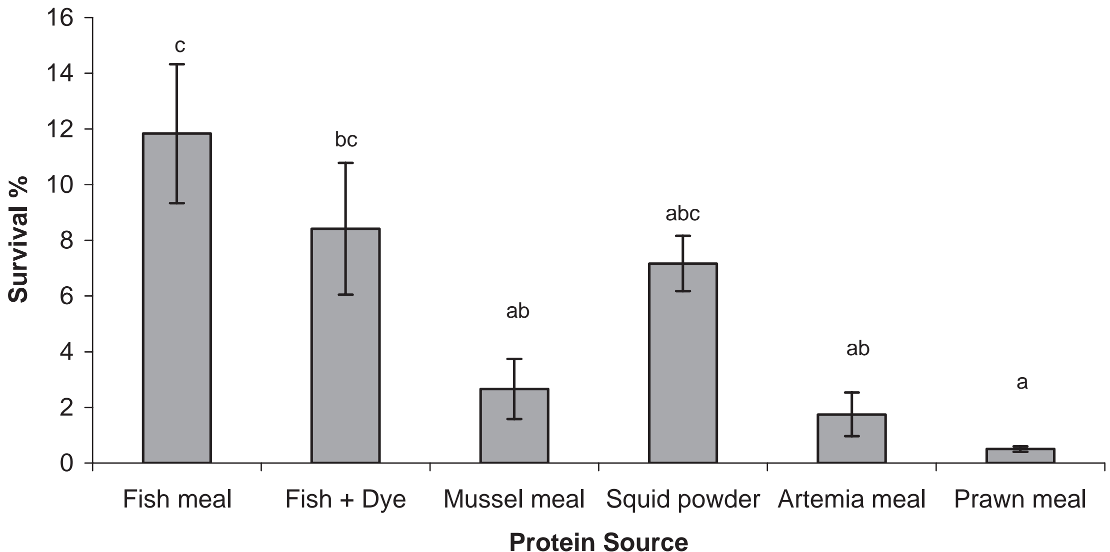

Figure 13. Survival of barramundi (*Lates calcarifer*) larvae, fed for 14 days on six isocaloric (20MJ/kg), isonitrogenous (48% protein) artificial diets, varying in protein source. Different superscripts [(a-c)] denote statistically significant differences (P < 0.05; ±S.E. n = 3).

Experiment 4. Refining the qualitative protein requirements of L. calcarifer *larvae fed microbound diets from 14 to 28 DAH.*

Fish fed the 66S/33F, 100F and 66F/33S had a significantly greater total length at the end of the experiment than all other experimental diets (Fig. 14). Fish fed the 100M and 66M/33F had a greater total length than those fish fed the 33/33/33 diet.

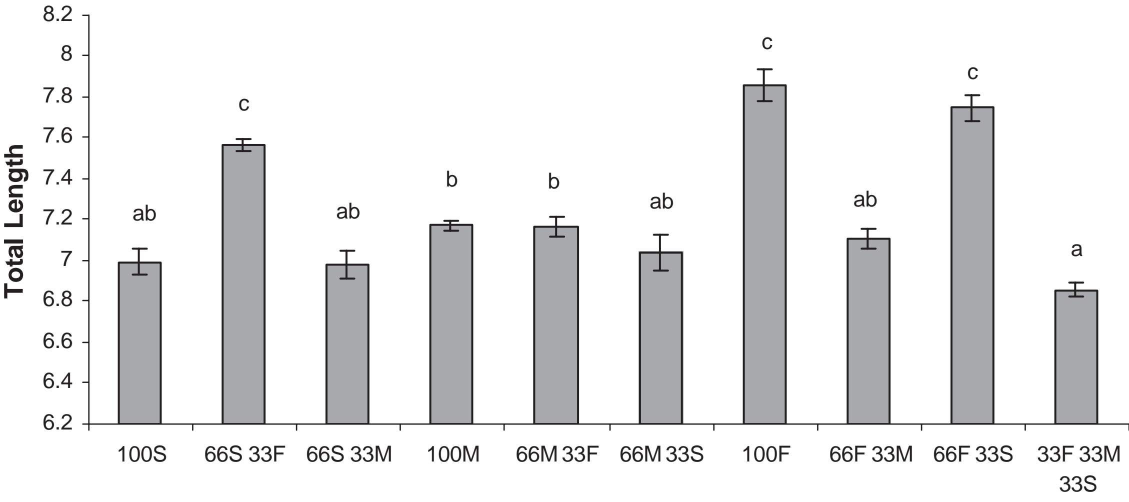

Figure 14. Total length of barramundi (*Lates calcarifer*) larvae, fed for 14 days on six isocaloric (20 MJ/kg), isonitrogenous (48% protein) artificial diets, varying in protein source. Different superscripts [(a-c)] denote statistically significant differences (P < 0.05; ±S.E. n = 3).

There was a significant difference in final dry weight between treatments, with those fish fed the 100F and 66F/33S diets having a significantly higher final dry weight than those fed the 100S, 66S/33M, 100M and 33/33/33 diets (Fig. 15). Fish fed the 66F/33S diet also had a significantly higher dry weight than those fed the 66M/33F diet. All other values were intermediate.

Those fish fed the 100F diet and the 66S/33F diet had a significantly higher T4 level than those fed the 100M, 66M/33S and 33/33/33 diet (Fig. 16). The 66F/33S and 100S diets also had a higher T4 levels than the 100M and 33/33/33 diets, with all other values being intermediate. There was a significant correlation between T4 and total length ($r^2 = 0.497$; $P < 0.01$) and between T4 and dry weight ($r^2 = 0.148$; $P < 0.05$).

Survival was variable, ranging from 2% to 50%, with no significant differences between treatments. However the 33% fish/33% mussel/33% squid diet had a particularly low and variable survival.

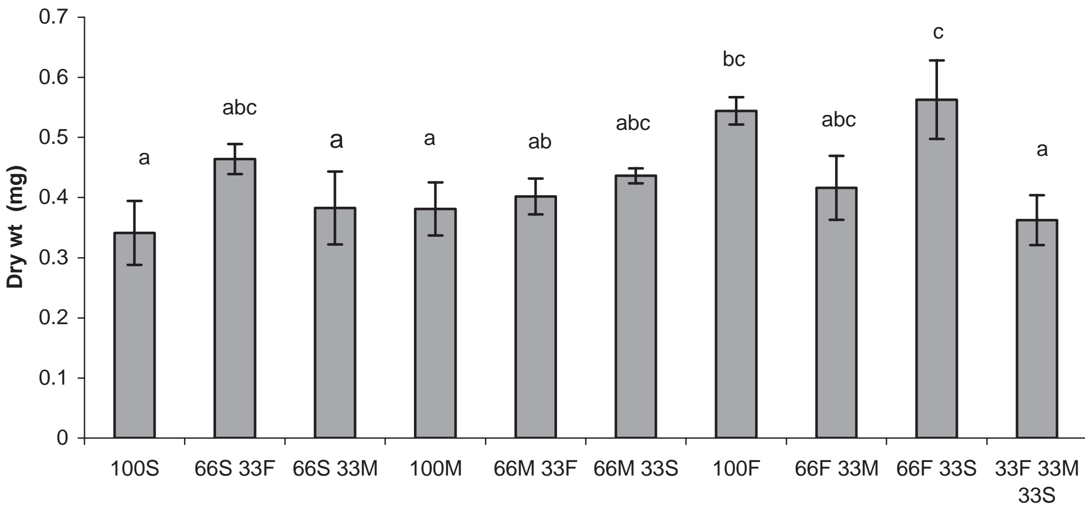

Figure 15. Final dry weight of barramundi (*Lates calcarifer*) larvae, fed for 14 days on six isocaloric (20MJ/kg), isonitrogenous (48% protein) artificial diets, varying in protein source. Different superscripts [(a-c)] denote statistically significant differences (P < 0.05; ±S.E. n = 3).

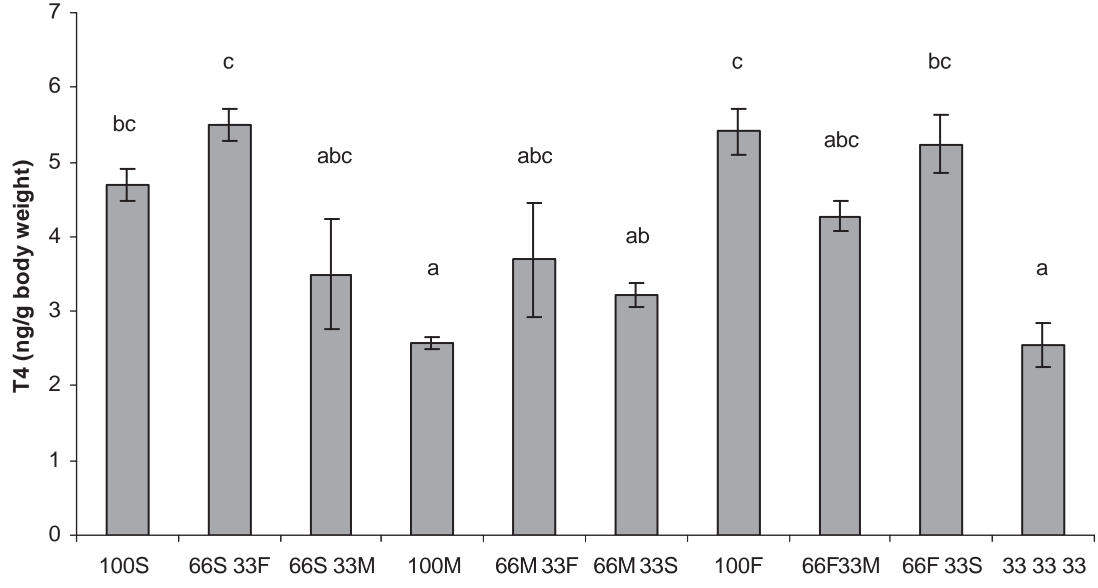

Figure 16. T4 level of barramundi (*Lates calcarifer*) larvae, fed for 14 days on six isocaloric (20MJ/kg), isonitrogenous (48% protein) artificial diets, varying in protein source. Different superscripts [(a-c)] denote statistically significant differences (P < 0.05; ±S.E. n = 3).

Experiment 5. Small-scale variations in fish meal and squid powder and the effect on growth, survival and thyroid hormone levels in barramundi larvae fed microbound diets from 14 to 28 DAH.

The total length of larvae fed the 90% fish meal, 10% squid powder diet had a significantly higher total weight at the end of the experiment than all other diets with the exception of the 80F/20S and 30F/70S diets (Fig. 17).

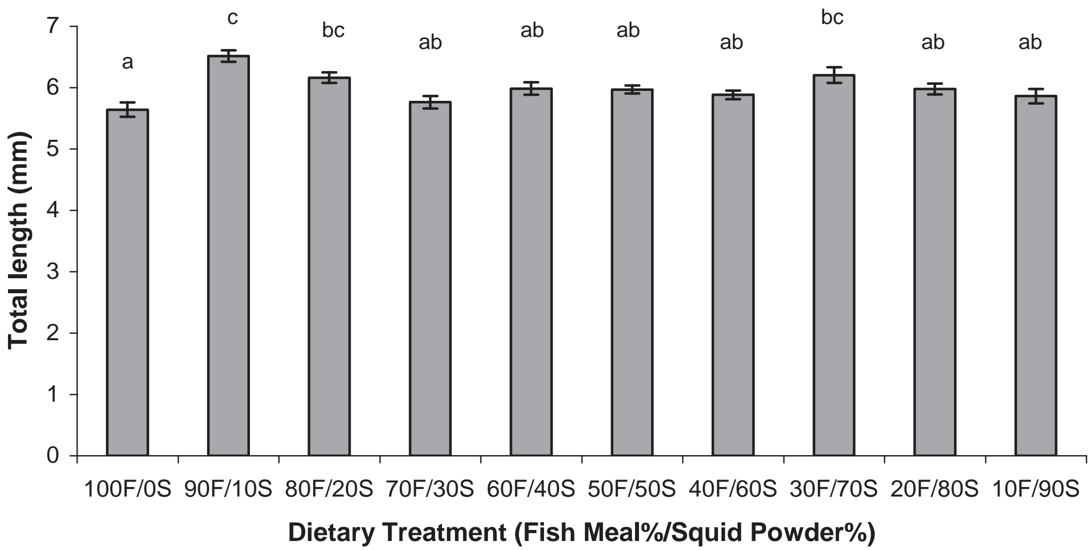

Figure 17. Final total length of barramundi larvae fed artificial diets containing ten different combinations of fish meal and squid powder from 14DAH to 28DAH. Treatments with a different superscript (a-c) were found to be significantly different from each other (one-way ANOVA; P < 0.05; ±S.E. n = 3).

The dry weight of fish fed the 90F/10S diet was significantly higher than all other treatments with the exception of those fish fed the 80F/20S treatment (Fig. 18). The 100F/0S, 60F/40S, 50F/50S and 10F/90S treatments had significantly lower dry weight than the 80F/20S dietary treatment. All other values are intermediate. No significant differences occurred between survival levels or carcass T4 levels in this experiment. Survival ranged from 33.6%, in the 60F/40S and 40F/60S diets, to 50%, in the 80F/20S diet.

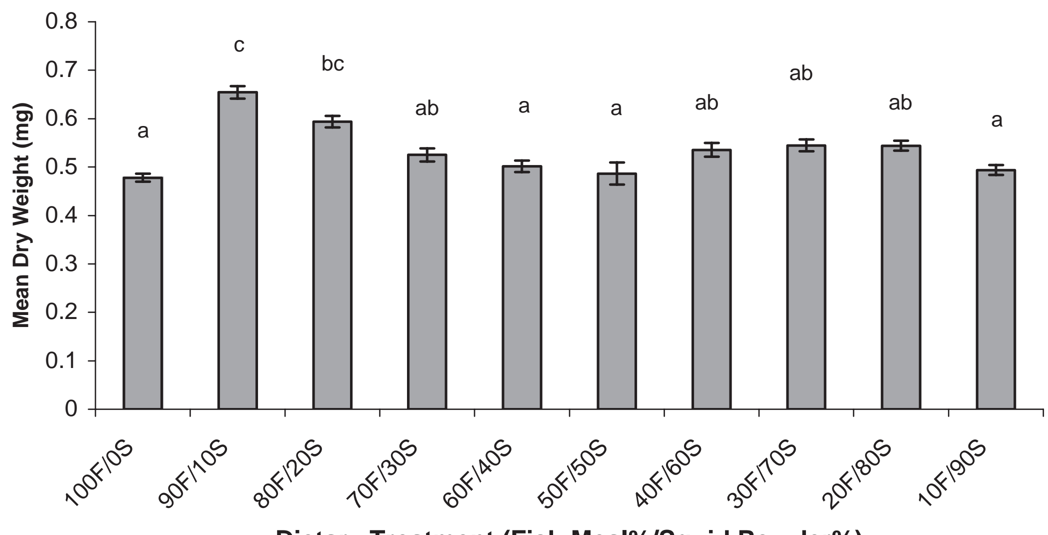

Figure 18. Final dry weight of barramundi larvae fed artificial diets containing ten different combinations of fish meal and squid powder, from 14DAH to 28DAH. Treatments with a different superscript (a-c) were found to be significantly different from each other (one-way ANOVA; P < 0.05; ±S.E. n = 3).

Experiment 6. The potential of krill meal to meet part of the qualitative protein requirement for barramundi fed microbound diets from 14 to 28 DAH.

Both dry weight and total length were significantly lower for larvae fed the diet with the highest krill meal inclusion level (Figs. 19 and 20). Significant linear regressions exist, relating decreased total length ($r^2 = 0.611$, $P < 0.01$), dry mass ($r^2 = 0.524$, $P < 0.01$) and carcass T4 level ($r^2 = 0.536$, $P < 0.01$) to increasing krill meal inclusion.

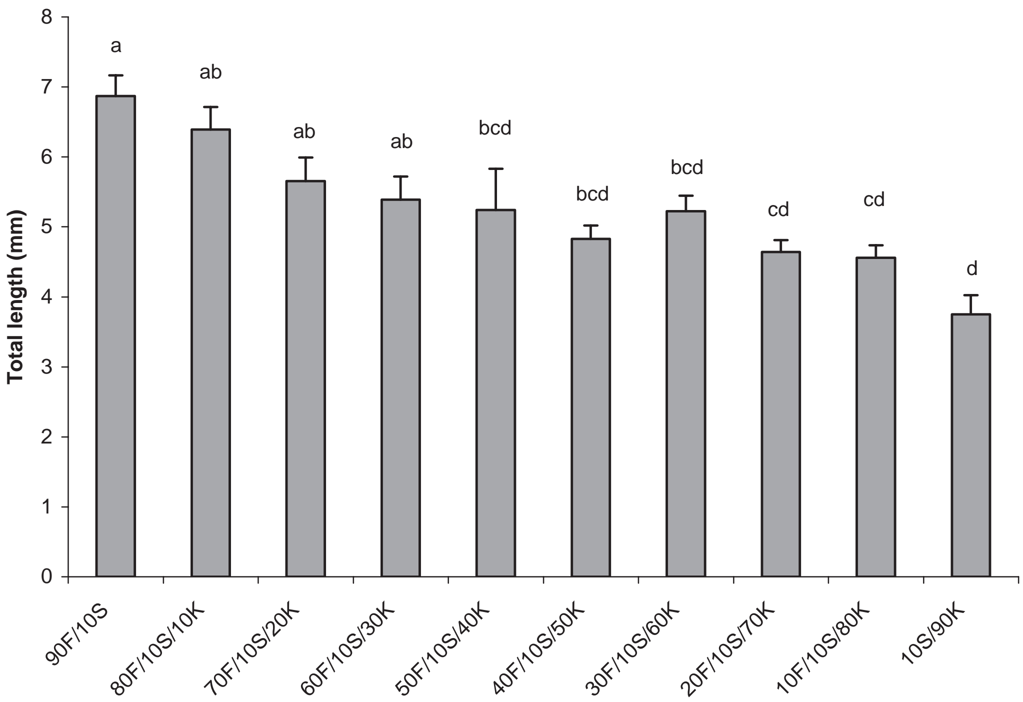

Figure 19. Final total length of barramundi larvae fed artificial diets containing ten different combinations of fish meal, squid powder, and krill meal from 14 DAH to 28 DAH. Treatments with a different superscript (a-c) were found to be significantly different from each other (one-way ANOVA; P < 0.05; ±S.E. n = 3).

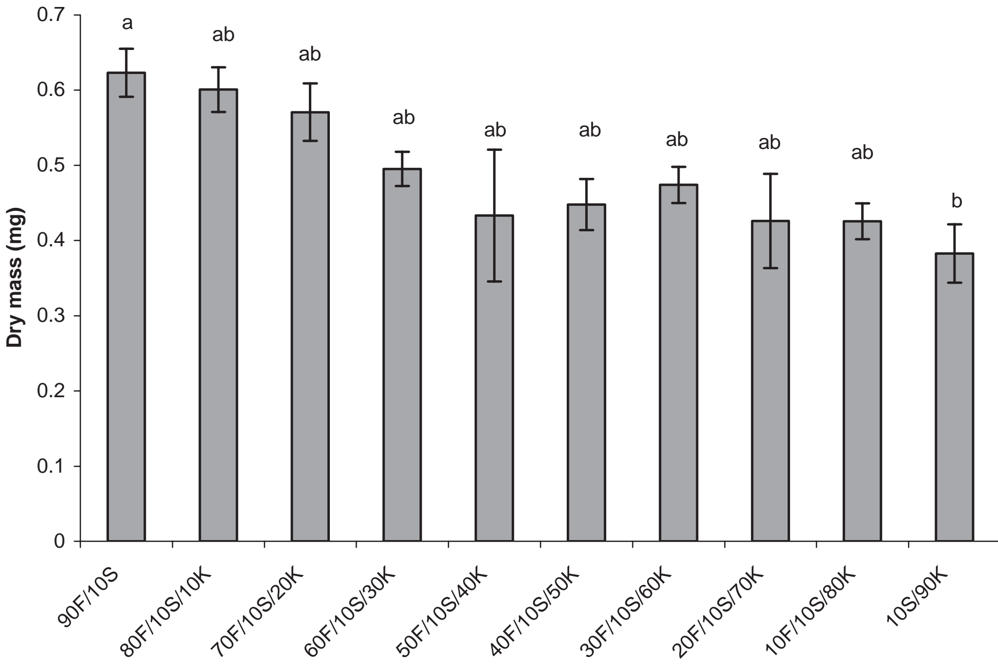

Figure 20. Final dry mass of barramundi larvae fed artificial diets containing ten different combinations of fish meal, squid powder, and krill meal from 14 DAH to 28 DAH. Treatments with a different superscript (a-c) were found to be significantly different from each other (one-way ANOVA; P < 0.05; ±S.E. n = 3).

