ENHANCED HATCHERY PRODUCTION OF STRIPED TRUMPETER, *Latris lineata*, IN TASMANIA THROUGH SYSTEM DESIGN, MICROBIAL CONTROL AND EARLY WEANING

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Book 1: Summary

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1 NON TECHNICAL SUMMARY

2004/221	Aquafin CRC - Enhanced hatchery production of Striped Trumpeter, *Latris lineata*, in Tasmania through system design, microbial control and early weaning

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OBJECTIVES:

1. Investigate the control of microbial communities in intensive larval fish culture using ozonation and probiotics.
2. Determine the optimal environmental parameters, and water quality systems and tank design for reducing hatchery mortality and malformations in finfish larvae.
3. To better understand "grey gut syndrome" and the ontogeny of the immune system, including linkages to developments with probionts and immunostimulants.
4. Evaluate formulated diets and their use in early weaning.
5. Evaluate the growth and survival of Striped Trumpeter post-larvae and juveniles reared under semi-commercial conditions.
6. Evaluate the possibility for the culture of Striped Trumpeter using alternative systems and/or sites.

Striped Trumpeter, *Latris lineata*, are one of Australia's finest eating fishes and are native to south-eastern Australia and New Zealand. They have long been considered the best candidate for diversifying sea cage culture in Tasmania. Research into the culture of Striped Trumpeter has been underway since the 1990's and the following report concludes 14 years of continuous research through the Aquaculture CRC (1994 to 2000) and Aquafin CRC (2001 to 2008). The unusually long post-larval or "paperfish" stage lasting up to nine months and requirements for oceanic conditions made them a difficult species to culture. The current CRC project (2005 to 2008) built on the excellent research foundation in three critical areas. First, variability and larval mortality was reduced through improved health and better control of bacterial diseases and parasites. Second, there was further development of live feed, weaning, and grow-out diets, nutritional and other factors, particularly those influencing malformations. Third, the larger-scale assessment of survival and growth of post-larvae and juveniles was undertaken on a semi-commercial scale and culminated in the grow-out of Striped Trumpeter in sea cages.

Improving larval survival was achieved through a better understanding of the microbial flora in culture tanks associated with fresh and frozen algae concentrates. New bacterial identification methods gave superior insights into the complex bacterial communities and how they change during larval rearing. Potential probionts improved survival in live feed cultures of rotifers. Growth and larval survival were better when the probionts were transferred to larvae via rotifers but not when added directly to the culture water. Development of novel filtration and ozonation systems delivered seawater to the marine fish

hatchery which totally removed the need for antibiotics. Ozonation of Striped Trumpeter eggs stopped the vertical transfer of nodavirus from broodstock to the larvae while water treatment prevented myxozoan parasites from becoming established in larval and juvenile fish. Larval feeding rate was optimised by changing the larval visual environment with greenwater, light intensity and tank colour.

Jaw malformations have been a recurrent obstacle in the hatchery production of high quality juvenile Striped Trumpeter. This is a problem shared with many marine finfish species including Yellowtail Kingfish. Nutrition and temperature were examined along with manipulation of the physical culture environment and larval behaviour to reduce jaw malformations. The onset of jaw malformation after metamorphosis in Striped Trumpeter follows changes in larval behaviour from an even distribution throughout the water column to close association with the tank walls, often with vigorous swimming into the walls known as 'walling' behaviour. Potential mechanisms for the influence of walling behaviour on jaw malformation are mechanical damage and poor nutrition, via reduced feed intake and increased energy expenditure. The project highlighted the often overlooked importance of hard-surface interactions in the growth and survival of cultured marine fish and demonstrated a cheap and effective technique for assessing tank background colour as a means of reducing malformations in cultured fish. Batches of fish with up to 76% commercially acceptable good jaws were produced.