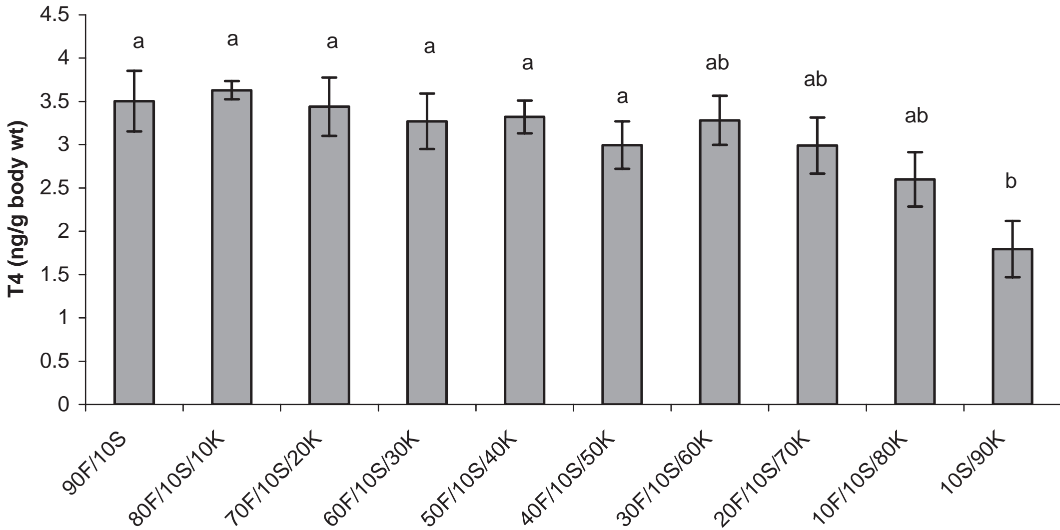

Figure 21. Carcass T4 level of barramundi larvae fed artificial diets containing ten different combinations of fish meal, squid powder, and krill meal from 14 DAH to 28 DAH. Treatments with a different superscript (a-c) were found to be significantly different from each other (one-way ANOVA; $P < 0.05$; ±S.E. n = 3).

The EAAI was generally high for all protein sources used, with mussel and prawn meals having the lowest values (Table 6). The AA/aa ratio was lowest for leucine in *Artemia* meal, methionine in fishmeal, lysine in krill meal, mussel meal and squid powder, and valine in prawn meal.

Table 6. Ratio of essential amino acids in protein sources to that of 21 DAH barramundi* (AA/aa) and essential amino acid index (EAAI).

Protein Source	Arg.	His.	Ile.	Leu.	Lys.	Met. (+ Cys.)	Phe. (+ Tyr.)	Thr.	Val.	EAAI
Artemia	1.00	0.98	1.00	0.86	0.91	0.91	1.00	1.00	0.96	0.96
Fish	1.00	1.00	0.94	0.94	0.93	0.83	0.98	0.95	0.95	0.95
Krill	1.00	0.96	1.00	0.92	0.80	1.00	1.00	0.98	0.96	0.96
Mussel	1.00	0.98	0.91	0.82	0.75	0.99	1.00	1.00	0.86	0.93
Prawn	1.00	0.87	0.94	0.94	0.86	1.00	1.00	0.87	0.82	0.93
Squid	1.00	0.95	1.00	0.96	0.80	1.00	0.97	0.97	0.81	0.94

* EAA profile of 21 DAH barramundi from (Rimmer et al. 1994)

1.3.1.4 Discussion

The total length and dry weight of fish larvae at the end of both experiments was directly related to dietary energy level and no effect of dietary protein content was evident. Dietary energy was manipulated by squid oil inclusion, and as such may also represent the availability of particular fatty acids. However the consistent increase in growth for larvae fed diets containing the higher utilisable dietary energy level, irrespective of the squid oil content, indicates that dietary energy is relevant to this study and has a significant effect on barramundi larvae growth. High dietary lipid levels have previously been shown to increase growth, survival and development in European seabream (Zambonino Infante and Cahu, 1999) and red drum (*Sciaenops ocellatus*) (Buchet et al. 2000), and the importance of utilisable dietary energy to such lipid requirements has previously been highlighted (Buchet et al. 2000).

All dietary protein inclusion levels supported similar rates of growth in barramundi larvae during this study. This suggests that the minimum protein requirement for optimal growth of barramundi larvae may not have been assessed in this study and would therefore be ≤ 45% of the total dry mass of the diet.

There was a significant correlation between T4 and total length in Experiments 1 and 2, and the regression relating carcass T4 level to dietary energy inclusion in Experiment 2. The growth stimulatory effects of T4 have been previously established for larvae of barramundi (Wan et al. 1997), among other species (Nacario, 1983; Lam and Sharma, 1985). Whole carcass T4 levels were found to be depressed in the lowest dietary protein and energy combination in Experiment 1. While there was no significant physical manifestation of this depressed T4 level, it may represent a decreased potential for growth.

The optimal source of protein found for barramundi larvae from 14 DAH to 28 DAH in this study came from a combination of fishmeal and squid powder, at 90% and 10% of total dietary protein, respectively. The first limiting amino acid in fish meal, when compared to the EAA profile of 21DAH barramundi, is methionine. Squid powder has relatively high levels of methionine, and is therefore likely to balance this deficiency. The first limiting amino acid in squid powder is lysine, which is likewise balanced by relatively high lysine levels in fish meal.

This study indicates that the optimal macronutrient profile for a microbound diet for barramundi larvae from 14-28 DAH contains at least 50% protein and 21MJ/kg dietary energy. The protein component of this diet should be composed of fish meal and squid powder, at a 9:1 ratio (total dietary protein basis). T4 appears to relate well to growth parameters in barramundi larvae, and may be representative of the capacity for larval growth.

1.3.1.5 References

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1.3.2 Influence of preparation method on physical characteristics, water stability, nutrient leaching and ingestion of micro-bound diet particles

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1.3.2.1 Introduction

Factors such as nutrient leaching, settlement rate and water stability of formulated food particles are greatly influenced by their physical properties (Kaushik, 1999; Durazo-Beltran and Viana, 2001; Dominy et al. 2003; Barrows and Lellis, 2000). In turn, the methods used to prepare diet particles play an important role in determining the physical properties and behaviour of food particles in the water column (Kaushik, 1999; Robinson, 2002; Barrows and Lellis, 2000).

Throughout this study and in the majority of prior research in this field, formulated micro-bound food particles for fish larvae have been prepared using oven drying. The diet mixture is oven dried before being pulverized and sieved into appropriate particle size ranges. Earlier experiments during this project showed that micro-bound diet (MBD) particles prepared in this way are negatively buoyant and have limited residence time in the water column. However, particle sinking rate is influence by the drying method used for MDB preparation; for example, MBD prepared by freeze-drying having a longer residence time than oven-dried particles. The residence time of a diet particle in the water column determines its availability to larvae, and is influenced by the method of preparation.

While leaching of some water-soluble nutrients (e.g. amino acids) from inert food particles is considered to be advantageous as a feeding stimulus (Yufera et al. 2002), excessive leaching of essential nutrients impacts negatively on nutritional value of such particles. A number of studies have shown that low-molecular weight water-soluble nutrients readily leach from inert food particles at a very high rate and this is considered a major limitation of inert food particles used as potential replacements for live foods fed to fish larvae.

One of the most important physical characteristics of an inert food particle is its ability to retain a desirable size and shape. It is energetically advantageous to feed as large a particle as the gape size of the animal will allow (Goolish et al. 1999). While chemoreception plays an important role in food searching, visual location is central to final capture of food particles and their ingestion. Successful inert food particles for fish larvae should be of optimal size, should retain that size after immersion and be easily distinguished by the rudimentary larval visual system (Cahu and Zambonino Infante, 2001). Furthermore, stable food particles, which have fallen to the bottom of larval culture tanks and will not be eaten, are more easily removed than a layer of minute particles and provide less surface area for bacterial proliferation.

Appropriate buoyancy is a persistent problem with inert food particles. Diet particles that settle to the bottom of culture vessel before larvae have a chance to ingest them are wasted. This increases costs associated with larval rearing and impacting negatively on water quality (Backhurst and Harker, 1988; Southgate, 2003). On the other hand, while it is possible for larvae to consume food particles from the water's surface, floating diet particles are less accessible, particularly in fully-stocked culture tank where the larvae are distributed throughout the water column. Additionally, due to the limited motion of early stage larvae, diet particles must be caught during their fall through the water column, as opposed to being pursued (Cahu and Zambonino Infante, 2001). Ideally, an inert food particle should be neutrally buoyant (Bengtson, 1993; Langdon, 2003), maintaining a constant position in the water column where it will remain available for ingestion until eaten.

Development of appropriate inert food particles marine fish larvae requires an understanding of larval feeding behaviours. A range of behaviours has been identified in fish larvae relating to food search, food location or capture and ingestion. Increased swimming speed, for example, has been linked with food search, often in response to chemical or visual sensing of prey (Mearns, 1986), while lunging (explosive leaps from a stationary position) is thought to be connected with attempted capture of prey (MacCrimmon and Twongo, 1980). The ultimate test of the success of an inert diet formulation, however, is the level to which it is ingested. This is influenced by all factors discussed above and, presumably, the diet ingested to the highest degree is that which has an acceptable rate of settlement, maintains its structural integrity long enough to be ingested and is attractive enough to the larvae to be consumed. Ideally, an inert food formulation should increased larval behaviour linked to food search or ingestion. Clearly, high quality inert food particles should also be digestible and their nutrients efficiently assimilated.

The majority of inert food particles used during this project (and those used in the majority of prior studies) have been prepared by pulverising a dried diet and sieving to an appropriate size range. Diet particles can also be prepared using marumerisation, where the diet is fed as a moist dough into a corkscrew extruder and the resulting strands fed into a marumariser which forms rounded pellets from the extruded diet. Recent acquisition of marumarisation equipment at the Australian Institute of Marine Science (AIMS) provided an opportunity to incorporate marumarised diet particles into experiment to determine the effects of preparation technique on the characteristics of diet particles.

The major objective of the following experiments was to assess the effects of drying technique (freeze-drying, oven-drying or moist) and method of preparation (crumbling or marumarised) on the physical characteristics, nutrient leaching, structural integrity and settlement rate of MBD. They also assessed the effects of these techniques on ingestion and assimilation of MBD by barramundi (*Lates calcarifer*) larvae. The results of these experiments will help optimise the performance of the prototype MBD developed during this project.

1.3.2.2 Materials and methods

The diet particle types produced used in the following experiments (with the exception of the ingestion and assimilation experiments), were:

- Freeze-dried Crumble Diet particles (FC)
- Oven-dried Crumble Diet particles (OC)
- Freeze-dried Marumarised Diet particles (FM)
- Oven-dried Marumarised Diet particles (OM)
- Moist Marumarised Diet particles (MM)

General diet preparation

The general diet used in this study was based on that of Partridge and Southgate (1999). The ingredients used in the diet, and the proportions in which they were used, are shown in Table 1.

Fish meal, squid powder and cellulose were combined in a beaker and mixed. Oil and lecithin were then added and mixed in. Remaining ingredients were added and combined thoroughly. Hot water was added to a beaker containing the gelatine until it was dissolved and this solution was added to the ingredient mixture.

For the 'crumble' diet, more hot water was added until the mixture was very viscous. This mixture was homogenized and the resulting 'slurry' spread thinly and evenly on plastic trays covered with aluminium foil. These trays were either placed in the oven at 50°C for 48 hours (oven-dried diet) or in the freeze drier at –5°C for 48 hours (freeze-dried diet). Once dried, diets were ground using a mortar and pestle and sieved through 700, 500, 400, 300 and 200 µm sieves. The target size range for the diets was 200–400 µm.

For the 'marumarised' diet, more hot water was to form a damp dough which was fed into a corkscrew extruder with a 300 µm die. The resulting strands were cut from the die at approximately 5 cm in length and fed into the marumariser. Resulting pellets were then dried in the same manner as the 'crumbled' diet, or left moist, and sieved to between 200-400 µm.

Experiment 1 – Physical characteristics of food particles

The five diet particle types, plus freeze-dried and oven-dried crumbled diet particles produced without cellulose, were photographed using scanning electron microscopy (SEM) to observe their external physical features. Diet particle samples were mounted on an aluminium stub and platinum-coated and images were collected at a probe current of 10nA using a JEOL JSM5410LV scanning electron microscope.

Experiment 2 – Soluble Nutrient Leaching

The method used to determine the level of soluble nutrient leaching from each of the five diet types was based on that of Bengtson (1993). Prior to the experiment, 25 x 300 ± 0.2 mg samples of each diet type were weighed into plastic screw top vials. The exact weight of each sample was recorded along with the weight of a corresponding filter paper. To ensure that only soluble nutrients from the diets and not smaller, broken pieces of diet particles, passed through, 90 mm Whatman GF/F filters were used with an aperture size of 0.45 µm. These were numbered prior to weighing. Each sample was added to 100 mL of 0.45 µm filtered seawater in a beaker. Leaching was determined at 1, 5, 15, 30 and 60 minute intervals (Baskerville-Bridges and Kling, 2000) and a control (water without diet) sample was collected at each time interval. A filter paper control was also prepared comparing

the difference in filter weight before and after drying. Five replicates were taken for each diet at each time interval (25 samples per diet). The filter apparatus consisted of a 90 mm wide ceramic Buchner funnel sitting on a 1 L Erlenmeyer flask with a side-arm connected to a vacuum.

After the prescribed period, beaker contents were poured onto the filter. The filter paper was removed from the funnel, placed on a plastic tray covered with aluminium foil and transferred to a drying oven at 50°C for 48 hours. After this time, they were weighed to the nearest 0.02 mg. The weight of soluble nutrients lost after immersion for each of the time intervals was determined according the equation:

$$A = \left[\frac{[(b+c)+d]-e}{(b+c)} \right] x\ 100$$

Where: A is the proportion of weight lost (%); b is the initial filter weight (mg); c is the initial diet weight (mg); d = is the weight gained by filter paper control (mg) and e is the final dry weight of filter and diet (mg).

The rate of amino acid leaching from the five diet types was determined by the methods of Baskerville-Bridges and Kling (2000). Diets were prepared as described above except that (for the purpose of detecting amino acids) glycine was substituted for 3% of the fishmeal component. Additional diets, containing no binder, were also made for determination of total potential for leaching from each diet type.

Prior to the experiment, 25 x 200 ± 0.2 mg samples of each of the five diet types were weighed into plastic screw top vials. Each sample was added to 100 mL of 0.45 µm filtered seawater in a beaker and processed as described above.

For each replicate, the water which passed through the filter was collected in and frozen until analysis. The level of amino acids present in the sample water, quantified by reaction with ninhydrin reagent, was considered to be that which had leached from the diet particles during immersion (Stryer, 1995; Baskerville-Bridges and Kling, 2000). This was determined for each replicate by taking a 3 mL sample and combining with 3 mL of ninhydrin reagent (0.35% w/v in ethanol). This mixture was then heated at 90°C for 15 minutes before cooling to room temperature. The absorbance of the resulting solution was recorded in a spectrophotometer at 570 nm (Baskerville-Bridges and Kling, 2000). Total potential leaching from each diet type was determined by analysing the binder-free diets in the same way at 1, 2 and 3 hour intervals and the proportion of amino acids leached from each diet was considered to be a function of total potential leaching. Glycine was used to construct a standard curve against which samples were compared to determine the amino acid content of the sample water.

The proportion of potential amino acid leaching from each of the diet particle types was determined according to the equation:

$$A = (b/c)\ x\ 100$$

Where: A is the proportion of potential leaching; b is the amino acid content of the sample water of the treatment replicates and c is the amino acid content of the sample water from those samples containing the corresponding binder-free particles.

For both soluble nutrient leaching and amino acid leaching, a univariate ANOVA was run (SPSS for Windows, Version 11.0) to determine the effects of length of immersion and diet type on leaching level. A further univariate ANOVA was run to determine the effects of preparation type and drying method on leaching.

Experiment 3 – Structural integrity of diet particles

A Malvern Mastersizer X grain size analyser (generally used for the measurement of sediment) was used to evaluate the degree to which diet particles disintegrated over time following immersion. The sample chamber of the analyser was filled with 500 mL of 0.45 µm filtered seawater. The diet/water solution was constantly cycled through the analyser and particle size measurements were taken after 1, 5, 15, 30 and 60 minutes. The instrument, through a computer program, produced a size-frequency distribution for particles ranging from 1 to 600 µm. Five replicates for each diet type were analysed in this way. Comparisons were then made between diet types in terms of the proportion of particles in the desirable size range (>150 µm) at a given time.

A univariate linear model (SPSS for Windows, Version 11.0) was run on the proportion of particles in the desirable size range over time to determine if there was a significant effect of diet type or time of immersion on the proportion of particles in the desirable size range. A further univariate linear model was run to determine if there was a significant effect of preparation technique or drying technique.

Experiment 4 – Particle settlement rate

The rate at which particles of each of the five diet types settled from suspension was determined, with some adjustments, by the method of Baskerville-Bridges and Kling (2000). An apparatus was constructed to allow samples to be taken from the water column at various depths (Fig. 1). This consisted of a 1 L plastic cylinder attached to a heavy wooden base (to keep the column stable and prevent agitation of the sample in the water during sampling). At 3 points along the length of the cylinder (100 mL, 500 mL and 900 mL), spacers, made from the open ends of 18 gauge needle sheaths, were attached. These spacers were designed to position the sample needles so the tip would take a sample from the centre of the water column. One 18-gauge needle was inserted into each of the three spacers attached to the side of the cylinder (Fig. 1).

The cylinder was filled with 1 µm filtered seawater to the 1 L mark. Following addition of the diet particles, sampling was undertaken every 30 seconds for 2.5 minutes. The diet particles were removed from the samples using numbered and pre-weighed 47 mm Whatman GF/C filters. Controls of water without diet particles were also taken.

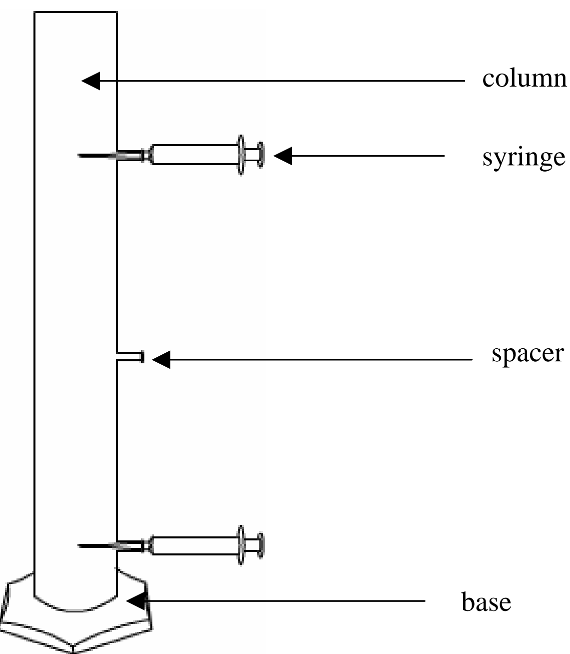


Figure 1. Apparatus used to determine particle settlement rates.

Three grams (± 1.3 mg) of each diet was weighed out and added to the settlement column. At the prescribed time intervals, a 5 mL sample was taken from each of the three sampling positions. These were filtered and resulting filter papers were transferred to a drying oven where they remained for 48 hours at 50°C. They were then re-weighed. Comparisons were made between the various diet types with reference to the pattern of settlement as well as the amounts of diet hypothetically available to the larvae at various times after diet immersion.

A univariate linear model was run (SPSS for Windows, Version 11.0) to determine if there was an effect of diet type or length of immersion on particle settlement rate. A further univariate linear model was run to determine if there was an effect of preparation and drying technique on the settlement rate.

Experiment 5 – Ingestion and Assimilation of diet particles

The barramundi (*L. calcarifer*) larvae used in this experiment were obtained from Blue Water Barramundi in Mourilyan, north Qld. The method used to quantify the levels of ingestion and assimilation of each of the five diet types was based on that of Partridge and Southgate (1999). Briefly, MBD labelled with ^{14}C were fed to 18 day old barramundi larvae for set periods of time. The ^{14}C content of the larvae was then measured, from which the level of ingestion of each of the five diet types and the degree to which each of them had been assimilated into larval tissues was determined. For more detailed information about this methodology see Partridge and Southgate (1999).