Weaning of post-larvae from live feeds to formulated diets had been a bottleneck to large-scale production of Striped Trumpeter juveniles. Experimentally, early weaning was achieved from 30 days post-hatch using new improved diets from Europe. The weaning of fish in production trials can now routinely be achieved by 40 days post-hatch. With the excellent improvements in the production of weaned larvae attention was focused on increasing the quality of post-larvae. Prior to the current project almost nothing was known about the optimal rearing conditions for post-larvae. Temperature has a profound effect on fish growth and determining the optimum temperature was important in understanding growth and defining further nutritional characteristics. The optimum temperature for rearing Striped Trumpeter post-larvae changes with age from 16^{o}C to 14^{o}C. The optimum lipid content and diet ration were also examined. Polynomial models predicted a feeding rate of 4 % biomass day^{-1} to be optimal. The increased understanding of post-larval rearing conditions was integrated into a new nursery rearing facility incorporating recirculation systems and temperature control, designed to produce batches of 10,000 post-larvae for future on-growing trials in sea cages.

As the project moved towards commercial-scale grow-out, knowledge of the immune system became more important. Striped Trumpeter were found to produce an antibody molecule similar to that of other teleosts. Initial investigations suggest that they begin to produce antibodies much later than other marine fish. No antibodies have been detected in fish less than six months old. These unusual findings could affect future husbandry and vaccination practices. The holding of fish at higher densities led to the discovery of two new previously undescribed parasitic copepods. The two species were fully described and their life histories examined with the aim of developing effective control and treatment regimes. The first species *Chondracanthus goldsmidi* was described from the gills and opercula and occurred in sufficiently high infestations in tanks to be considered a potential problem. Trials were conducted to establish the efficacy of freshwater, hydrogen peroxide and in-feed medications. The second parasite *Caligus nuenonnae* n. sp. was described from the skin but was not a significant health issue.

Research culminated in sea-cage grow-out trials using hatchery produced Striped Trumpeter. Around 2,500 juvenile fish were stocked in a commercial salmon farm within the Huon River region and on-grown for up to two years. The fish were fed formulated diets in three 20 m sea cages. The trials provided the first hatchery-reared Striped Trumpeter grown to market size in sea cages. Overall survival was good ranging from 66 to 94% and the average weight of harvested fish was 1430±9g. HOG (head-on, gilled, gutted) yield was excellent at 94%. Growth and survival may have been higher at a more marine dominated site. The water salinity was often below full salinity during the winter and sea temperature ranged from 9.4^{o}C to 18.9^{o}C averaging 13.9^{o}C. Over 2 t of fresh farmed fish were sold and market feedback was highly positive. A basic economic analysis of Striped Trumpeter farming suggested it would take 14 years to reach 5,000 t production at which point cumulative profit could eclipse cumulative spend.

The project delivered timely scientific and production outputs. An important component of the project involved post-graduate training. The project supported five post-graduate students. Much of the research has been published, and all of it has been subjected to scrutiny through conferences and workshops. The research results and techniques developed have direct application to other marine species and the Striped Trumpeter team have worked with leading researchers in Australia and overseas to apply the techniques developed on a range of marine species including Yellowtail Kingfish and Southern Blue-fin Tuna.

OUTCOMES ACHIEVED TO DATE

The three main planned outcomes from the project were in large measure achieved:

1. Australian aquaculture has been provided with more systematic ways to control microbial communities, and to evaluate, identify and produce probionts for use in improving hatchery survival rates in finfish. Microbial control has been achieved in culture through the innovative use of ozonated seawater in both live feeds and larval rearing.

2. There will ultimately 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.

3. The Atlantic salmon industry has moved significantly closer to diversification into new species, particularly Striped Trumpeter through sea cage trials using cultured fish. In time this should improve their profitability while reducing their risks, hence ensuring their long-term sustainability.

KEYWORDS: Striped Trumpeter, *Latris lineata*, finfish hatchery technology, probiotics, ozone, larval fish malformation, larval weaning, Tasmania

2 ACKNOWLEDGEMENTS

This project formed part of the Research Program of the CRC for Sustainable Aquaculture of Finfish ("Aquafin CRC"), and employed funds invested out of the CRC's Commonwealth grant and by University of Tasmania and the Tasmanian Government and other Participants of the CRC. We thank the Aquafin CRC CEO Dr Peter Montague and Subprogram Leader Mr Steven Clarke, Tasmanian Aquaculture and Fisheries Institute (TAFI) Director, Professor Colin Buxton and FRDC's Executive Director, Dr Patrick Hone, and Programs Manager, Crispian Ashby for their strong support of the project. We thank Mr Pheroze Jungalwalla, Executive Officer of the Tasmanian Salmonid Growers Association, for his encouragement and long-term support.

The industry partner Huon Aquaculture Group (HAG) owned by Mr and Mrs Bender provided substantial in-kind support to conduct farm trials. We appreciate the technical support of Dr David Morehead who ran the on-farm trials and never wavered in his optimistic endorsement of Striped Trumpeter as an excellent new species for aquaculture. The feeding, handling and management of fish on the farm was undertaken by Andrew Bourke, Robert Churchill and Colin Johnson.

A Striped Trumpeter Working Group was formed at the start of the project to maximise the chances of success and to provide advice to the Tasmanian Government. It provided advice and support and we thank Prof Colin Buxton (TAFI), Dr Stewart Fielder (NSW Fisheries), Mr Pheroze Jungalwalla (TSGA), Mr Wayne Hutchinson and Mr Steven Clarke (SARDI) for participating.

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3 BACKGROUND

A major bottleneck in the development of many new marine fish species for aquaculture in Australia is the production of high quality juveniles (Battaglene and Fielder 1997). According to the Australian Fisheries Statistics in 2008 (ABARE 2009) only three species of fish are cultivated from eggs and reared in sea cages in large numbers. They are, in order of production: Atlantic salmon in Tasmania at 24,248 t; barramundi in Queensland at 2,464 t and yellowtail kingfish in South Australia at 3,801 t. Clearly, the largest production and associated infrastructure is in Tasmania. However, the rapid growth of the Atlantic salmon industry is under threat from increasing costs (particularly food), control and management of disease and predation (especially AGD, Amoebic gill disease, and seals), competition from overseas producers and environmental changes (climatic) (Battaglene and Cobcroft 2003). Experience in other countries indicates the need for the Tasmanian Atlantic salmon industry to support research programs on other commercially valuable finfish species, to provide diversification opportunities and avoid excessive reliance on a single species, Atlantic salmon. A successful industry for Striped Trumpeter would complement the existing industry for Atlantic salmon and provide an alternative should there be a catastrophic disease introduction or major market slump in demand. The Tasmanian Government has long supported the development of an alternative white-fleshed species to augment and diversify the production base. It understands the long-term nature of new species' development and has been highly involved in establishing the salmon industry where it remains a partner in the major hatchery SALTAS (Salmon Enterprises of Tasmania).

Striped Trumpeter, *Latris lineata*, were chosen as the best candidate for diversifying sea cage culture in Tasmania in the late 1980's. They are highly prized as one of the best eating fishes in Australia, are also greatly esteemed as sashimi in Japan and have firm white flesh, which is both tasty and fatty (Yearsley 1999). Market research by Tassal Limited into the flesh qualities of "farmed Striped Trumpeter" (wild-caught juveniles reared in captivity to adulthood) has been very encouraging and they rank alongside Australia's best white-fleshed fish in controlled taste tests (Pakes Research 2000). Another potential market advantage is the very high omega-3 polyunsaturated fatty acids (PUFA) concentrations in the flesh of "farmed fish" (Nichols et al. 2001). They also have a limited distribution in southern Australia from Sydney to Kangaroo Island and around Tasmania and New Zealand. Once plentiful in Tasmania the fishery for Striped Trumpeter has now declined from over 100 tonnes to around 50 tonnes per annum. Striped Trumpeter were selected not only for their marketing qualities, but also their docile nature, lack of cannibalism, ability to take formulated feeds and be held in captivity at high densities. However, they have a complex and extended larval phase with a nine month "paperfish" stage and to date they have not proven easy to culture. The knowledge base for Striped Trumpeter is one of the best for a new aquaculture species in Australia. They were one of only two fin-fish species studied for the entire seven years of the Aquaculture CRC (1994 to 2000), following a stringent national screening process. The research providers comprised the Department of Primary Industries and Fisheries, Marine Research Laboratories (MRL), University of Tasmania's School of Aquaculture at Launceston and the CSIRO Marine Research. In 1998 research on Striped Trumpeter was combined under the Tasmanian Aquaculture and Fisheries Institute (TAFI) with continued input from CSIRO. In 2000 the Tasmanian Government, the then largest salmon company Tassal Ltd, and the research providers combined to support the continuation of research through the Aquafin CRC. The research strategy of Aquafin CRC was developed explicitly to deliver the essential technologies needed by the Australian

finfish farming industry. The industry partners agreed to invest in a CRC, defined the major goals which they believed a CRC could best achieve, and clearly indicated the weight of effort which should be applied to each of these goals. For five years Tassal Limited was an industry partner with TAFI and CSIRO, providing financial support to both CRC projects. Tassal Limited had a signed MOU (Memorandum of Understanding) with TAFI and CSIRO providing preferential access to methodologies and fish arising from the project. However, in 2003 they withdraw from the project after going into receivership and following major industry restructuring. They have formally relinquished claims to preferential access to methodologies and fish.