Because the marumariser has many small parts, which are difficult to clean, it was not possible to produce ^{14}C-labelled marumarised diet particles. Only freeze-dried and oven-dried crumbled diet particles were produced and tested. The formulation and preparation of these diets was the same as that of the crumbled diet outlined for Experiment 13.1 with the exception that 58% of the fishmeal component of the diet was replaced with ^{14}C labelled rotifers.

Each 2 L tank used in the experiments was filled with 600 mL lightly aerated 1 µm filtered UV-treated seawater. Groups of thirty 18-day-old *L. calcarifer* larvae were placed into each tank. After stocking, larvae were starved for approximately 14 hours to become acclimatised.

After feeding on the experimental diets, larvae from each tank were removed by pouring the contents through an immersed 800 µm sieve. The larvae were retained on the sieve and washed with distilled water to rinse any remaining diet from them. They were then transferred into a 20 mL scintillation vial and killed by immersing the vial in a container of crushed ice for one hour. Any remaining water in each vial was removed and 1 mL of solubilizing fluid (Soluene 350™, Packard) was added to dissolve the larval tissue. The vials were placed in an oven at 50°C for 48 hours to complete solubilisation. Five mL of scintillation cocktail (Hionic-Fluor™, Packard) was added after this time and the ^{14}C content of the larvae was determined using a β scintillation counter.

Larval radioactivity controls

In order to be confident that any ^{14}C detected in the larvae was due to ingested and/or assimilated ^{14}C-labelled diet, two control treatments were run in conjunction with the feeding experiments: (1) dead larvae with all other factors also being the same as the feeding experiments; and (2) live larvae held in the "radioactive" water without food. Both control treatments were replicated five times.

Ingestion of ^{14}C-labelled MBD

Larvae in each treatment were fed 3 mg of MBD every 10 minutes for one hour. They were then processed according to the procedures outlined above. Sample readings were corrected with consideration of the controls and ^{14}C content of the diets, according to the equation below, and expressed as µg of diet ingested per larva.

$$A = \frac{[(b-c) / d]}{e} \times 1000$$

Where: A is the total ingestion or assimilation (µg of diet per larvae); b is the ^{14}C content of larvae in sample (counts per minute); c is the sum of the averages of the two controls for that diet type; d is the specific activity of the diet and e is the number of larvae per replicate. Five replicates of each diet type were used.

Assimilation of ^{14} C-labelled MBD

The ^{14}C labelled MBD was fed to the larvae at a rate of 3 mg every 10 minutes for one hour, after which the larvae were fed a 'cold chase' diet for 7 hours. This diet was the same as the crumble diet used in Experiment 13.1. Larvae were processed as outlined above and the degree of assimilation of the diet (in µg of diet per larvae) was calculated, with consideration of the controls and diet-specific activity (detailed above). Five replicates per treatment were used.

Feeding Behaviour

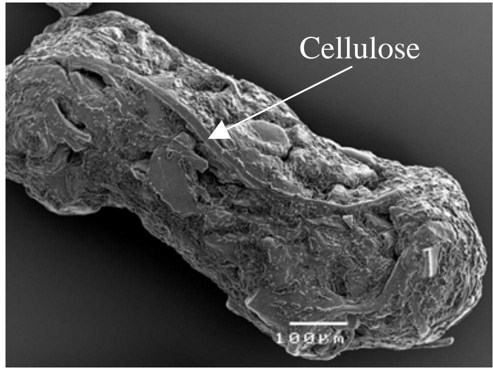
The behavioural responses of barramundi larvae to the presence of each of the five diet types were determined using methods based on those of Temple et al. (2004). Twenty-day old larvae were starved for one day prior to the experiment. Ten larvae were stocked into a 250 mL beaker containing 1 µm filtered seawater (i.e. a density of 40 larvae per litre) and allowed to acclimate in the beaker for three minutes. After this time, 50 ± 5 mg of the diet type being tested was added. One larva was randomly selected in the beaker and was observed for three minutes. During this time, swimming duration, number of strikes and number of pauses was recorded (Temple et al. 2004). 'Swimming' was considered to be a forward momentum of the larva, assisted by movement of the caudal and/or pectoral fins and was measured using a stopwatch. For each pause, the watch was stopped until swimming resumed to gauge the amount of time spent swimming. 'Striking' was defined by short, sharp bursts of speed, often in a direction different to that which was previously being travelled. 'Pauses' were times at which swimming stopped completely. Five replicates of each diet type were sampled in this way. A control treatment containing larvae without food was also run for comparison.

A one-way ANOVA was run on to determine if there was a significant difference between the volumes of ^{14}C labelled freeze-dried and oven-dried diet particles ingested by larvae. A second one- way ANOVA was run to determine if there was a significant difference between the volumes of ^{14}C labelled freeze-dried and oven-dried diet particles assimilated by the larvae. A univariate ANOVA was run on each of the observed behaviours to determine if there was any significant effect of either preparation technique or drying technique on induction of feeding related behaviours in the larvae.

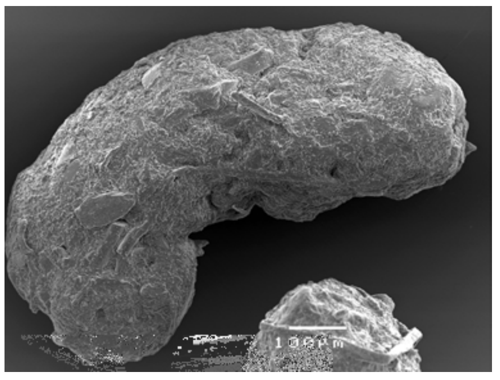
1.3.2.3 Results

Experiment 1 - Physical characteristics of food particles

Scanning electron micrographs of each of the seven particle types are shown in the figures below. These micrographs show the physical features of the particles such as surface texture and shape.



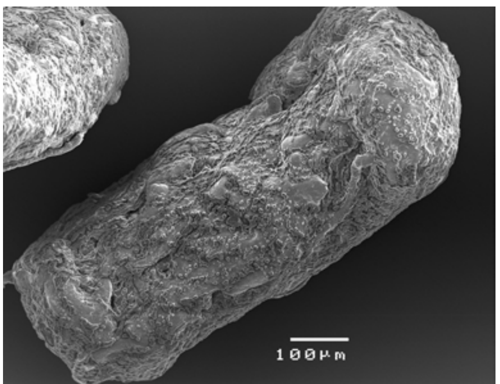
Scanning electron micrograph of a freeze-dried marumarised diet particle.



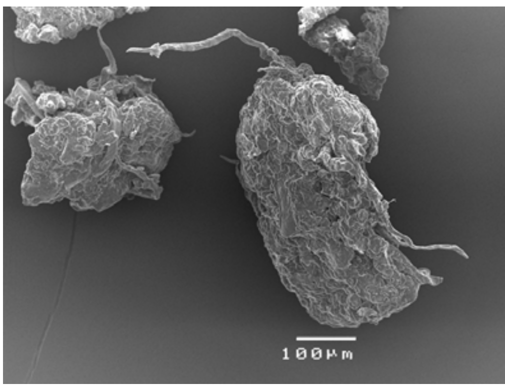
Scanning electron micrograph of a moist marumarised diet particle.

The 150x magnification of a freeze-dried marumarised (FM) diet particle, shows that it is elongated with a fairly uniform width. The surface texture is pitted with some larger hollows and obvious ridges (e.g. what appears to be a strand of cellulose running the length of the particle).

The 150x magnification of a moist marumarised (MM) diet particle, shows that it is of a similar shape to the FM particle, being elongate and of a uniform width. The surface texture is rough with few gaps or pits and, while smaller ingredient particles in the matrix are evident, they do not protrude considerably.



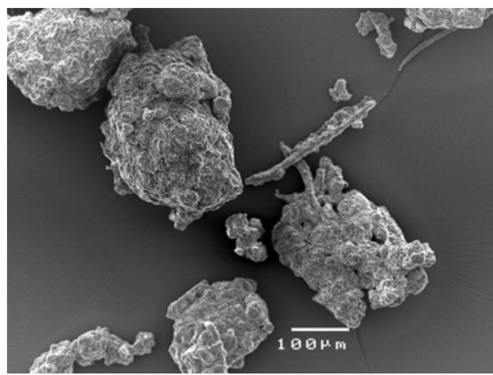
Scanning electron micrograph of an oven-dried marumarised diet particle.



Scanning electron micrograph of a freeze-dried crumbled diet particle.

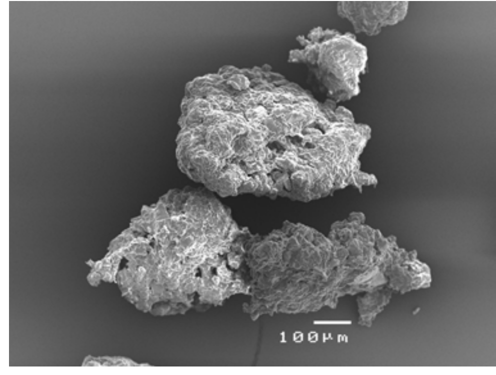
The 150x magnification of an oven-dried marumarised (OM) diet particle, shows it to be of a similar shape to both the FM and MM particles, again being elongate and of a uniform width. As with the MM particle, the surface texture is rough but devoid of any major protrusions.

Conversely, the 150x magnification of a freeze-dried crumbled (FC) diet particle, shows that the ‘body’ of the FC particle is approximately 2/3 the length of the marumarised particles and has a number of conspicuous protrusions, namely strands of cellulose each of which are roughly $^1/_4$ to $^1/_3$ the length of the particle. The surface texture is similar to that of the FM particle with some obvious lumps and gaps between ingredient particles.

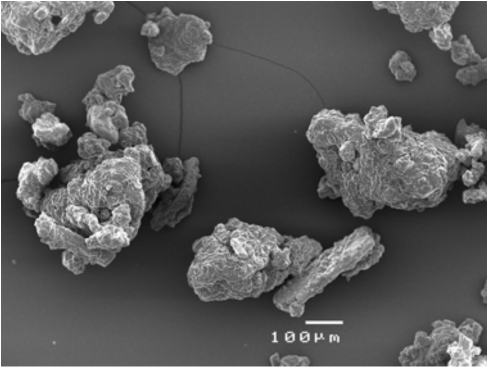


Scanning electron micrograph of oven-dried crumbled diet particles.

The 150x magnification of an oven-dried crumbled (OC) diet particle, shows that, while similar in width, is considerably shorter than the other particle types. Like the FC particle, the surface texture of the OC particle is very rough, with large gaps between ingredient particles and visible strands of cellulose protruding which are approximately ¼ the length of the particle itself.



Scanning electron micrograph of a freeze-dried cellulose-free diet particle.



Scanning electron micrograph of an oven-dried cellulose-free diet particle.

The 150x magnification of a cellulose free FC particle, shows that it is approximately the same size and shape as that of the OC particle, being considerably shorter than the marumarised particles. The surface texture of this particle is rough with large pits and many protruding bumps.

The 150x magnification of a cellulose free OC particle, shows that this particle, while of a similar size and shape to the cellulose free FC particle, appears to be more densely packed as very few individual ingredient particles are visible. The surface texture of this particle is rough with a small number of pits.

Experiment 2 – Soluble Nutrient Leaching

Fig. 2 shows the proportion of dietary ingredients which were lost from each of the five diet particle types at five time intervals over a one hour period. All diets followed a similar pattern of nutrient loss with a sharp increase between one minute and 15 minutes after immersion. A peak in nutrient loss can be seen at the 15-minute mark, after which no further increases were determined. This pattern is consistent for all diet particle types. After 30 minutes of immersion, only the FC and

OM particles continued to leach water-soluble nutrients. It can also be seen that the OM and FM particles leached a noticeably lesser proportion of total nutrients than particles of the other three diet types. There was found to be a significant effect of both length of immersion ($F(4,100) = 168.328$, $P < 0.05$) and diet type ($F(4,100) = 122.543$, $P < 0.05$) as well as preparation technique ($F(1,120) = 51.017$, $P < 0.05$) and drying technique ($F(2,120) = 24.971$, $P < 0.05$) on the level of soluble nutrient leaching but no significant interaction between the preparation technique and drying technique ($F(1,120) = 0.447$, $P > 0.05$).

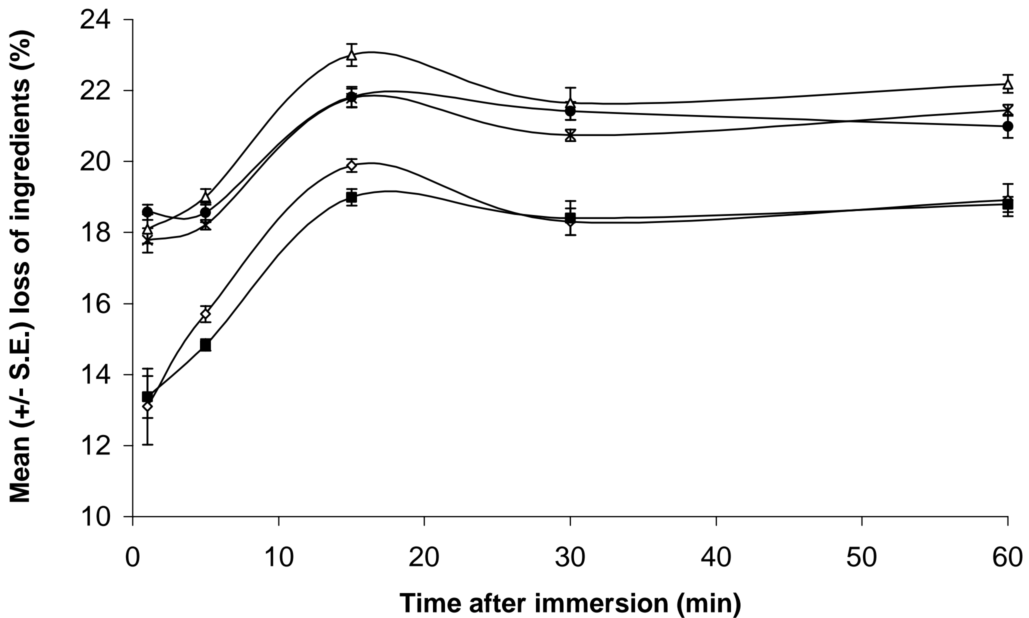


Figure 2. Mean (± S.E. n = 25) proportionate loss of water soluble nutrients from five particle types: freeze-dried marumarised (◇); oven-dried marumarised (■); moist marumarised (△); freeze-dried crumbled (x); oven-dried crumbled (◆) over time.

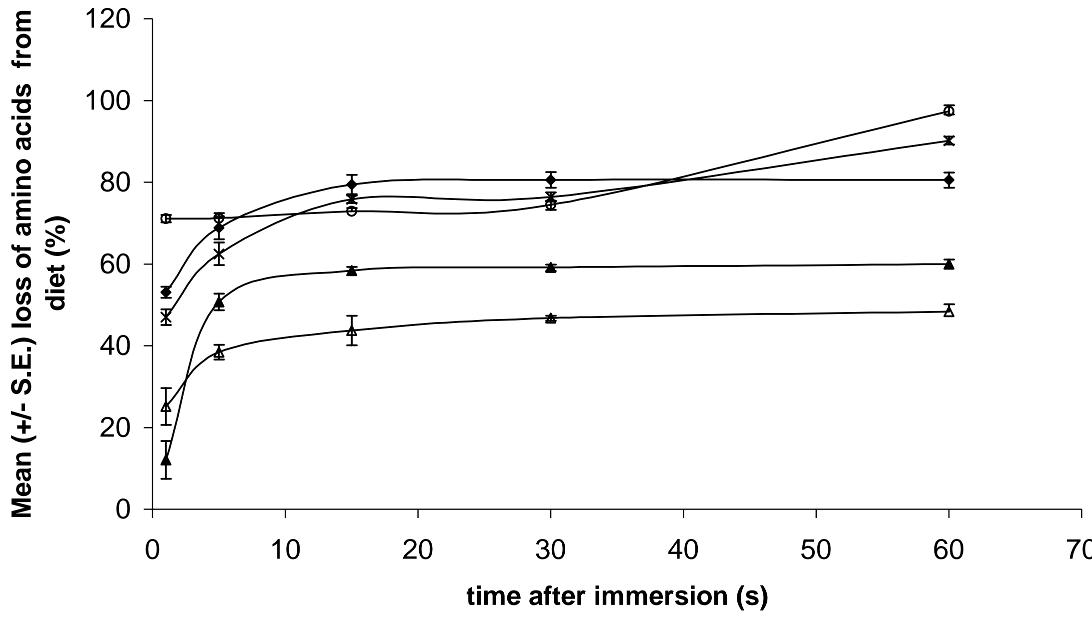


Figure 3. Mean (± S.E. n = 25) proportionate loss of amino acids from five particle types: freeze-dried marumarised (△); oven-dried marumarised (◆); moist marumarised (▲); freeze-dried crumbled (◇); oven-dried crumbled (x) over time.

Fig. 3 shows the proportion of loss of amino acids over time. Each diet particle type had a distinct pattern of leaching. Between 12% (moist marumarised diet) and 71% (freeze-dried crumbled diet) of potential amino acid leakage had occurred by the end of the first minute of immersion. Particles of all three drying treatments of the marumarised diet particles peaked in amino acid loss after 30 minutes of immersion. Particles of the crumbled diet continued to leach amino acids until sampling was terminated after 60 minutes when over 97% of amino acids had leached from the freeze-dried particles and 90% from the oven-dried particles. There was a significant effect of both length of immersion ($F(4,99) = 65.435$, $P < 0.05$) and diet type ($F(4,99) = 83.251$, $P < 0.05$) as well as preparation technique ($F(1,119) = 21.239$, $P < 0.05$) and drying technique ($F(2,119) = 8.330$, $P < 0.05$) on the level of amino acid leaching as well as a significant interaction between the two ($F(1,119) = 26.97$, $P < 0.05$).

Experiment 3 - Structural integrity of diet particles

Over 90% of OM and MM particles remained in the desirable size range (>150 µm) after one minute compared with FM (63%), OC (40%) and FC (4%) diet particles (Fig. 4). After five minutes immersion, while the proportion of MM particles in the desirable size range had decreased to 73%, it remained the diet type with the highest proportion, followed by OM (59%) → FM (20%) → OC (4%) and finally FC diet particles (3%).

Less than 1% of OC diet particles remained in the desirable size range after 15 minutes (Fig. 4). FC particles (2.43%) showed a higher proportion in the desirable size range after 30 minutes (Fig. 4) than FM particles (1.75%). Otherwise, MM remained the diet with the highest proportion in the desirable size range across all time intervals, followed by OM.

There was a significant effect of immersion time ($F(4,99) = 179.104$ $P < 0.05$) and diet type ($F(4,99) = 119.479$, $P < 0.05$) on the proportion of diet particles in the desirable size range as well as a significant interaction ($F(16,99) = 19.694$, $P < 0.05$) between the two. Additionally, there was a significant effect of both preparation ($F(1,119) = 20.836$, $P < 0.05$) and drying technique ($F(2,119) = 6.020$, $P < 0.05$) on the same as well as a significant ($F(1,119) = 2.530$, $P < 0.05$) interaction between the two.

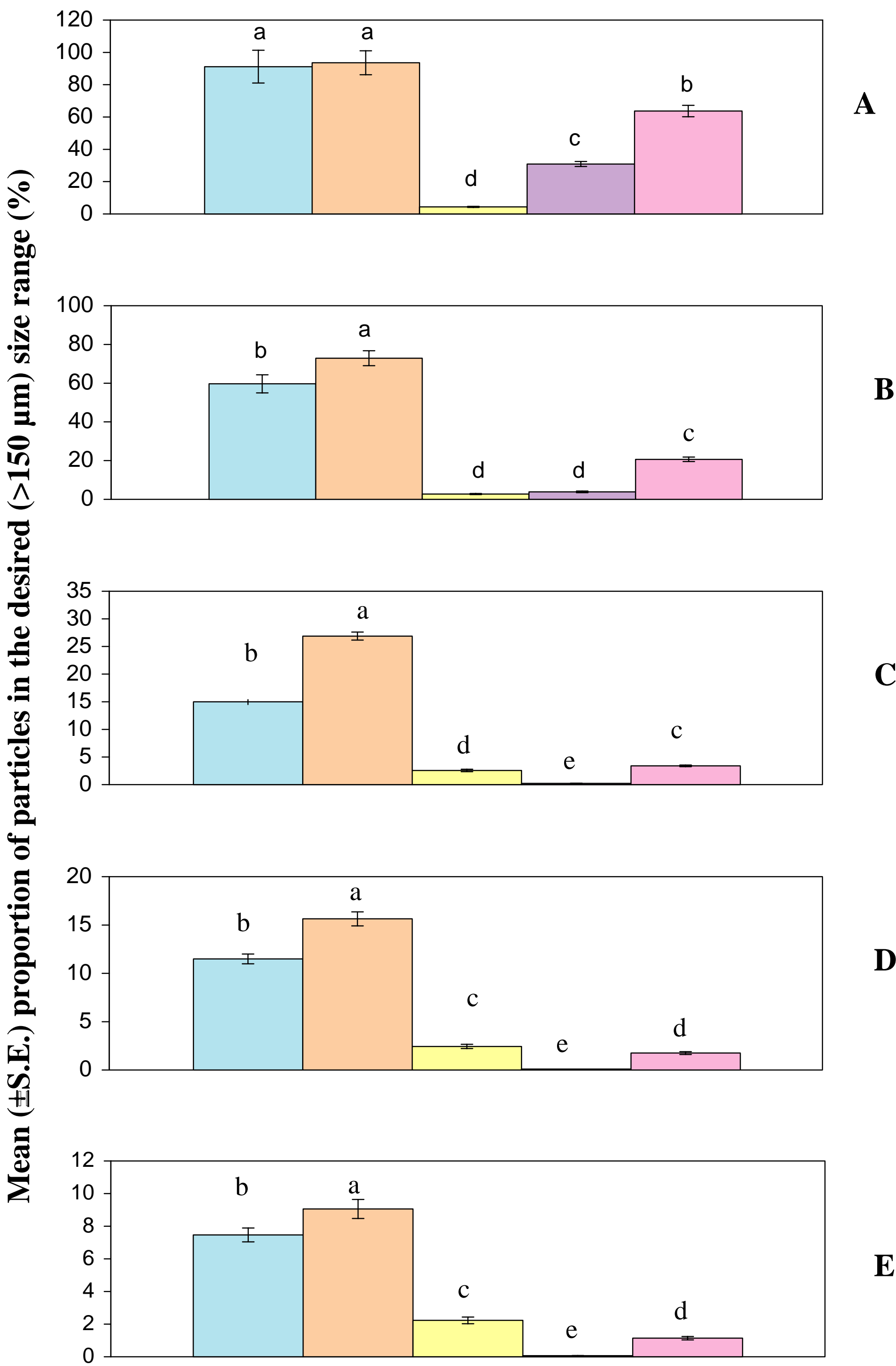


Figure 4. Mean (± S.E. n = 5) proportion of five particle types (from left to right): oven-dried marumarised (OM); moist marumarised (MM); freeze-dried crumbled (FC); oven-dried crumbled (OC); freeze-dried marumarised (FM) in the desired size range of >150 µm over five time intervals after immersion in water: 1 min (A); 5 min (B); 15 min (C); 30 min (D) and 60 min (E). Means with the same superscript are not significantly different from one another ($P > 0.05$).

Experiment 4 – Particle settlement rate

The mass of particles at each sampling position over time is shown in Fig. 5. For all diet particle types, with the exception of MM, the mass of particles in the top and middle sampling points decreases between 30 and 60 seconds after immersion in water. This is accompanied by an increase in the mass of particles in the bottom sampling point, except in FM particles where the mass of particles in the bottom sampling point decreases. The mass of particles present at the top and middle points continued to decrease for the crumbled particles. That of the bottom point increases in FC particles only. The mass of MM diet particles is very low after 30 seconds of immersion and then starts to increase. This increase can be seen for the FM and OM particles after 90 seconds of immersion in water. At this same point, the mass of particles in all three sampling points was even for the FC diet particles.

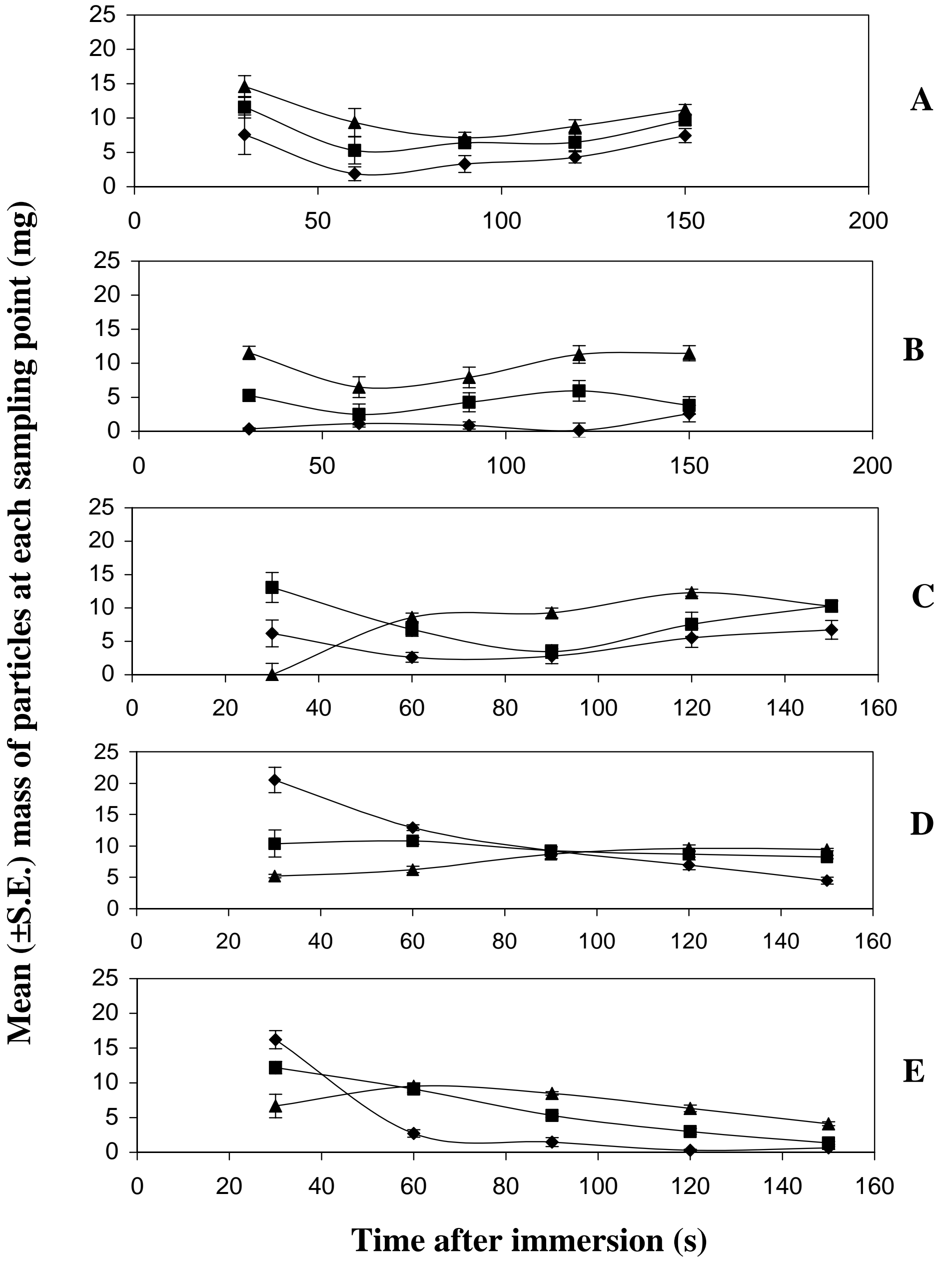


Figure 5. Mean (± S.E. n = 5) mass of five particle types: freeze-dried marumarised (A); oven-dried marumarised (B); moist marumarised (C); freeze-dried crumbled (D); oven-dried crumbled (E) at three points in the water column: top (◆); middle (■) and bottom (▲) at five sampling times over 2.5 minutes. Bars

with the same superscript, within each time interval, are not significantly different (P > 0.05).

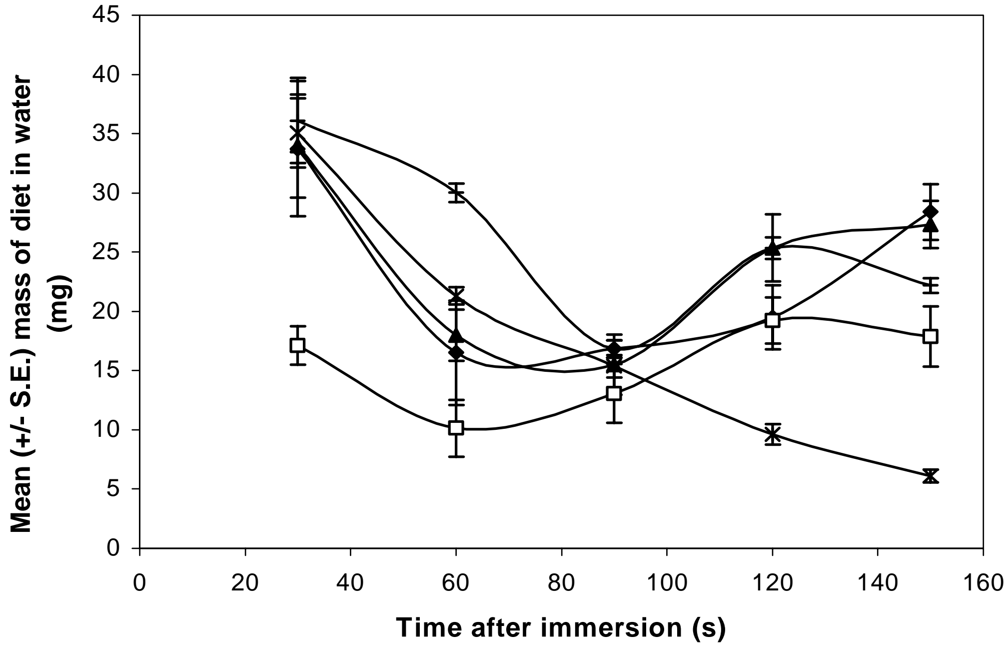


Figure 6. Mean (± S.E. n = 5) total mass of five particle types: freeze-dried marumarised (◆); oven-dried marumarised (▲); moist marumarised (□); freeze-dried crumbled (+); oven-dried crumbled (x) in the water column over time.

The mass of each diet particle type remaining in the water column over time is shown in Fig. 6. All diets, with the exception of the MM particles, showed their greatest mass for any time interval at 30 seconds after immersion in the water with the highest mass of particles being FC, followed by oven-dried crumbled, then OM → FM with a low mass of MM particles present in the water column. The mass of oven-dried crumbled diet particles in the water decreased steadily until the final sampling point. Freeze-dried crumbled and marumarised and oven-dried crumbled diets showed a decreasing presence from 30 sends to 90 seconds post-immersion, after which they increased slightly in an apparent 'second wave' of particles. This second wave of particles is apparent a little earlier for the MM diet as shown by the fact that the mass of moist marumarised particles in the water column decreased between 30 and 60 seconds, then increased again up to 120 seconds after immersion before decreasing. At the final sampling time, the greatest mass of particles were those of the freeze-dried marumarised diet, followed by oven-dried marumarised → freeze-dried crumbled → moist marumarised and finally with the oven-dried crumbled diet having the lowest mass of particles in the water column. There was a significant effect of both length of immersion ($F(4,100) = 23.446$, $P < 0.05$) and diet type ($F(4,100) = 20.816$, $P < 0.05$) on the mass of particles in the water column. There was also a significant effect of drying technique ($F(2,120) = 11.173$, $P < 0.05$) but no significant effect of preparation technique ($F(1,120) = 0.157$, $P > 0.05$). A significant interaction between the two ($F(1,120) = 11.599$, $P < 0.05$) was also shown.

Experiment 5 – Ingestion and Assimilation of diet particles

The levels of ingestion and assimilation of freeze and oven-dried crumbled diet particles by barramundi larvae is shown in Fig. 7. Oven-dried particles were both ingested and assimilated to a slightly higher degree than freeze-dried particles. Conversely, the efficiency of assimilation of the freeze-dried particles was higher than that of oven-dried particles. Statistical analyses, however, showed neither diet was significantly different in terms of ingestion and assimilation ($P > 0.05$).

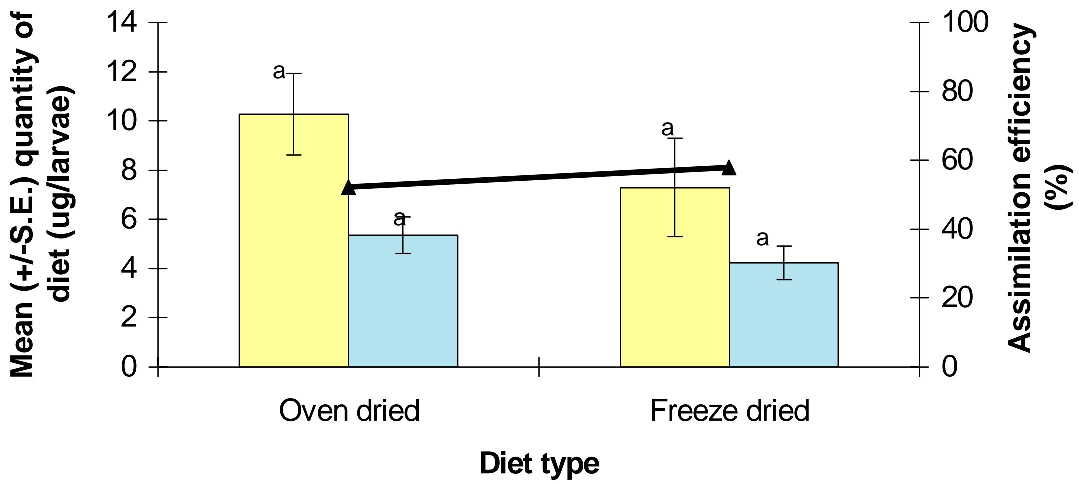


Figure 7. Mean (± S.E. n = 5) ingestion (dots) and assimilation (check) of the two drying techniques: Oven-drying and Freeze-Drying, by 18 day old barramundi larvae. Means with the same superscript are not significantly different (P > 0.05).

Larvae in treatments fed the freeze-dried crumbled diet spent a greater amount of time swimming than those fed the other diets (Fig. 8). This was followed by freeze-dried marumarised, oven-dried crumbled, oven-dried marumarised and finally moist marumarised diet particles. Larvae in treatments receiving food showed a noticeably higher swimming activity than those in the control. There was a significant effect of drying technique on time spent swimming ($F(2,20) = 5.422$, $P < 0.05$) but no significant effect of preparation technique ($F(1,20) = 3.706$, $P > 0.05$) and no interaction between the two ($F(1,20) = 0.035$, $P > 0.05$).

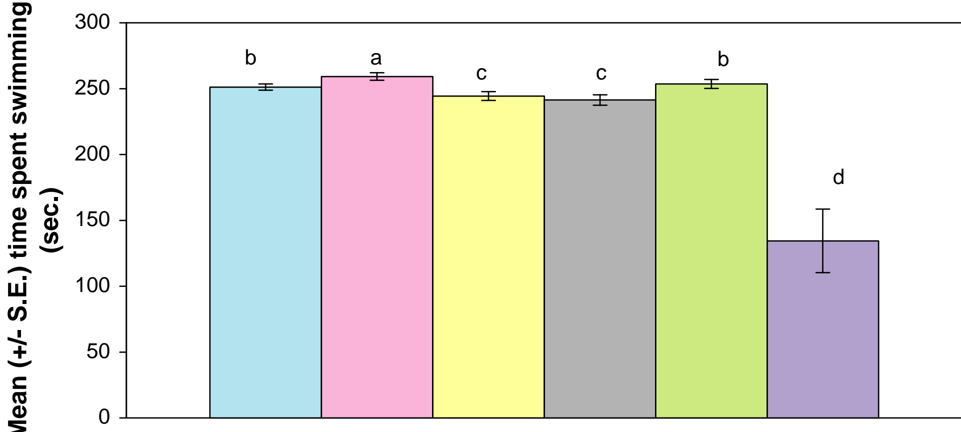


Figure 8. Mean (± S.E. n = 5) time spent swimming by 18 day old barramundi larvae fed one of the five particle types (from left to right): oven-dried crumbled (OC); freeze-dried crumbled (FC); oven-dried marumarised (OM); moist marumarised (MM); freeze-dried marumarised (FM) and a control. Means with the same superscript are not significantly different (P > 0.05).

In terms of the number of strikes the larvae made when fed each diet type (Fig. 9), there does not appear to be much of a difference between diets. Oven-dried crumbled particles elicited a marginally higher number of strikes than other diets and moist marumarised, the lowest number; however, there was no significant effect of either drying ($F(2,20) = 1.152$, $P > 0.05$) or preparation ($F(1,20) = 0.308$, $P > 0.05$) technique on the number of strikes and no significant interaction between the two ($F(1.20) = 0.244$, $P > 0.05$). All diets elicited a significantly higher number of strikes than observed for larvae in the control treatment.

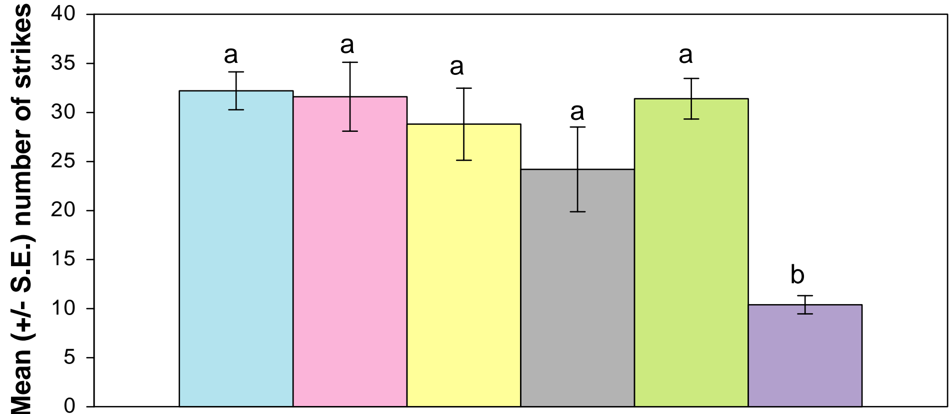


Figure 9. Mean (± S.E. n = 5) number of 'strikes' by 18 day old barramundi larvae fed one of the five particle types (from left to right): oven-dried crumbled (OC); freeze-dried crumbled (FC); oven-dried marumarised (OM); moist marumarised (MM); freeze-dried marumarised (FM) and a control. Means with the same superscript are not significantly different (P > 0.05).

Fig. 10 shows the number of pauses made by larvae within three minutes of being fed the various diet types. Only a very negligible difference between the five types was evident. Larvae receiving the freeze-dried crumbled diets paused slightly more often than those fed the other diets but there was no significant effect of either drying ($F(2,20) = 0.694$, $P > 0.05$) or preparation ($F(1,20) = 0.231$, $P > 0.05$) technique on the number of pauses and no significant interaction between the two ($F(1,20) = 0.004$, $P > 0.05$). Larvae in the control treatments paused a lot less frequently than those in treatments receiving food, although this is also a result of the fact that they were observed to spend more time motionless than larvae in the fed treatments so each pause was a lot longer.

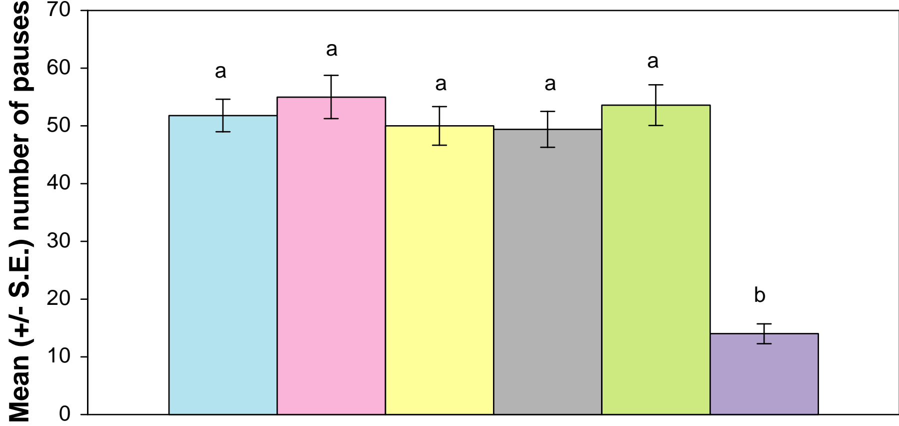


Figure 10. Mean (± S.E. n = 5) number of 'pauses' by 18 day old barramundi larvae fed one of the five particle types (from left to right): oven-dried crumbled (OC); freeze-dried crumbled (FC); oven-dried marumarised (OM); moist marumarised (MM); freeze-dried marumarised (FM) and a control. Means with the same superscript are not significantly different (P > 0.05).