The earlier Aquafin CRC project 1B.4 with the support of FRDC (Fisheries Research and Development Corporation) (2001/206) started in 2001 and finished in 2004. It built on the increasingly sophisticated R&D program to develop Striped Trumpeter as an aquaculture species. Excellent progress had been made in understanding and controlling reproduction, arguably the best for any species in Australia. Broodstock collected from the wild are routinely spawned year-round through temperature and photoperiod control (Morehead 1997). Problems with early larval rearing have also been overcome and egg incubation and early larval rearing protocols have been established. The mortality peak associated with first-feeding has been reduced using better live feed production techniques (Brown et al. 1998: Battaglene and Brown 2006) and improving water quality particularly at the air/surface interface (Trotter 2003). Improved swimbladder inflation rates have been achieved using new surface cleaners, optimal temperatures and light conditions (Trotter 2003). Problems with jaw malformations, common to all batches of post-larvae in this and many other species, improved but remain a difficulty (Cobcroft et al. 2001). Some malformations (scoliosis) may be linked to myxozoan, *Kudoa neurophilia*, infections (Grossel et al. 2003). The project met all production targets and over a thousand 100 day-old post-larvae were produced in 2002, 2003 and 2004. Early larval survival has been greatly enhanced with up to 80% survival to Day 20 and 3.5% survival to Day 100. Still the bottleneck to juvenile production remains mortality from flexion to metamorphosis, caused by an apparent primary metabolic disorder with a nutritional and/or health basis. Recent research has focused on investigating the link between deficiencies or imbalances in the three essential PUFAs - docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) - found in live feeds (Sargent et al. 1999). Several commercially available live feed enrichments have been tested on a production scale. Novel experimental emulsions and biochemical approaches have been developed allowing the dietary requirement for DHA to be identified in larvae (Bransden et al. 2005). A significant relationship was found between dietary DHA and larval grey gut syndrome (bacterial gut degeneration), possibly suggesting the importance of DHA as an immunomodulator in early developing Striped Trumpeter larvae. Copepods have been cultured as a supplement to traditional live feeds enriched on novel emulsions and have improved larval rearing success (Morehead et al. 2005). A range of novel diets containing large doses of vitamins C (ascorbic acid) and E (a-tocopherol) have been tested in live feeds and fed to larvae. Increased feed ration has reduced the presence of urinary calculii, increased survival, dry weight and viable larvae. New commercial particulate diets from Europe have shown promise as a weaning diet for post-larvae. Partial weaning may be possible from Day 50 but more research is required to establish the earliest age at which larvae can be weaned. Weaning protocols also need to be devised that maintain adequate water quality. Controlled experiments using antibiotics have demonstrated that high bacterial loads are an important factor in larval mortality. Antibiotics improved survival, increased digestion, and reduced the incidence of grey gut syndrome and the presence of urinary calculii, resulting in more viable larvae. Efforts in 2003 focused on producing fish without antibiotics using a new water filtration and ozonation treatment

system. Improved water quality allows greater control of the early bacterial problems, often encountered in Striped Trumpeter culture, and enables better interpretation of experimental results by limiting confounding influences.

The first Aquafin CRC Striped Trumpeter project clearly delivered timely scientific and production outputs. The research results and techniques developed have direct application to other marine species and the Striped Trumpeter team worked with leading researchers in Australia, Scotland, Mexico and Spain to apply the techniques developed on a range of other marine species. The project was reviewed in 2003 by an independent international panel (Anon 2003), and as part of the second year Aquafin Review. Full review reports are available from the Aquafin CRC, but the following extract summarises the highly favourable findings: "The review team was impressed by the high standards of the research and the methodological approach to the Striped Trumpeter project. The research team operates from a strong scientific base and exhibits a high level of dedication to the challenging research. The research team in the opinion of the external reviewers has covered all bases and has encompassed relevant larval rearing and production information from around the world." Both reviews made a series of recommendations for future research that have been incorporated into the current project. In a shared view of the future it was agreed that research was required in three key areas to take full advantage of the momentum and resources established. First, the health of post-larvae needed to be improved through better control of bacterial diseases and parasites. Research in this area is by no means unique to Striped Trumpeter and a general lack of understanding of factors influencing larval survival and growth combined with poor seawater supplies means that many Australian hatcheries and research facilities struggle to reliably supply seedstock. Indeed, many commercial hatcheries routinely used large quantities of prophylatic antibiotics. Bacterial load reduction using antibiotics is not acceptable in terms of fish, environmental or human health and the catastrophic effects of indiscriminate use of antibiotics in aquaculture are well known (Weston 1996). In the past 10 years there has been growing interest in the use of probiotics to improve the health of aquatic animals. Use of probiotics shows increasing promise as a strategy that will reduce hatchery mortality and has been used successfully in crustacean hatcheries (Gomez-Gil et al. 2000). However, the diversity of species raised in hatcheries and the specifics of the bacterial interactions means that there is no universal solution or magic bullet. The development of probiotics needs to go hand in hand with better system design and water quality management, including ozonation, to achieve microbial control. The second key area is the further development of live feed, weaning, and grow-out diets, leading to a better understanding of nutritional and other factors, particularly those influencing malformations. The third, is the larger-scale assessment of survival and growth of post-larvae and juveniles on a semi-commercial scale, leading ultimately to the grow-out of Striped Trumpeter in sea cages.

This report is presented in two books. The first as a series of scientific abstracts framed by introductory information about the project and a summary of the research conducted. The full research papers are provided in Book 2 and are either published or intended for publication. The full paper citation (or stage in publication) and authorship details are provided. Important information relating to the project is also included in Appendices 1 to 3. The first research Chapter 7 provides a detailed review of research into the culture of Striped Trumpeter at the start of the project, the next four Chapters 8-11 relate to the selection of potential probionts and microbial differences in green and clearwater culture. Chapters 12 and 13 examine the effects of turbulence, algal-induced turbidity and tank colour on larval growth, survival and behaviour. Chapter 14 reports on early larval weaning and the use of new micro diets. Chapters 15 and 16 investigate optimal temperatures, lipid levels and

rations for culture of post-larvae. Jaw malformations and the important role of larval behaviour in relation to walling are experimentally examined in detail in Chapters 17 and 18. The characterisation of immunoglobulin in Striped Trumpeter is provided in Chapter 19. The identification and control of parasites affecting Striped Trumpeter post-larvae and juveniles is examined in Chapters 20 to 23. The final research Chapter 24 provides a summary of the farm trials giving details of the growth and survival of juveniles stocked into sea cages. The Intellectual Property from the current project 2004-221, 1B.4(b) and the earlier project 2001-206, 1B.4(a) has been assigned to UTAS taking effect from 1st July 2008 to allow development of new projects (Appendix 1 and 2). Ethical approval to conduct the research was provided by the University if Tasmania Animal Ethics Committee under permits A8191, A8705, A8719, A8975.

4 NEED

The project was essential for four main reasons. First, experience in other countries, now rapidly developing new species like Atlantic cod, turbot, haddock and halibut, indicates the need for a new coldwater species to complement the existing salmon industry in Australia, which is facing significant challenges. Major stakeholders, the Tasmanian Government and Tasmanian Salmonid Growers Association fully supported the development of Striped Trumpeter as an alternative species, recognising that it was a long-term investment. The Tasmanian Aquaculture Research Advisory Group and TasFRAB (Tasmanian Fisheries Research Advisory Board) sanctioned the project as a top priority. Overcoming the Striped Trumpeter larval mortality bottleneck was identified in the Tasmanian Fisheries and Aquaculture Strategic Research Plan 2005-2009 as a high priority. Second, the project addressed the two objectives of the Aquafin CRC, Production Subprogram (Subprogram Hatchery Technology) by increasing the availability of species suitable for aquaculture and improving the quality of fingerlings for farm stocking. It also filled an expertise gap for the CRC following the Tuna Propagation Program demise by further developing a team capable of tackling difficult to rear marine fish and tapping into research institution in-kind contributions. The Aquafin CRC Board and JMAC (Joint Management Advisory Committee) requested the submission of the proposal. Third, there was an identified need for the research at a generic level where there was a necessity for both, a more systematic way to match the nutritional profile of live feeds with the requirements of new species of marine larvae (FRDC Hatchery Feeds R&D Plan high priority), and the development of system design and probiotics for the control of disease or improved health of hatchery and farmed aquatic animals (key area for research (7.2.2) in the Aquatic Animal Health Subprogram Strategic R&D Plan 2002-2007). Fourth, it facilitated capacity building and collaboration among the key institutions developing new marine species and an excellent training opportunity for post-graduate students, hatchery technicians and scientists.