1.3.2.4 Discussion

Physical characteristics of food particles

The size, shape and surface texture of the MBD particles produced in this study varied considerably according to the preparation and drying technique. While all particles were approximately the same width, the marumarised particles were two to three times the length of the crumbled diet particles. Presumably, during the sieving process, marumarised particles were able to orient themselves to fit through the sieve lengthwise. The effect of this difference in length between crumbled and marumarised particles on their ingestion may be significant. However, to a point, width, not length, is the critical dimension for diet particle selection (Blaxter *in* Hunter, 1981).

A factor that is likely to have a significant effect on some of the physical properties investigated in this study is surface area to volume ratio. One of the greatest weaknesses of MBDs is their rapid loss of water-soluble low molecular weight nutrients (Lopez-Alvarado et al. 1994). This loss of nutrients has been correlated with their high surface area to volume ratio (Barrows, 2000; Langdon, 2000). Clearly, the greater the surface area of a diet particle, the more contact the dietary ingredients have with the surrounding water and the greater the opportunity for nutrients to leach. Surface area to volume ratio increases with the decreasing size of the diet particle and it is also increased by the presence of ridges, valleys and pits in the surface of a particle. The 'ideal' inert food particle, therefore, would be as smooth as possible.

None of the diets particle types assessed in these experiments were smooth, however their surface textures did differ noticeably. The deep pits, numerous ridges and relatively large gaps of the freeze-dried particles (both marumarised and crumbled) increase the surface area to volume ratio compared with oven-dried particles and the moist marumarised particle. The results of this study indicate that surface texture is a function drying technique and, on this basis, it could be assumed that either oven-drying or not drying at all (for marumarised particles) is the best method of preparing MBDs to reduce leaching.

Particle length differed greatly amongst treatments and also affects the surface area to volume ratio. The longer a particle, the lower the surface area to volume ratio. A greater number of smaller particles are required to deliver a given volume of nutrients but would have a greater surface area available for nutrient loss. From this perspective, marumarisation, which produced consistently longer particles than crumbling, may be best technique for preparing diet particles in order to reduce leaching from MBDs.

Another physical feature which differed between the particle types tested is the presence or absence of visible strands of cellulose. Inclusion of cellulose in MBDs assists in binding the dietary ingredients; however, cellulose strands visibly protruded from some of the diet particles, effectively increasing the size of the particle. This may hinder particle ingestion. The crumbled diet particles produced without cellulose were of a comparable size to those with cellulose (not including the protruding strands) for both freeze and oven-dried particles. This suggests that cellulose has negligible, if any, effect on binding success in MBDs in the studied size range. It may be advantageous, then, to eliminate cellulose inclusion in MBDs for larval fish. Further testing, however, is required to determine whether cellulose improves structural integrity or other aspects relating to diet performance.

Particle density is an important factor in determining many of the physical properties (particularly sinking rate and particle stability) of inert food particles (Rout and Bandyopadyay, 1999; Barrows, 2000). The density of particles examined in this study was not determined; however, the results indicate that freeze-drying reduces particle density. Freeze-dried treatments of both marumarised

and crumbled diet particles had noticeably more gaps and cracks than their oven-dried counterparts. Furthermore, after drying and prior to crumbling, the freeze-dried diet made up 4-5 times the volume of an equivalent amount of oven-dried diet. During the freeze-drying process, moisture in the particles is first frozen before sublimating in the 'drying' phase. As the moisture freezes it expands and subsequent sublimation results in air pockets, which replace the ice and presumably reduce diet density. Preparation technique may also play a part in particle density determination. This was particularly noticeable in the differences between the oven-dried marumarised and oven-dried crumbled particle where the former had a noticeably smoother surface texture and were devoid of the protrusions and gaps evident in oven-dried particles. Settlement rate has been correlated in many studies with the density of food particles where less dense particles generally sink at the slower rate (Rout and Bandyopadhyay, 1999; Barrows and Lellis, 2000).

The prevalence of cracks and clear differentiation of diet ingredients was more evident in the freeze-dried MBD particles than in oven-dried or moist particles prepared by the same method. This may also have some bearing on particle integrity in the water column as these potential weak points may cause breakage of the particle when immersed and agitated. This hypothesis is supported by the findings of Barrows (2000) who observed that surface densification caused by marumarisation seemed to increase particle stability.

Soluble Nutrient Leaching

One of the greatest weaknesses of current inert food particles is their high rate of nutrient loss (Onal and Langdon, 2000; Langdon, 2003). A number of studies have shown that as much as 80% of the free amino acids in MBDs leach out within the first few minutes of immersion in the water (Lopez-Alvarado et al. 1994; Baskerville-Bridges and Kling, 2000). In this study, the majority of potential amino acid loss from particles of each diet type occurred 1-5 minutes after immersion when between 38% and 71% of free amino acids had leached from the diets. An interaction between preparation and drying techniques was apparent because freeze-dried marumarised particles leached the least amount of amino acids while the freeze-dried crumbled particles leached to the highest degree. Crumbled diet particles leached noticeably more than the marumarised particles, regardless of drying technique, indicating that preparation technique has the greatest effect in determining the level of amino acid loss from microbound particles. Of the marumarised diet particles, those prepared by freeze-drying showed relatively low rate of amino acid loss.

A low level of amino acid leaching is beneficial as an attractant for chemoreception of food particles by larvae (Yufera et al. 2002). The freeze-dried marumarised particles leached 25% of their amino acid content within one minute of immersion but did not leach more than 48% after one hour. Compared to other particle types, freeze-dried marumarised particles leached more than enough amino acids to be attractive to larvae while retaining considerably more amino acids at the end of the experiment. A similar conclusion can be drawn on reflection of the levels of water-soluble nutrient leaching. The results from this experiment also show that particles of the freeze-dried marumarised diet, along with the oven-dried marumarised diet, leached the least amount of nutrients. This indicates that in terms of reducing soluble nutrient leaching, freeze-drying or oven-drying marumarised particles is the best method of manufacture.

Structural integrity of diet particles

The capacity of an inert food particle to retain its original size and shape in the water column for as long as possible is integral to delivering the nutrients necessary for growth and development of larval fish whilst minimising the energy required for feeding. Furthermore, high levels of particulate matter in tanks (as would be seen when diet particles disintegrate), has been shown to cause gill damage in fish (e.g. Barrows and Lellis, 2000). Unfortunately, a lack of structural integrity is one

of the biggest weaknesses of MBD (Planas and Cunha, 1999). A number of studies have been undertaken to investigate the use of different binders and production techniques in an effort to reduce disintegration of particles in the water column. Person Le Ruyet et al. (1993), for example, compared zein and alginate binding agents and found that both gave relatively poor results. Durazo-Beltran and Viana (2001) found that the protein source used in the different diets tested had more of an effect on particle stability than the type or concentration of the binder. These findings contrast with those of Langdon (2003) and Guthrie et al. (2000) who reported that binder type affected particle stability. The MBD used in this study incorporated a binder inclusion of 3% (gelatin). This was determined by Partridge and Southgate (1999) to be the optimal type and inclusion level of binder in weaning MBDs for barramundi larvae.

Of the diet types tested, moist marumarised particles maintained the greatest degree of structural integrity and showed consistently higher proportions of particles in the desirable size range throughout the sampling period. Oven-dried marumarised diet particles showed the next highest degree of structural integrity throughout, followed by freeze-dried marumarised particles until 30 minutes post-immersion, where the proportion of freeze-dried marumarised particles fell below that of freeze-dried crumbled particles. Results indicate that the method of preparation, rather than the method of drying, had the greatest effect on structural integrity in MBDs. This may be due to the fact that marumarisation causes 'surface densification' which has been linked to particle stability (Barrows, 2000). Moist particles likely performed best as a result of not having any of the cracks evident in the dried particles as a result of the drying process (see above). The fact that oven-dried marumarised particles maintained a high degree of structural integrity but oven-dried crumbled particles disintegrated more rapidly supports the hypothesis that preparation technique has a greater influence on particle integrity than drying technique.

Particle settlement rate

The rate at which diet particles settle from suspension is integral to maximising ingestion of that diet; the greater the availability of the particle, the more chance larvae have of locating and ingesting it (Baskerville-Bridges and Kling, 2000). While the 'ideal' inert diet particle would be neutrally buoyant, a shorter-term goal is to produce a diet particle which sinks at the slowest possible rate. Particle density can be relatively easily manipulated with slowing of MBD particle sinking rate by reducing density (Barrows and Lellis, 2000). Marumarisation causes particles to be considerably more dense than crumble type particles (Barrows and Lellis, 2000), particularly those prepared by freeze-drying (see above).

This study showed a significant effect of both diet type and time on the mass of diet particles in the water column, as well as a significant interaction between the two. This indicates that the amount of food available to larvae is dependent on a combination of the type of diet and immersion time. Furthermore, while there was a significant effect of drying technique and a significant interaction between drying and preparation technique, there was no significant effect of preparation technique itself. This suggests that settlement rate depends more on the drying technique than the method of diet preparation. The results indicate that use of either oven-dried or freeze-dried marumarised particles would maximise the amount of diet particles available to fish larvae.

Ingestion and assimilation of diet particles

Neither freeze-drying nor oven-drying of crumbled MBD produced a diet particle that was ingested or assimilated significantly more than the other. Oven-dried particles were ingested and assimilated at a marginally higher rate than freeze-dried particles but showed lower assimilation efficiency. This may reflect (as shown above) that freeze-drying increases the surface area of diet particles by creating cracks and pits in the surface which weaken the structural integrity of the particle and result

in greater disintegration. This resulting high surface area to volume ratio of freeze-dried particles, however, may have aided in digestion and subsequent assimilation of the diet in providing a greater area for digestive enzyme activity.

Barlow et al. (1993) observed that barramundi less than 17-18 mm total length (the size range used in this study) swam actively through the water column when searching for food, as observed in this study. This may be due to the fact that, as suggested by Doving and Knutsen (1993), marine fish larvae respond to the concentration of metabolites emanating from prey organisms in a manner that is optimal for searching for food organisms. However, the behavioural responses of larvae to the various diet particles tested in this study were not very conclusive. An earlier part of this study showed that crumbled particles leached more than marumarised particles and, of the crumbled diets, those prepared by freeze-dying leached more than oven-dried particles. Oven-dried and moist marumarised diets promoted less strikes than the other diet types, indicating that larval behavioural responses may reflect the degree of leaching loss from the particle types. However, these differences were not significant. Similarly, the numbers of pauses made by larvae in all five treatments were not significantly different.

The data generated by these experiments clearly shows that methods of preparation and processing have a major influence on the behavioural and physical characteristics of inert food particles. While these data are useful towards development a suitable particle type for presentation of formulated diets to marine fish larvae, they indicate that the major limitations of current techniques are nutrient leaching and particle buoyancy. Further research is required to address these issues.

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Development of marine fish larval diets to replace *Artemia*

PART 3

Sagiv Kolkovski

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1.0 *ARTEMIA*

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Introduction

The brine shrimp *Artemia* is one of the most commonly used and suitable live feeds in the early life stages of fish and crustacean aquaculture due to its nutritional value and size (Lavens and Sorgeloos, 2000; Storrup and McEvoy, 2003). During the last twenty-five years the Great Salt Lake in Utah, USA has been the main supplier of *Artemia* cysts to the world aquaculture market (Lavens and Sorgeloos, 2000). Unreliable harvest yields of *Artemia* cysts from this source over recent years have placed considerable pressure on the aquaculture industry and could limit future prospects for expansion and development (Sorgeloos et al. 2001; Storrup and McEvoy, 2003). These supply problems and associated high prices for cysts have led to further exploration of new locations for cyst production. Despite this, producers struggle to meet the requirements of the rapidly growing global aquaculture industry (Lavens and Sorgeloos, 2000; Sorgeloos et al. 2001). In 1997, some 6,000 hatcheries required over 1,500 metric tonnes of dry cysts annually (Abatzopoulos et al. 2002), and with the global expansion of aquaculture in the last decade these numbers are likely to have doubled. Some 80 – 85% of the cyst sales went to shrimp hatcheries, the remainder to marine finfish larviculture (Abatzopoulos et al. 2002). Over 95% of more than 3000 metric tonnes of adult *Artemia* required for the aquaculture industry and aquarium trade are harvested from a restricted number of natural sources (Sorgeloos and Lavens, 1996). The forecast for global cyst and adult *Artemia* supply is discouraging, therefore *Artemia* culture rather than wild harvesting is an important alternative to improve supply (Sorgeloos et al. 2001, Teresita et al. 2003). Smaller quantities (1-20 tonnes per annum) of high quality cysts might in the future be provided on a more continuous basis worldwide from managed ponds and saltworks to supplement cyst supply. These cyst products can provide interesting opportunities for localised commercial development (Abatzopoulos et al. 2002).

Cognis Pty Ltd, operate a 520 hectare *Dunaliella salina* production facility for the extraction of beta-carotene. The ponds are located at Hutt Lagoon, which is a naturally occurring salt lake system (Fig.1). *D. salina* is grown at >200 ppt salinity in shallow (<500 mm) ponds that are artificially enriched with nutrients. *D. salina* is relatively pest and disease free when grown at high salinity. At sea salinity *D. salina* is green with normal chlorophyll, but high salinity triggers the production of beta-carotene and changes the algae's colour to red. However, a major problem encountered within the production process is *Artemia franciscana*, which occurs at Hutt Lagoon and can devastate *D. salina* numbers by using it as a food source. As with any intensive monoculture system, the mass production of a single food source can lead to 'mass outbreaks' or 'plagues' of a pest species when conditions are right.

Figure 1. Cognis production ponds.

Although considered to be a pest for *D. salina, Artemia* is considered to be a valuable resource to the world aquaculture production of prawns, marine finfish, and other species. *Artemia* is used in three forms for aquaculture, as adult brine shrimp (biomass), either live or frozen, and as cysts, which are dried and canned and may be stored for years. Live *Artemia* sells wholesale for about $90.00 AU / kg, frozen biomass sells wholesale for $ 20.00 - $40.00 AU / kg on the Australian market, while dried *Artemia* cysts currently fetch $ 190.00 AU / kg and have fetched as much as $ 570.00 AU /kg.

Therefore, the removal of *Artemia* from *D. salina* production ponds can have a two-fold benefit, by increasing production of *D. salina* and concurrently producing a new line of valuable products. This led the R&D activities at Hutt Lagoon to concentrate on two opposite directions. One is the development of systems and methods for culturing *Artemia* biomass and cysts and the other is eliminating them from the lagoon.

Identification of local *Artemia* cyst sources

Artemia *cysts evaluation*

Artemia cysts samples were collected from two different sources in Western Australia:

Sample 1: Hutt Lagoon, Port Gregory

Sample 2: Cargill Salt, Port Headland

The cysts samples were sent to the *Artemia* Reference Centre, University of Ghent, Belgium for analysis. The cysts were then tested for size, hatching and fatty acid profile (Fame) as well as DNA fingerprints.

Table 1. Cysts physical and biological properties.

	Cyst diameter (µm)	Cyst count (cyst/g)	Hatching percentage (at 24 h)	Hatching percentage (at 48 h)
Sample 1	262.19 ± 9.26	224,333 ± 1,453	58.24 ± 2.39	87.48 ± 1.39
Sample 2	240.48 ± 9.87	251,649 ± 12,018	87.13 ± 0.95	

Table 2. Fatty acid results from *Artemia* cysts.

	20:5w3 EPA (%)	20:5w3 EPA (mg/g DW)	22:6w3 DHA (%)	22:6w3 DHA (mg/g DW)	Sum w3 (%)	Sum w3 (mg/g DW)
Sample 1	14.6	23.6	0.1	0.2	15.2	24.7
Sample 2	8.6	13.5	0.1	0.1	9.3	14.5

Comments from The* Artemia *Reference center, Belgium:

Sample 1: Although the cysts diameter suggests that the sample is not *Artemia franciscana*, DNA fingerprints confirmed that the cysts sample is indeed a *franciscana sp*.

Sample 2: The cysts sample is closely related to the Shark Bay sample (from *Artemia* Reference Center database), and is distinct from the Port Gregory and Dampier samples. It may be a mixture of parthenogenetic and *A. franciscana* population.

The results showed that the Port Gregory cysts have a very good nutritional profile while the hatching is asynchronous over 48 h. Cargill salt cysts hatching profile was significantly better (87.1% in 24 h), however, the nutritional profile was inferior compared to the Port Gregory cysts. The asynchronous hatching of the Port Gregory cysts can be explained by the low quality sample. The sample was collected from the banks of the ponds where it was exposed to the elements for an unknown period. The overall quality of the cysts is greatly affected by harvesting and processing conditions (Bosteels et al. 1995; Lavens and Sorgeloos, 1996; Bossier et al. 2003; Kolkovski et al. pers. comm. current project). Subsequent samples taken during the 2004 bloom showed that the cysts have very good hatching patterns (88 ± 4%, at 24h), superior to reported hatch rates from commercially available AAA grade Inve® cysts in Australia.

Cargill Salt Pty Ltd (now part of Rio Tinto group) were reluctant to develop any project involving *Artemia* cyst and biomass production, fearing it may interfere with their saltworks. Cognis Pty Ltd operating at Hutt Lagoon in Port Gregory has a natural population of *Artemia* and was interested in expanding their production to include *Artemia*. The company cultures *D. salina* for beta-carotene extraction in extensive lakes within the lagoon. *D. salina* is a very suitable diet for growing *Artemia.* Due to the availability of large quantities of the algae, there is significant potential to farm *Artemia* for cyst and biomass production at this site. Therefore, this location was chosen for a preliminary *Artemia* cyst and biomass pond production experiment and is the proposed location for future related experiments and development of commercial scale production in FRDC (2004/238).

1.1 Pilot-scale pond production

During June 2003 the DoFWA conducted an *Artemia* cyst and biomass pond production trial in collaboration with Cognis Pty Ltd at their beta-carotene production site. The objective of the trial was to assess the potential and identify the problems with mass production of *Artemia* cysts and biomass. Based on the results from this trial, further systems and experiments will be developed in a follow up project.

Six specialised ponds were built by Cognis. A new filtration system was designed and developed by DoFWA to allow continuous filtration of the pond water in order to remove cysts and *Artemia* nauplii. The ponds were inoculated with *Artemia* nauplii at two stocking densities. Brine containing *D. salina* (*D. salina* brine) from one of the extensive production lakes was pumped for 8 h per day. After commissioning the system and initiating the trial, DoFWA staff remained on site for two weeks in order to conduct system troubleshooting and train Cognis staff.

The ponds and filters were operating continuously. The company staff checked the ponds and filters Mon – Fri to prevent overflows and measurements were taken while DoFWA staff were on site. On the third week the filtration system needed to be modified and DoFWA staff returned to make the modifications required. The filters operated for a further 9 weeks until the trial was finalised by the DoFWA staff at day 93.

1.1.1 Methods

System

Ponds

The experimental system consisted of six 18 m³ PVC lined ponds, 250 mm deep, each with a 50 mm inlet, plumbed to a 200 mm manifold, with the option of supplying *D. salina* brine or seawater (Fig 2).

Figure 2. Experimental pond after *D. salina* addition.

Inlet protocol

Day 1: 3500 l of seawater was added to each pond and 500 l of *D. salina* brine.

Days 2– 11: 1000 l of *D. salina* brine added each day to each pond.

Days 12 – 98: ponds operated as semi-flow though, 25 l min^{-1} of *D. salina* brine added for 8 h day^{-1} (8:00am – 4:00pm).

Filtration to remove and separate nauplii and cysts

First stage: Filtration within the pond was used to remove cysts and nauplii (Fig. 3). The filter consists of two 1.5 m sections of submerged 150 mm PVC pipe with 500 µm screen. The pipe had eight 200 x 150 mm sections cut out and covered with 500 µm mesh. The two sections are joined with a tee piece containing a submersible light (Fig. 4) to attract the nauplii. The outlet is connected to the end of the first stage filter and gravity fed to the second stage filter.