5 OBJECTIVES

1. Investigate the control of microbial communities in intensive larval fish culture using ozonation and probiotics.
2. Determine the optimal environmental parameters, and water quality systems and tank design for reducing hatchery mortality and malformations in finfish larvae.
3. To better understand "grey gut syndrome" and the ontogeny of the immune system, including linkages to developments with probionts and immunostimulants.
4. Evaluate formulated diets and their use in early weaning.
5. Evaluate the growth and survival of Striped Trumpeter post-larvae and juveniles reared under semi-commercial conditions.
6. Evaluate the possibility for the culture of Striped Trumpeter using alternative systems and/or sites.

6 REVIEW OF STRIPED TRUMPETER CULTURE

Publication Title: Advances in the culture of Striped Trumpeter larvae: a review

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Publication status: Aquaculture (2007) 268:195-208

6.1 Abstract

Striped Trumpeter, *Latris lineata*, was chosen as the best new candidate for sea cage culture in Tasmania in the late 1980s. It has a complex and extended post-larval or 'paperfish' stage lasting up to 9 months and has historically proven difficult to culture. Excellent progress has been made in understanding and controlling reproduction and broodstock are spawned year-round through photothermal control. Problems with early larval rearing have been overcome and egg incubation and early larval rearing protocols have been established. A mortality peak associated with first-feeding has been reduced using better live feed production techniques and improved water quality. Using antibiotics showed that high bacterial loads were an important factor in larval mortality. A new water filtration and ozonation system has removed the need for antibiotics. Larval nutrition research focused on the link between potential deficiencies or imbalances in the three essential PUFA in live feeds: docosahexaenoic acid, eicosapentaenoic acid and arachidonic acid. Novel experimental emulsions were applied with dose response experimental designs to identify the dietary requirement for selected PUFA and vitamins. Despite the advances in live feed enrichments, the live feeds, particularly *Artemia*, were found to have sub-optimal lipid profiles. Copepods were cultured, as a supplement to traditional live feeds, and improved larval rearing success. Costs to scale up production and to control extensive cultures presently restrict the usefulness of copepods. Important breakthroughs have occurred in health with the detection and control of nodavirus, myxozoan and bacterial disease. Ozone disinfection of eggs and sterilisation of hatchery seawater have been important control measures. Another bottleneck to production has been mortality of larvae from notochord flexion to metamorphosis. System changes to reduce nocturnal movements and a better understanding of optimal live feed densities, and weaning onto formulated diets, have improved survival and growth. High rates of jaw malformation remain a challenge and no definitive cause has been established. Reduced rates of malformations have been associated with one or a combination of high feed rates, lower larval densities and temperatures, and reductions in 'walling' behaviour. Future research is aimed at finding ways to reduce malformations, develop probiotics and early weaning strategies, control parasites and scale-up production to assess performance of juveniles in sea cages.

See **Book 2 - Chapter 7** for full study details.