Figure 3. First stage filter in pond to remove cysts and nauplii.

Figure 4. Light to attract nauplii.

Second stage: External to the pond, another filter was used to retain nauplii and allow cysts to pass through (Fig. 5). The filter consists of a 240 l barrel containing a 250 µm screen filter. The filter was made from a 1 m section of 150 mm PVC pipe, with six 200 x 150 mm sections cut out and covered with 250 µm mesh. The inlet from the first stage filter is directed into the bottom of the barrel. Water exiting the barrel passes though the screen and out the 50 mm outlet at the top of the barrel connected to the third stage filter.

Figure 5. Second stage filter to remove nauplii.

Third stage filter: External to the pond, this filter was used to retain cysts (Fig. 6). The overflow from the second stage filter is directed into a 100 µm screen bucket contained within a second 240 l barrel. This screen bucket was designed to collect the cysts, however due to problems with blockage, the filtration system was modified during the trial. The screen bucket was removed, the 50 mm inlet remained at the top and a 50 mm outlet was placed at the base, using an outside overflow pipe to maintain water level. The high salinity of the brine ensures the cysts float and do not exit via the outlet at the base.

Figure 6. Third stage filter to retain cysts.

1.1.2 Hatching

Artemia cysts were incubated and hatched for 4 h, in a 3,000 l stainless steel tank with a conical base (Fig. 7). The tank had vigorous aeration from the base at the centre and the temperature was maintained at 28°C with the use of a submersible titanium heater. The methods used for hatching and estimating hatch rate followed those given in Lavens and Sorgeloos (1996).

Figure 7. 3,000 l tank used for hatching.

1.1.3 Stocking

Six ponds were stocked in May 2003 at 2 rates, 18 million *Artemia* nauplii pond^{-1} and 9 million *Artemia* nauplii pond^{-1} (3 replicates of each). These stocking rates were designed to achieve 1000 *Artemia* l^{-1} and 500 *Artemia* l^{-1} when the ponds were filled to 18 m^3 on day 12.

1.1.4 Feeding

The *Artemia* were fed *D. salina* brine, pumped in from the processing plant inlet channel. This ensured that optimal *D. salina* concentrations and quality were met. A total of 1,000 l of *D. salina* brine day^{-1} was pumped into each experimental pond, a flow rate of 25 l min^{-1} from days 1 – 11. For the remainder of the experiment *D. salina* brine was pumped into each pond for four h day^{-1} at 25 l min^{-1}.

1.1.5 Measurements

D. salina

Algae counts were performed on days 1 – 15, for pond water before and after addition of *D. salina* brine, to calculate the cells consumed by the *Artemia* over the time period between pumping.

Water quality

Dissolved oxygen (D.O.), pH, temperature and salinity were measured using a WTW Multiline P4 meter. Temperature data loggers (Thermacron®) were used to measure at 5 min intervals throughout the trial.

Adult* Artemia *length and individuals bearing cysts

Adult *Artemia* were collected from each pond periodically in triplicate, and length measurements and the proportion of individuals bearing cysts (IBC) were determined using a stereoscope at 100X magnification.

Artemia *biomass and survival*

At 98 days post hatch (dph) the biomass was harvested from each pond and screened. Production (kg pond^{-1}) and weight were determined using gravimetric analysis. The *Artemia* survival in the ponds was determined according to the methods in Lavens and Sorgeloos (1996).

Artemia *nauplii produced*

The ponds and filters were sampled periodically throughout the trial in order to determine nauplii production rates. During final harvest, the biomass from each pond was screened to separate nauplii and adult *Artemia*. Nauplii numbers were estimated according to the methods in Lavens and Sorgeloos (1996).

1.1.6 Results

At 82 dph it was observed by the Cognis staff that mortalities were occurring within the ponds. The DoFWA staff were not able to finalise the trial until 93 dph. This subsequently compromised the quality of the results. The brine (220 ppt, salinity) preserved the mortalities quite well and estimates were made as to the numbers of *Artemia* in the ponds. There was a higher percentage of preserved mortalities than than live *Artemia* in both the high density ponds (HD ponds) and low density ponds (LD ponds) 75 ± 7% and 84 ± 14% respectively.

Feed consumption

The consumption of *D. salina* cells was monitored over a 23 h period for the first 12 days post hatch (dph) and was consistently higher (Fig. 8) in the HD ponds estimated to be stocked at 1,000 *Artemia* l^{-1} than in the LD ponds estimated to be stocked with 500 *Artemia* l^{-1}. These ponds were estimated to have 18 million *Artemia* and 9 million *Artemia* respectively. *D.salina*, in the HD ponds, was consumed at a lower average rate (*D. salina* cells *Artemia*$^{-1}$) than in the LD ponds, (Fig. 9). At 12 – 15 dph the flow regime was operated as flow-though (8 h day^{-1}) and the *D. salina* densities were higher in all ponds. The algal consumption was monitored over a 16 h period and increased relative to concentration across all ponds. The *Artemia* in the HD ponds continued to consume higher rates of *D. salina* per pond and lower rates per *Artemia* than the *Artemia* in LD ponds.

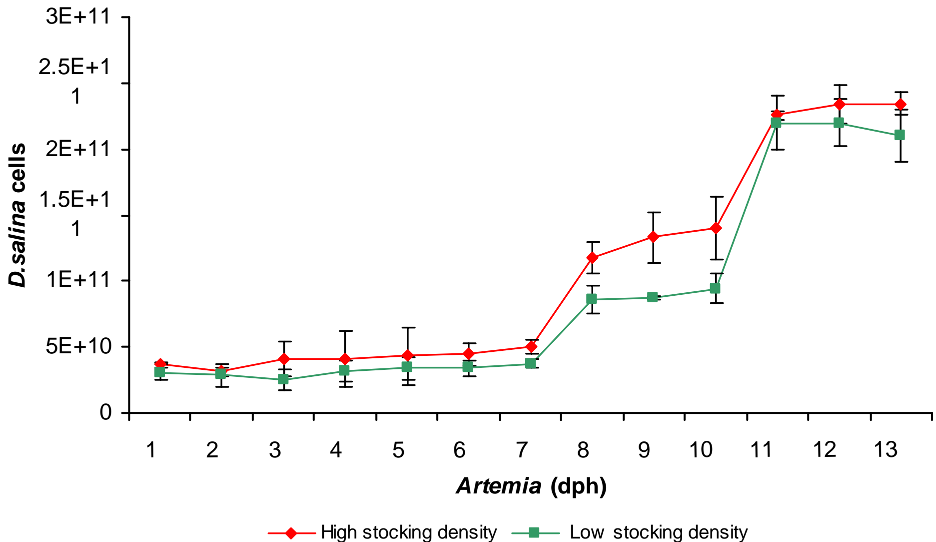

Figure 8. Total *D. salina* consumed in treatments (Average ± SE, n = 3), assuming no natural increase in *D. salina* by reproduction.

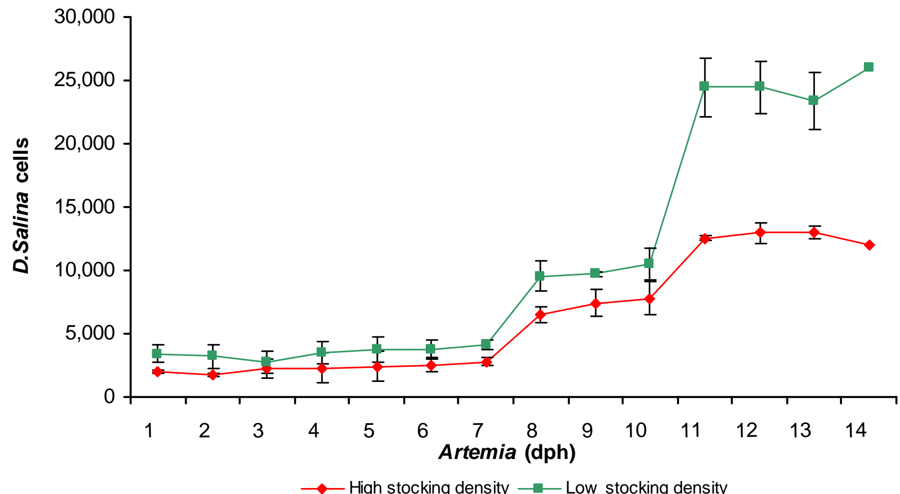

Figure 9. *D. salina* consumed per *Artemia* in treatments (Average ± SE, n = 3), assuming no natural increase in *D. salina* by reproduction.

Artemia *size and weight*

The average *Artemia* length was higher in the LD ponds for all days measured with the exception of the measurements taken 2 dph and 98 dph, (Fig. 10). The final measurements were 8.05 mm ± 0.39 mm and 7.94 ± 0.5 mm for HD and LD ponds respectively. The average total biomass was higher (not significant $P > 0.05$) in the ponds stocked at the high stocking density than the LD ponds, (3814.9 ± 651.9 kg and 2325.3 ± 1295.3 kg respectively).

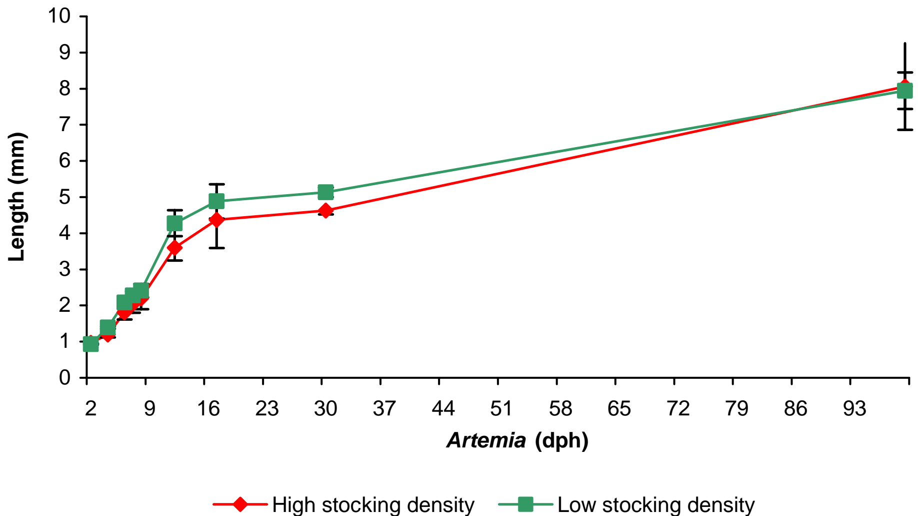

Figure 10. *Artemia* length in treatments (Average ± SE, n = 3).

Artemia survival

Survival percentage was not significantly different for both HD ponds and LD ponds (2.19 ± 0.006% and 2.13 ± 0.0002%, respectively). The average percentage of mortalities recovered in relation to original stocking estimates from treatments HD ponds and LD ponds respectively were 6.84 ± 0.011% and 5.41 ± 0.017% . At 82 dph high mortalities were occurring across all ponds and continued until the final harvest (98 dph).

Table 3. Total harvested nauplii, total harvested adult *Artemia* and proportion of nauplii to adult *Artemia*.

	Total harvested nauplii	Total harvested adult *Artemia*	Proportion nauplii to adult *Artemia*
High density ponds			
Pond 1	7,407	211,111	3.51%
Pond 3	8,000	380,000	2.11%
Pond 6	10,000	459,167	2.18%
Low density ponds			
Pond 2	0	8,000	0%
Pond 4	14,000	564,444	2.48%
Pond 5	0	2,041	0%

Cyst bearing individuals

The percentage of IBC *Artemia* was estimated from the live remaining *Artemia* at 98 dph. The percentage of IBC's was 43.07 ± 4.14% average and 47.93 ± 11.35% from HD and LD treatments respectively. This difference was not significant, ($P > 0.05$). Nauplii were found in all three HD ponds and one LD pond. The ponds in which the nauplii were found had between 211,111 and 564,444, adult *Artemia*. The two ponds where no nauplii were harvested also had the lowest total numbers of surviving adults harvested 8000 and 2041 in total (Table 3).

Cyst harvesting

Technical difficulties were experienced harvesting cysts. Cysts became trapped in foam produced on the pond surfaces and also adhered to the exposed parts of the pond walls, impeding their movement through the first stage filter.

Figure 11. Cyst bearing *Artemia franciscana* 30 days post hatch.

Water quality

Water quality parameters were monitored for the first 16 days of the trial. The D.O. levels found in the ponds ranged from 5.66 mg l^{-1} to 6.78 mg l^{-1} (Fig. 12). The pH ranged from 7.47 to 8.41 during the period monitored (Fig. 13). Salinity ranged from 40 ppt to 210 ppt, the salinity gradually increased throughout the trial (Fig. 14). The average temperature for ponds was 21.08 ± 0.1°C and 21.1 ± 0.1°C (Table 4).

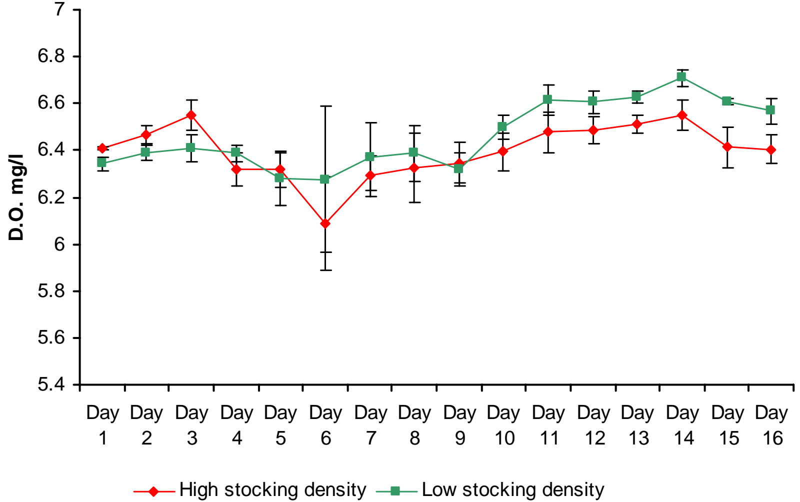

Figure 12. Dissolved oxygen levels (Average ± SE, n = 3) in the ponds, 1 – 16 dph.

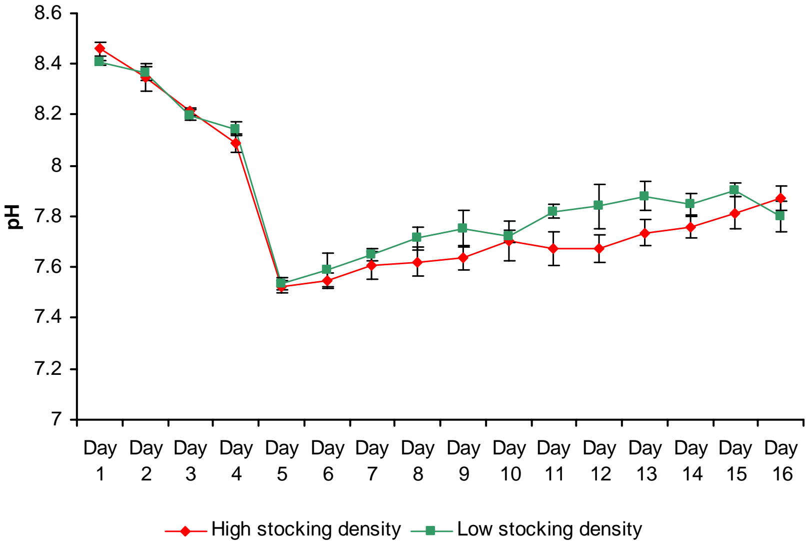

Figure 13. pH levels (Average ± SE, n = 3) in the ponds, 1 – 16 dph.

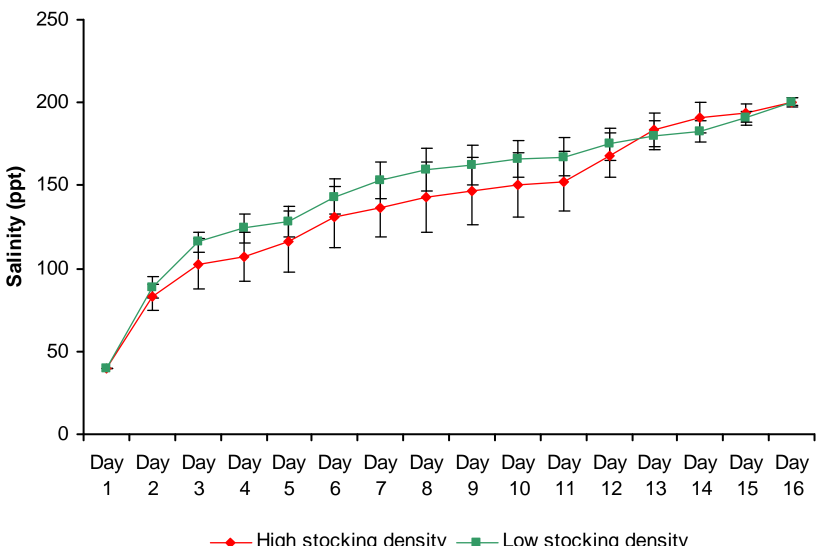

Figure 14. Average salinity levels (Average ± SE, n = 3) in the ponds, 1 – 16 dph.

Table 4. Average, minimum and maximum pond temperatures.

	High density ponds	Low density ponds
Average temperature °C	21.08 ± 0.1	21.1 ± 0.1
Average minimum °C	15 ± 1.3	13.8 ± 0.3
Average maximum °C	31.3 ± 3.7	34.5 ± 0.5

1.1.7 Discussion

As expected, the *D. salina* in HD ponds was depleted at a higher rate than in the LD ponds. This suggests that the *Artemia* in the HD ponds may have been food limited, reflected in the higher growth rates shown in the LD ponds. Food concentration is a major factor influencing growth and survival of *Artemia* cultures (Evjemo and Olsen, 1999, Camara et al. 2004).

The average final total biomass was higher in the HD ponds, although not significantly. There was a high degree of variation within treatments, largely due to the mortalities occurring (post 82 dph). High potential for cyst production has been demonstrated in the high percentage of IBC's, and low numbers of nauplii, and it was not affected by *Artemia* density. It is suspected that high salinity promoted oviparous reproduction, resulting in cyst production rather than oviviparous reproduction, which produces nauplii. Ponds were managed with salinity starting low and increasing throughout the trial in an effort to trigger cyst production, simulating natural conditions in which cysts are produced i.e. a lake drying up, the salinity increases and triggers cyst production.

The first and second stage filters operated effectively, however daily maintenance was required at a flow rate of 25 l min^{-1}. The high faecal loading produced by the *Artemia* quickly blocked the 100 µm mesh of the third stage filter. In order to improve efficiency, air was added to the first and second stage filters, but the third stage filter needed redesign. The screen was completely removed and a floatation tank design was employed instead. The aim was for the filters to be of very low maintenance; this was the result following modifications.

Large amounts of foam were produced on the surface of each pond which trapped cysts and *Artemia,* causing *Artemia* mortalities (Fig.15). This was identified as a problem that needs to be addressed for any future trials. A filter, with more efficient surface skimming capabilities, may alleviate this problem.

The cause of the mass mortalities is unknown. DoFWA staff were not present when the mortalities started occurring and therefore unable to monitor the conditions within the ponds. It is presumed that the *Artemia* reached the end of their life cycle.

Figure 15. Pond 1, foam produced on surface.

This initial trial has led to the development of a new project. A purpose built research / commercial *Artemia* cyst and biomass production system will be constructed on site at Hutt Lagoon. The system will consist of 18 x 20 m^3 fibre reinforced plastic (FRP) tanks, either within an enclosure or with individual covers, to reduce risk of wind blown cysts contaminating *D. salina* ponds, with cysts, and tanks with debris. The tanks will be operated as semi-flow though or full-flow though with the water and feed supply from the *D. salina* production lakes. The concept of the three-stage filtration system will be further developed, with the aim to continuously filter and collect nauplii and cysts while retaining a stable adult population within the tanks.

1.2 *Artemia* bloom at Cognis: Opportunity to develop cyst and biomass harvesting and processing techniques

1.2.1 Introduction

In June 1999, an outbreak of *Artemia* occurred at Hutt Lagoon in the *D. salina* grow-out ponds. This event recently recurred in July 2004, and as a result the production numbers of *D. salina* have fallen to levels that have led to the beta-carotene extraction process temporarily being shut down.

In both cases the massive hatch out of *Artemia* followed a period of heavy rainfall. This consequently washed the cysts that had been blown onto the shore in previous years, into the pond. During periods of low wind, a layer of relatively fresh water sits on top of the brine and serves to hydrate the cysts, which initiates the hatch. The *Artemia* quickly acclimate to the brine and are able to reproduce after 10 to 14 days. This phenomenon occurs most years, however not usually to this order of magnitude.

1.2.2 *Artemia* cyst collection

Artemia cysts were collected opportunistically from the brine when wind driven aggregations were formed. Predominantly cysts were collected, at points passing though culverts joining the lakes to an inlet channel leading to the site's production plant. The nature of the cysts floating in the brine allowed the use of a floating boom collector to hold the cysts in place whilst being collected. A thick layer of cysts results on the surface (Fig. 16) that be manually collected using scoops.

Figure 16. *Artemia* cysts collected in culverts.

This method ensures selective collection of high quality cysts. A considerable wind driven aggregation of cysts is needed to collect cysts efficiently in the culverts. 200 kg wet weight (ww) of cysts were collected in this manner by DoFWA staff. A further approx (ww) 200 kg cysts were collected by Cognis staff from the culverts, trapped in wind-agitated foam and from the shoreline, and these collected cysts were mixed together and stored in a cool room in moist conditions for one month. Storage of cysts is ideally done in brine until drying, therefore cysts dried on the shoreline are not preserved in the optimum conditions (Fig.17).

Figure 17. Shoreline cysts.

These cysts were not found to be as viable, in terms of synchronous hatching as those collected by the DoFWA staff when dried using the same method.

1.2.3 *Artemia* biomass collection

Pumping and Filtration

During the preliminary investigation, pond water was pumped from the ponds at sites that were identified as having high numbers of *Artemia*. A clear 5 l beaker was used to sample the water at each site. When there was a significant number of *Artemia* in the sample (>10 *Artemia*), the site was investigated further by pumping water using a diaphragm pump at 40 l min^{-1} through a 500 μm barrel screen. Pumping continued for 1.5 h from the surface water of a site observed to be of average density, under very low wind conditions. The barrel was then drained though a 250 μm screen resulting in only 120 g of biomass being collected, which was 30% dead *Artemia* and detritus. However, when pond water was pumped from 200-300 mm below the surface negligible biomass was collected. This indicates that there is somewhat lower numbers of adult *Artemia* in the lakes than first thought, as *Artemia* were seen to inhabit the upper 100 mm of the water column.

This is probably due to the *Artemia* moving to the surface water where it is warmer and more oxygenated, and/or where the *D. salina* congregates. Therefore, the estimated weight of *Artemia* biomass in the pond would be 33.3 g m^{-3}, which would be an estimated total of 8000 kg (calculated by using the upper 200 mm of lake volume). Considering that the majority of *Artemia* were found in the upper 200 mm of the water column, faster recovery of biomass from the ponds may be possible by draining the ponds into pumping channels over a drain board that blocks water from a depth of 200 mm to the bottom.

The biomass that was sampled from one of the ponds was taken from a relatively clean area. Even so, the biomass contained an estimated 30% detritus and dead *Artemia*. However, in order for the biomass to be of commercial value it needs to be clean (< 5% detritus and dead biomass). The separation of live biomass from dead biomass and detritus will be a challenge. If, as planned, the whole lake is pumped though a 250 µm contra-sheer filter, this will be an expensive exercise (due to pumping costs) and there will probably be considerable fouling of the fresh biomass removed. The contra-sheer screen is 6 m long, 1.9 m high and 2 m wide (Fig. 18). The sides are curved in towards the centre and covered with 250 µm mesh. The filtration method is passive, water is pumped into the centre and overflows out the top and over the curved sides. The water pushes particles >250 µm into channels at the base for collection. The screen can cope with a flow rate of 1000m^3 h^{-1}. DoFWA together with Cognis staff are currently considering the best course of action regarding the removal of fouling from biomass that has been pumped. However, more viable options are to be investigated.

Figure 18. Contra-sheer screen.

Manual harvesting

For small scale harvesting (up to 500 kg of biomass d^{-1}) a dip net may be used to scoop *Artemia* from the surface pond water, where an aggregation has been formed by wind action. During preliminary investigation a pool net with 500 µm mesh size was used to collect *Artemia* of high quality (Fig 19), which was relatively clean and with a high percentage (>90%) being alive. *Artemia* were concentrated on the net by scooping through the mass, then placed into 20 l buckets at the shoreline and subsequently carried to a 900 l bulk transport container on a car trailer and taken to the Cognis plant for processing (Fig. 20). This method achieved considerably higher quality *Artemia* biomass and removed more *Artemia* from the *D. salina* pond at a much-reduced cost. The same time input for manual collection and pumping yielded 216 kg and 120 g respectively. However, if a wind driven aggregation was pumped through an appropriate filter, it is assumed that a much greater yield would be possible. Therefore, appropriate wind conditions are needed to concentrate the *Artemia* to one side or preferably a corner of a pond in order to efficiently collect the biomass for sale.

Figure 19. Manual harvesting using a dipnet.

Figure 20. Manual harvesting transport container and collection.

1.2.3 Cyst and biomass collector

Pilot collector

Harvesting cysts and biomass from the shoreline during times of wind driven aggregations is limited to the duration of the wind blowing in any one direction (Fig. 22). A method was required to collect cysts and biomass and hold the collection until the cysts and biomass can be manually harvested. Therefore a cyst collector was designed and implemented by the DoFWA staff. From the northwestern corner of the lake, two arms directed south and east (along the banks of the ponds) were used to corral the wind driven cysts to the collector pen. The pen was designed as a sloping barrier with a bottomless box behind it. The front on the box sloped away from the incoming cysts and biomass, and protrudes about 25 – 50 mm above the surface of still water. Therefore, the wave action laps cysts over the lip of the holding pen and the excess water was returned to the lake through the open bottom of the pen. The pen sides were 250 mm below the surface of the water in order to hold the cysts and biomass at the surface and prevented them from being washed through the pen by the disturbance of the wave action.

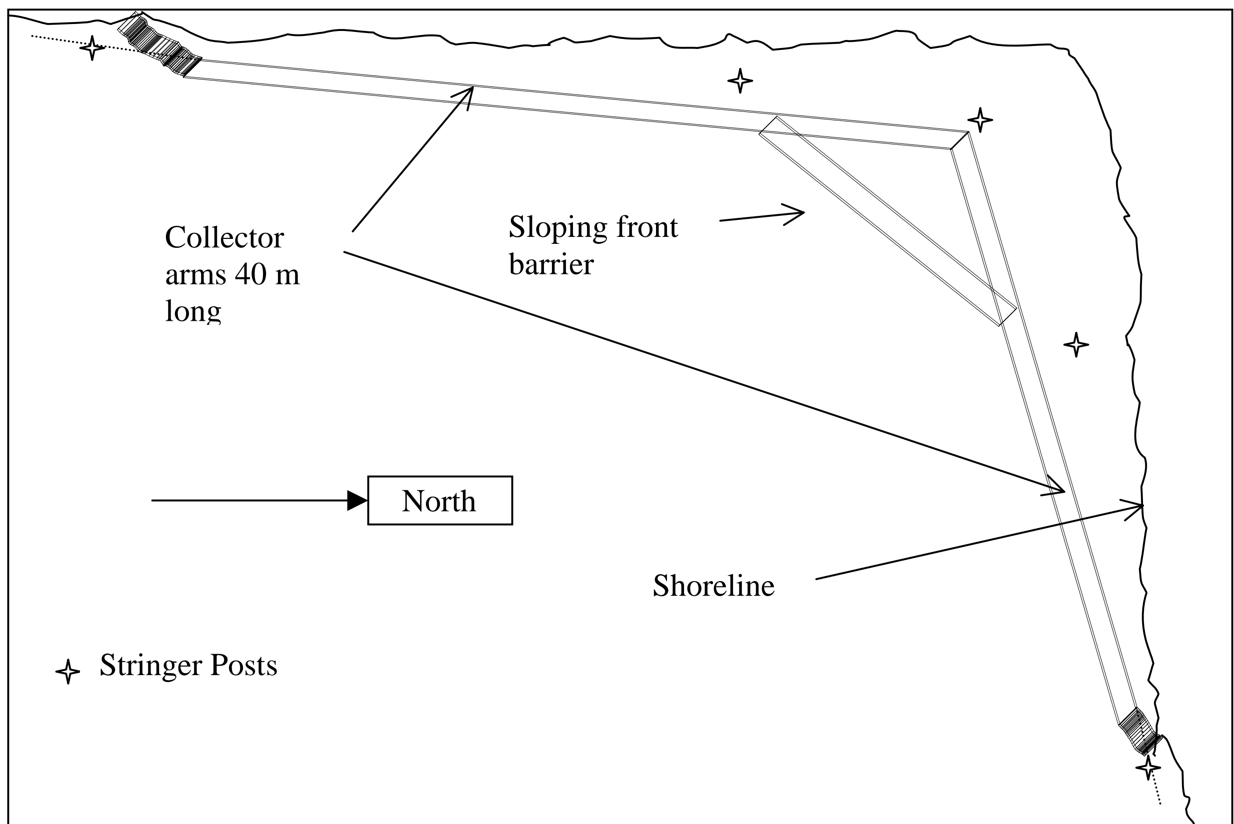

Figure 21. Schematic diagram of cyst collector.

There were five stringer posts used for the construction of the cyst collector. One post was at the apex of the collector and two others are used at the ends of the collector arms to draw them straight, approximately 40 m away from the centre post. The other two posts were used at either end of the sloping front section of the collector pen, approximately 15 m long. High tensile wire was strung between each post, having first been woven though the material at 1 m spans, 100 mm down from the top edge. The material used was polypropylene, 1 mm thick and 400 mm high. The arms were pinned in a vertical orientation using 1.2 m long reinforcing bar (12 mm diameter) on either side of the material. However, the ends are pulled away from the wire stringer and secured by pins and rocks to the shoreline. The front barrier was secured at a 60° angle from vertical, facing away from the on blown cysts, using the same technique (Fig. 21).

The installation of the collector proved to be successful collecting 8 metric tonnes of *Artemia* in the week it was operational (Fig. 23 and 24). The lake levels were subsequently dropped the following week to raise salinity levels and the collector became in operational, as the water level became too low. Modifications to this design are being considered, in order to cope with fluctuating water levels.

Figure 22. Shoreline conglomeration of cysts and biomass.

Figure 23. Biomass / cyst collector installed.

Figure 24. Biomass / cyst collector operating.

1.3 Cysts processing

The overall quality of the cysts is greatly affected by harvesting and processing conditions (Lavens and Sogeloos, 1996; Bossier et al. 2003). Proper processing and drying of *Artemia* cysts is of crucial importance to obtain a storable product with maximal hatching quality (Bosteels et al. 1995). Washing the cysts prior to drying is essential to remove salts that will crystallize and damage the dry cysts. As freshwater is used, the cysts will partially hydrate, if the cysts remain hydrated for too long the embryos will eventually reach an irreversible state of hatching metabolism that needs to be avoided (Bosteels et al. 1995, Lavens and Sogeloos, 1996). Therefore it is recommended that washing of the cysts is to be completed within thirty minutes and the drying started (Lavens and Sogeloos, 1996).

Drying temperatures < 35°C are recommended and can be up to 60°C when cysts are dehydrated (Bosteels et al. 1995, Lavens and Sogeloos, 1996). The brine storage dehydrates the cysts therefore these cysts may withstand higher drying temperatures. The upper temperature tolerance level of cysts also varies between both batches and strains (Lavens and Sogeloos, 1996). Best hatching viability is obtained by reducing the moisture content of the cysts to 10% within 8 h. Prolonged drying (i.e. > 24) h results in a decreased hatching percentage. It is suspected that very short drying times i.e. < 3 h may promote the quality of the cysts. Little is known about the actual relationship between water content and subsequent shelf life, however between 3% and 8% water content is generally targeted (Lavens and Sogeloos, 1996).

Drying using a fluidised bed dryer is regarded as the most efficient and versatile method followed by rotary drying (Bosteels et al. 1995, Lavens and Sorgeloos, 1996). The rotary dryers operate in a similar manner to a clothes dryer and the medium heat setting on domestic clothes dryers is approximately 35°C, which is the optimal drying temperature stated by Lavens and Sorgeloos, (1996) for the majority of cysts. Cysts can be contained in a pillowcase (tied at the end) and dried using a domestic clothes dryer. A Simpson 39S455 clothes dryer with three heat settings was purchased and each setting was trialed for drying the cysts that had previously been stored in a cool room. The three heat settings on the dryer were trialed for drying the cysts (Fig. 27 and Table 5). A temperature data logger was placed in the pillowcase with the cysts to record the temperature every minute throughout the drying process. The cysts were checked each hour for dryness and the drying time was recorded.

1.3.1 Quantity of cysts to dry

One kg of wet cysts were rinsed using fresh water though a vacuum flask. The cysts were dried in the clothes dryer in a pillowcase (method I). One kilogram was found to be the maximum quantity of cysts able to be dried evenly in the clothes dryer.

1.3.2 Drying method II

This method was trialed as a means of drying larger quantities of cysts at once. Sections of 200 l polyethylene barrels were welded together and placed on top of an industrial ventilation fan (Fig. 25). A 100 µm screen was mounted horizontally 300 mm above the fan inside the barrels. The screen was reinforced with polypropylene grid above and below to keep it rigid. A clear door was placed above the screen for access and viewing. To prevent cysts collecting in the centre of the screen a dome was inserted causing the cysts to fall to the outside of the screen (Fig 26).

Figure 25. Drying method II.

150 mm flexible ducting was attached to the top of the barrels with a flange. At the end of the ducting a bag made from 100 µm screen with a drawstring was attached to collect the cysts.

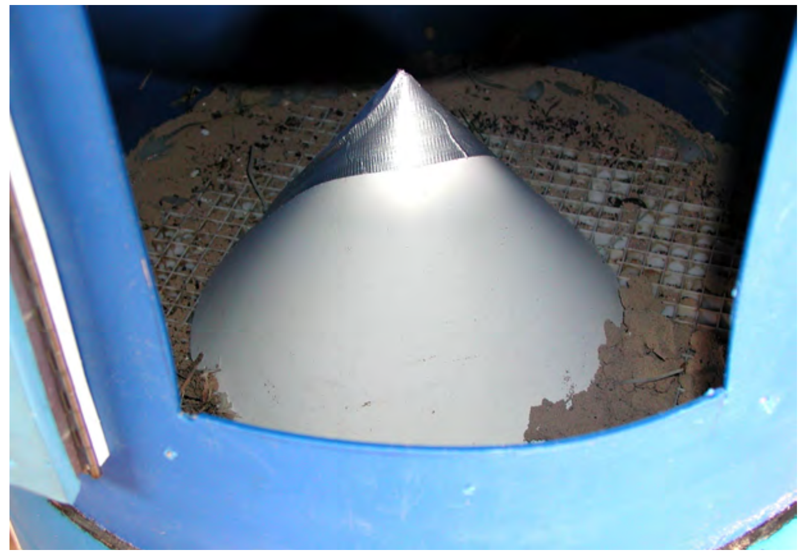

Figure 26. Dome used in drying method II.

The dryness of the cysts is determined by the height of the column and the strength of the fan, as the cysts become lighter with moisture loss they become further suspended in the air until they reach the overflow point at the top and exit though the ducting and are subsequently caught in the screen bag. The height of the column can be adjusted in order to control the final moisture content of the cysts. The Hutt lagoon cysts had higher final moisture content than the Inve® cysts (commercially available in Australia) 16.85 ± 0.22% and 8.97 ± 0.1% respectively, but the hatching viability was still high. In order to maintain cysts viability for long-term storage it is anticipated that the final moisture content of the cysts will need to be reduced.

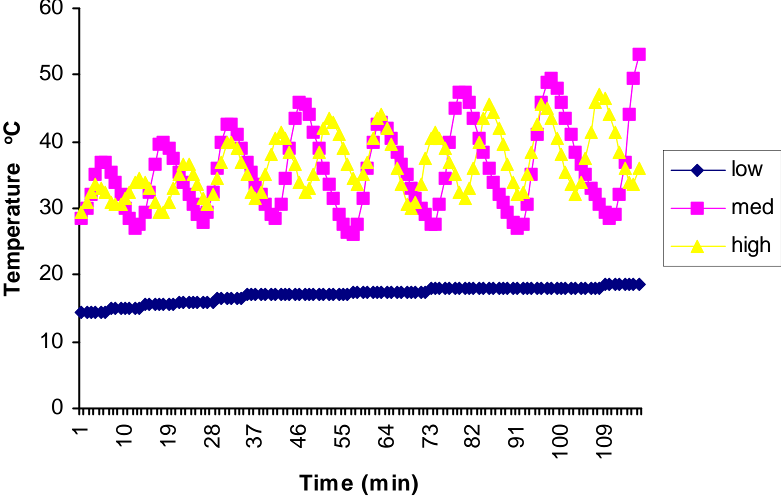

Figure 27. Temperature of cysts when being dried in the clothes dryer.

Table 5. Clothes dryer minimum and maximum temperatures for each setting.

	Clothes dryer heat settings		
	low	**medium**	**high**
Minimum temperature (°C)	14.5	26	29.5
Maximum temperature (°C)	18.5	53	47

1.3.3 Hatching

Duplicate 50 g samples of cysts from each group were used to inoculate 50 l culture tanks and hatched according to the methods described in Laven and Sorgeloos, 1996. The percentage hatch rate was measured. The system used for hatching is described in Kolkovski et al. (2004). All drying treatments were tested hatching viability decapsulated and non-decapsulated, except those from drying method II.

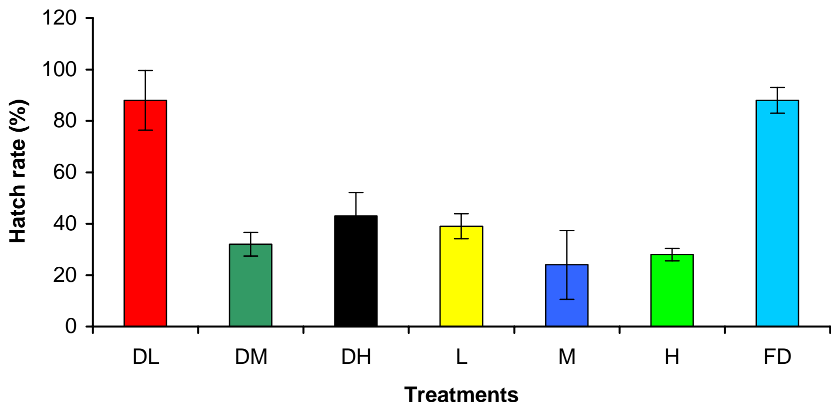

Figure 28. Hatch rates of Hutt Lagoon cysts dried using different methods.

Treatments

DL: Decapsulated cysts dried on low in the clothes dryer

DM: Decapsulated cysts dried on medium in the clothes dryer

DH: Decapsulated cysts dried on high in the clothes dryer

L: Non-decapsulated cysts dried on low in the clothes dryer

M: Non-decapsulated cysts dried on medium in the clothes dryer

H: Non-decapsulated cysts dried on high in the clothes dryer

FD: Decapsulated cysts dried using drying method II (unheated fan)

The two methods found to be the most successful in terms of cyst quality for drying, were the clothes dryer on low temperature and drying method II. The medium and high temperature settings on the clothes dryer were not successful. The medium and maximum settings were either too high for the cysts to withstand or the cysts were dried for too long on these settings. The temperature ranges were 26 – 53°C and 29.5 – 47°C for the medium and high dryer settings respectively. Having a more constant temperature range and a lower maximum on high could explain the better hatch rates for the cysts dried on high than medium (Fig. 27). Shorter drying times at high temperatures may have resulted in better results. The low temperature setting proved to be successful and therefore the fan method was used at ambient temperature (Fig. 28). Any debris found with cysts was removed after drying, by sieving the cysts though a 300 µm screen which retains the debris and allows the cysts to pass through. There is potential to upscale drying method II to dry larger quantities of cysts.

1.4 Processing biomass

Artemia biomass collected from the *D. salina* production ponds was rinsed with chilled (4°C) fresh water at the Cognis plant. *Artemia* was held for up to 1 h in the 900 l container, prior to the initiation of the rinsing process, the *Artemia* floated and formed a thick-concentrated layer on top of the brine. The volume of *Artemia* to brine was kept at approximately 33% *Artemia*. The *Artemia* floating on the brine were easily separated from the brine by simply draining the container from the bottom. *Artemia* were then rinsed with potable fresh water on a 250 µm screen until the rinse water coming from the sieve was clear (Fig. 29). The salinity was constantly monitored and when it declined to <5ppt, rinsing was complete. The rinse water was maintained at 4°C by a chiller unit, which quickly chilled the *Artemia* for transport to Geraldton Fisherman's Co-operative (1.5 h journey, by road) for blast freezing in 1 kg blocks in trays normally used for freezing rock lobster paste (Fig. 30 and 31).

The collection and freezing process was repeated on several occasions by both DoFWA and Cognis staff. Throughout the course of the bloom 10,000 kg of *Artemia* was collected, of which 1.5 t x 10^3 kg was frozen in 1 kg blocks and professionally packaged by the staff at Geraldton Fisherman's Co-Op. A W.A. company 'W.A. Artemia' currently distributes these 1 kg blocks commercially for Cognis. Potential estimated wholesale income from biomass sales is approximately 1.5 t x 10^3 kg x $10 kg = $15,000.

Figure 29. Washing brine from *Artemia* with chilled fresh water.

Figure 30. Pouring *Artemia* biomass into Rock lobster paste trays at GFC.

Figure 31. Stacking trays ready for blast freezing at GFC.

1.4.1 *Artemia* products and samples

Hydrolysate

Artemia is highly attractive to marine finfish larvae as a feed and was found to secrete amino acids and other compounds that act as feed attractants (Kolkovski et al. 1997, Kolkovski 2000, Kolkovski et al. 2000). Therefore it is anticipated that *Artemia* hydrolysate has a high potential for commercialisation as part of a protein source in microdiets, supplying free amino acids and peptides in a more easily assimilated form than whole proteins as well as acting as a feed attractant (Kolkovski, 2001). Other hydrolysates such as krill hydrolysate have been found to have high attractability for marine finfish larvae and it is believed that *Artemia* hydrolysate has high commercial potential in fish and prawn feeds (Kolkovski et al. 2000, Kolkovki and Tandler, 2000). The *Artemia* hydrolysate also has potential as bait (or inclusion in bait) for commercial and recreational fishing.

To hydrolyse the *Artemia*, a fermentation process was developed. This process was modified from the process developed by the Department of Agriculture, Western Australia (Smith, 1996). The amount and proportions of bacteria strains were adjusted to suit the *Artemia* biomass. *Artemia* was hydrolysed in 50 l temperature controlled tanks described in Kolkovski et al. 2004 (Fig. 32). Seven different combinations of ingredients including bacteria, molasses and formic acid were trialed (table 6). The hydrolysis process was initiated by adding the *Artemia* biomass, bacteria and molasses. Another method was trialed in which only formic acid was used to reduce the pH. The pH was monitored until it stabilised at pH 3.8 (in the treatments other than the formic acid treatment), indicating that the process was complete (Fig. 32). All the methods using bacteria (1-6) proved to be superior to the method using formic acid. The pH in acid digestion did stabilize and was lower but did not successfully hydrolyse the *Artemia.* The two types of bacteria used were freezed-dried *Lactobacillus* sp. (LB-12) and *Oenoccus* sp. (YC-380). *Lactobacillus* sp. initiated the process more effectively while the *Oenoccus* sp. was more efficient at hydrolysing once started. The *Lactobacillus* sp. were used as the sole bacteria in 2 treatments and combined with the *Oenoccus* sp. in three treatments. The amount of molasses and bacteria was also varied (table 6).

Table 6. Treatment details for hydrolysate trial.

Tank	Bacteria type	Bacteria amount	molasses (%)	molasses(kg)	*Artemia*	*Artemia*,(kg)	Formic Acid 85% (ml)
1	LB-12	50u	5	1	95%	19	
2	LB-12	50u	10	2	90%	18	
3	LB-12/YC-380	25u/25u	5	1	95%	19	
4	LB-12/YC-380	25u/25u	10	2	90%	18	
5	LB-12/YC-380	50u/50u	10	2	90%	18	
7						20	750

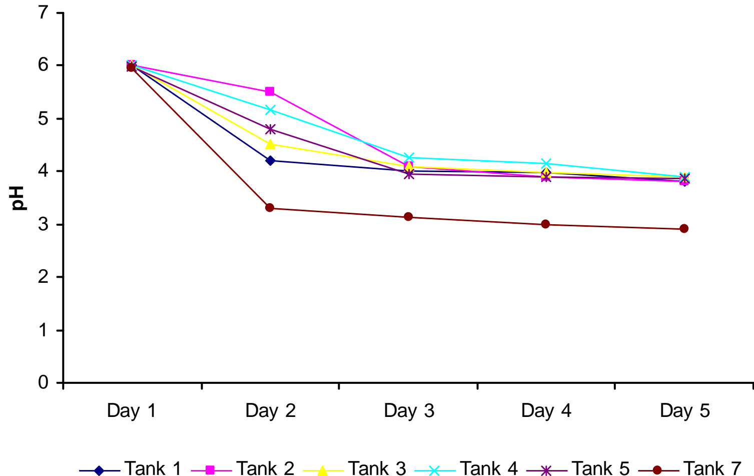

Figure 32. pH during process of making *Artemia* hydrolysate.

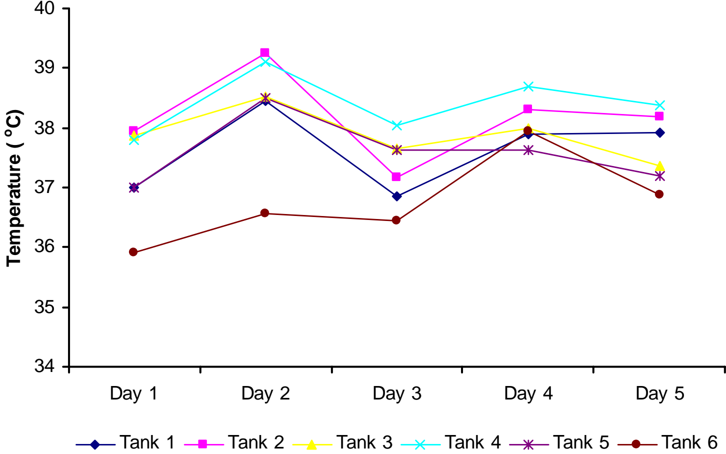

Figure 33. Tank temperatures whilst making the *Artemia* hydrolysate.

Two kilogram dry weight of *Artemia* hydrolysate has been freeze-dried to be sent to NIWA (National Institute for Water and Atmosphere) in New Zealand to be incorporated into microdiets as a feed attractant. It is anticipated that DoFWA will also manufacture experimental weaning micro-diets using *Artemia* hydrolysate.

Figure 34. Hydrolysate.

'Pickled' Artemia

'Pickled' *Artemia* is the commercial name used for *Artemia* preserved in brine. The collected *Artemia* have been found to remain in-tact for 2 months when stored in brine (>200 ppt salinity) and stored at < 4°C, although some reduction in lipid and protein content was observed. *Artemia* are currently being stored in a cool room preserved with the brine and sodium metabisulphite, a food grade preservative, at a rate of 2.8 ppt, to determine the length of time that such a product can be stored without deterioration. W.A. *Artemia* is already distributing a small amount (hundreds of litres) of the pickled *Artemia* to the ornamental fish market for market appraisal (Fig. 35).

Figure 35. Pickled *Artemia*.

Frozen Artemia

These were frozen in a blast freezer at Geraldton Fisherman's Co-Op (GFCO) as described in the processing section of the report. Sample blocks have been sent to various industry users and are currently available as a commercial product (Fig. 36 and 37).

During the project, the PI established links between GFCO and Cognis to establish commercial collaboration for the processing and freezing of the *Artemia* biomass. GFCO expressed their willingness to be involved in future development of commercial production of *Artemia* through their existing seafood processing plant at Geraldton. These links between the two companies should facilitate the commercialisation of the *Artemia* production.

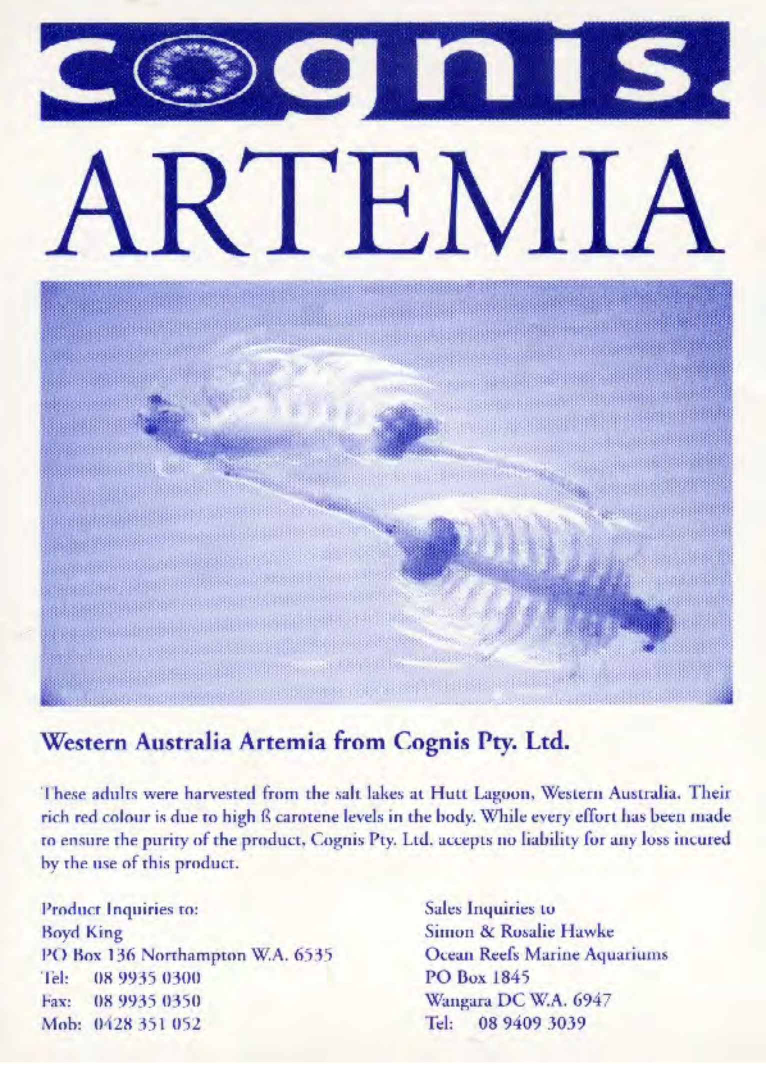

Figure 36. *Artemia* biomass sticker.

Figure 37. Frozen Biomass.

1.4.2 *Artemia* biomass and cysts characteristics

Artemia was analysed for protein, fat, ash and fatty acid analysis on a dry matter basis, all samples were rinsed and sent for analysis frozen apart from the Hutt Lagoon brine and SMS preserved *Artemia* (Chemistry Centre of WA). It was found that the Hutt Lagoon nauplii have a very good nutritional profile in terms of protein and HUFA's when compared with commercially available Inve® nauplii. The Hutt lagoon "Pickled" *Artemia* had lower lipid and protein levels to that of the frozen product (Fig. 38) and this issue will be addressed in the following project FRDC (2004/238).

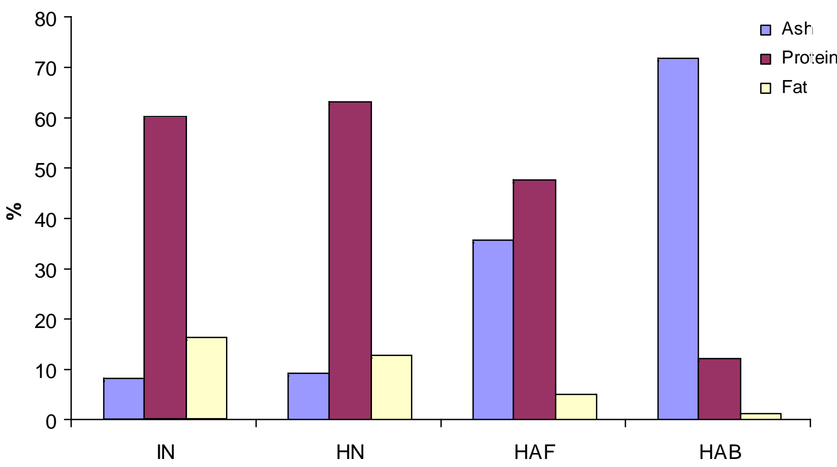

Figure 38. Chemical analysis *Artemia* on a dry matter basis.

Treatments

HAF: Hutt Lagoon frozen lake harvested adult *Artemia*

HAB: Hutt Lagoon brine and SMS preserved lake harvested adult *Artemia*

IN: Inve® newly hatched nauplii

HN: Hutt Lagoon newly hatched nauplii

Table 7. Fatty acid analysis.

	Sample	IN	HN	HAF	HAB
Name	Notation	Percent of Total Fatty Acids			
Linoleic	C18.2 cis-9,12	6.2	8.1	8	7.5
gamma-Linolenic	C18.3 cis-6,9,12	0.7	0.5		
Linolenic	C18.3 cis-9,12,15	30.2	29.1	20.5	22.2
Arachidic	C20.0	4.8	5.2	3.6	3.6
cis-11-Eicosenoic	C20.1 cis-11	0.6	0.7		
Eicosadienoic	C20.2 cis-11,14		0.6	0.6	
Arachidonic	C20.4 cis5,8,11,14	0.7	0.4		
Eicosatrienoic	C20.3 cis-11,14,17	1	1.8	1.2	1
Behenic	C22.0	1	1.2	1.4	1.6
Eicosapentaenoic	C20.5 cis-5,8,11,14,17	2	3	1.8	1.4
Docostetraenoic	C22.4 cis-7,10,13,16	0.3	0.3	0.8	0.2

1.4.3 Industry stakeholders already using Cognis *Artemia*

MG Kailis, Exmouth Hatchery W.A. – Using pickled and frozen *Artemia* for Rock lobster research; AQWA, Hillarys W.A. – using pickled and frozen *Artemia* for a host of species including leafy seadragons; Culture for Conservation, Challenger TAFE Fremantle W.A.- using pickled and frozen *Artemia* for seahorses and marine finfish; Southwest Seahorses, Kalbarri W.A. – using pickled and frozen *Artemia* for seahorses; Sunkissed Yabbies, Gingin W.A. – using pickled and frozen *Artemia* for feeding yabbies and freshwater fish; Marine farms Ltd, Exmouth W.A., Mahi Mahi hatchery and

growout facility – using cysts for nauplii in Mahi Mahi larval rearing; NIWA, New Zealand – trialing the freeze dried hydrolysate in experimental microdiets for marine finfish larvae; Tropical Marine Science Institute, University of Singapore – using frozen *Artemia* to feed seahorses; University of Malaysia – trailing the pickled *Artemia* for fresh water ornamental fish; W.A. *Artemia* – supplying frozen *Artemia* to the domestic and commercial market.

1.4.4 Eradication of shoreline cysts in Cognis algal production ponds

Currently the DoFWA and Cognis are investigating methods to eradicate *Artemia* cysts from the shoreline. One assessed the wetting of cysts (with freshwater) to achieve a state of irreversible hatching metabolism and subsequently drying them to reduce hatching viability. The aim is to reduce the hatching viability of the cysts on the ponds banks to as close to zero as possible.

Three 20 g samples of Cognis cysts (dried, with a known hatch rate of >85%) were sprayed with fresh water directed from 3 x 4 mm irrigation sprinkler heads at a flow rate of 5.3 l min^{-1}. An area of 5.6 cm^2 (for each 20 g sample) was sprayed for 12 h and then allowed to dry at ambient temperature for 72 h. The samples were subsequentley hatched in the *Artemia* system (Kolkovski et al. 2004), according to the methods in Lavens and Sorgeloos, 1996. The hatch rate had reduced to an average of 4.86 ± 0.01% for the sprayed cysts (Fig.39).

Subsequently Cognis set up a truck with a water tank and spray bar and sprayed cysts on a concentrated area of the shoreline keeping them wet for 8 h. The cysts were then left to dry for a period of 72 h. Samples from the shoreline pre and after the wetting were sent to DoFWA to be tested for hatching viability. The cysts were hatched following the same protocol as the cysts sprayed for 12 h by DoFWA. The hatch rate of the shoreline cysts had reduced from 33.82 ± 0.006% to 26.98 ± 0.01% (Fig. 39). As this was not seen to be a significant reduction, the wetting affected by the wind, longer periods of wetting are currently being applied to compensate for wind-driven drying and the hatch rate of the cysts will be tested.

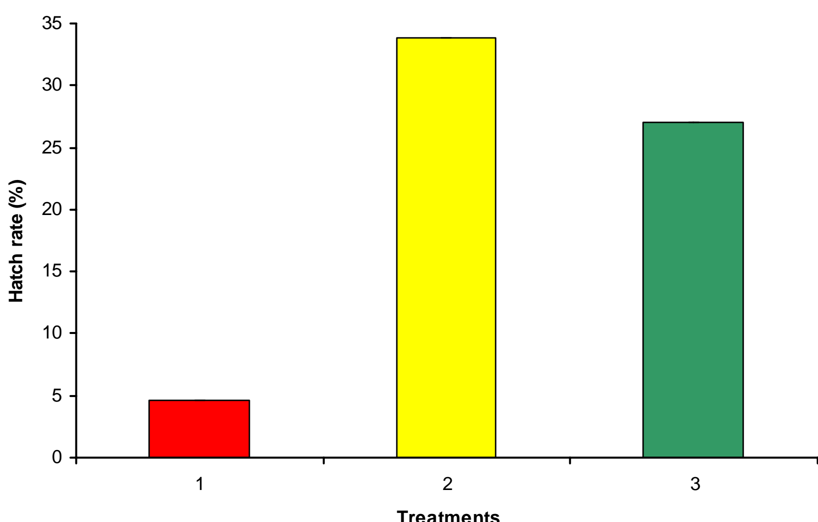

Figure 39. Cognis cyst hatch rates results from wetting trials.

Treatments

1. Cysts wet in the laboratory for 12 h and dried for 72 h
2. Pond shoreline cyst hatch rate pre wetting on shoreline
3. Shoreline cysts after 8 h wetting and 72 h drying on shoreline

1.5 Recommendations to reduce the risk of further blooms at Cognis

It is thought that the current practice of eradicating cysts with acid or physically removing them when they are noticed on the shoreline, will only serve to remove a small percentage of *Artemia* from the lakes. It might be possible that, along the 4 km shoreline of each pond, the cysts build up over the 4-year period and give the potential for *Artemia* to reach epidemic numbers. There have been two such outbreaks of *Artemia* in the ponds in 4 years and during these events a large number of cysts are produced. In these two cases the built up numbers of cysts are more easily identified and are therefore removed or killed with acid. However, during the years between *Artemia* 'outbreaks', cysts are not noticed. Therefore, they are not sufficiently removed from the system until such an event occurs.

An alternative, ongoing and low maintenance management practice would be to use cyst collectors in the ponds. This would continually remove cysts from the system thus minimizing the impact of future *Artemia* outbreak events. Cyst collectors could also provide an easy way to identify periods of high cyst production within the ponds. The identification of these periods would serve to help recognize contributing environmental factors towards the promotion of cyst production by *Artemia*. This in turn would be of benefit to research into intensive biomass and cyst production.

It is suggested that two cyst collectors will be placed in each pond in the corners down from prevailing winds. Therefore a cyst collector would be placed in the northeast corner to collect with the aid of the southwesterly afternoon breeze, and the other collector would be placed in the northwest corner to collect with the aid of the southeasterly morning breeze.

The design of each collector needs to be relatively inexpensive and simple to assemble on site. The collectors are made as two parts, one part is designed to hold the cysts once they have been trapped and the other part is designed to sweep the cysts to the holding pen. The tendency for the cysts to float on top of the brine should be exploited in order to control their movement towards the pen and hold them there. Within FRDC project (2004/238), a monitoring program at the Hutt Lagoon site, may lead to a model that predicts future outbreaks of *Artemia* and helps develop methods to avoid or control such outbreaks.

1.6 Acknowledgements

This component of the project was carried out as a collaborative project with Cognis Australia Pty Ltd. Special thanks to Mr Boyd King, Hut Lagoon site manager (Cognis) for his contribution to the project. Dr Greg Maguire, Department of Fisheries WA, provided support, valuable comments and reviews throughout the project. Thanks to John Heine for editing and proofing and Sandy Clarke for formatting and putting together this publication.

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