7 BACTERIAL FLORA IN LIVE OR CONCENTRATED ALGAE AND CLEARWATER

Publication title: Bacterial flora and larval performance in the culture of Striped Trumpeter *(Latris lineata)* larvae using live or concentrated algae or clearwater

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Publication status: unpublished

7.1 Abstract

Marine fish larvae are susceptible to mortality caused by bacterial infection during larval rearing. This study investigated performance and cultivable bacteria of Striped Trumpeter larvae reared to 15 days post-hatch (dph) in clearwater or greenwater based on live microalgae or a concentrated algal paste (instant). BTB-Teepol (BTBT) agar had better recovery of environmental Vibrios than TCBS and was used as the presumptive *Vibrio* selective media. Larval growth was higher in live algae (7.1 ± 0.3 mm standard length, SL, 330 ± 28 µg larva^{-1} dry weight, DW) than in clearwater (6.6 ± 0.3 mm SL, 268 ± 22 µg larva^{-1} DW) or instant algae (6.6 ± 0.3 mm SL and 232 ± 30 µg larva^{-1} DW). However, survival was higher in both live algae and clearwater (35 ± 9 and 38 ± 5%, respectively) than in instant algae (7 ± 4%). Mortality of larvae reared in instant algae was consistently high from 12 dph and coincided with significantly higher cultivable bacteria in the larvae (presumptive *Vibrio* 0.2 colony forming units [cfu] µg^{-1} larva DW at 3 dph to 650 cfu µg^{-1} larva DW at 15 dph) and in larval culture tanks (presumptive *Vibrio* peak at 8 x 10^5 cfu ml^{-1} at 7 dph and decline to 2 x 10^3 cfu ml^{-1} at 15 dph). Bacterial isolates (n = 515) were classified as presumptive Vibrios (39%), with eight well-defined species and eight unspeciated types with distinct phenotypes (75% similarity level), and glucose non-fermenters (61%), with 19 unspeciated types with discrete phenotypes (82% similarity level). Potential pathogenic isolates included *Vibrio anguillarum* and *V. ichthyoenteri*, and a strain with probiotic properties, *V. alginolyticus*, was identified. There was a correlation between bacterial flora of the larvae and the culture environment, demonstrated by a more complex flora associated with larvae held in live algae compared to instant algae or clearwater. Live microalgae is recommended for greenwater culture of Striped Trumpeter to provide for good growth and survival. Concentrated algal paste products should be used with caution as greenwater for marine fish larval rearing as they may lead to potentially harmful bacterial proliferation and larval mortality.

See **Book 2 - Chapter 8** for full study details.

8 MICROBIAL COMMUNITIES CULTIVATION-INDEPENDENT APPROACHES

Publication Title: Microbial communities of post hatch Striped Trumpeter (*Latris lineata*) larvae, held under different rearing conditions, determined using cultivation-independent approaches

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Publication status: unpublished thesis

8.1 Abstract

A knowledge of the microbial bacterial community of the larvae, seawater and live feeds was believed to be necessary in order to develop an understanding of what represents a healthy microbial ecology relevant for larval fish rearing success. 16S rRNA bacterial clone library and terminal restriction fragment length polymorphism (TRFLP) analysis was utilised to examine the microbial community associated with Striped Trumpeter (*Latris lineata*) larvae cultured under different "green water" conditions. It was discovered that the larvae-associated microbial diversity was restricted but varied considerably between culture conditions. Most bacteria detected belonged to class *Alphaproteobacteria* (predominantly the *Roseobacter* clade), *Gammaproteobacteria* (genus *Psychrobacter* and *Pseudoalteromonas*) and *Actinobacteria* (primarily genus *Microbacterium*). No association was found between larval survival and microbial community structure. Similar results were obtained using TRFLP analysis, though it was found that the larval microbial community was distinct from the bacterial community present in the surrounding water.

See **Book 2 - Chapter 9** for full study details.

9 ASSESSMENT AND TRACKING OF BACTERIAL PROBIONTS

Publication Title: Assessment and tracking of bacterial probionts within a Striped Trumpeter (*Latris lineata*) larvae rearing system

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Publication status: unpublished thesis

9.1 Abstract

Potential probiotic candidates were identified by using antimicrobial *in vitro* plate testing against known pathogenic *Vibrio* species, with six out of 25 isolates tested selected for further testing. In *Artemia* challenge trials, it was determined that *Pseudoalteromonas agarivorans* ST18 and *Aliivibrio fischeri* ST7 had the least effect on *Artemia* survival. To further assess the probiotic capability of strains ST18 and ST7, rotifer and *Artemia* cultures were challenged with pathogenic strain *V. proteolyticus* V760 mixed with strains ST18 or ST7. Strain ST18 was found to have a probiotic effect in that cultures containing both V760 and ST18 were not significantly different from the control system but produced significantly better survival compared to the pathogen-only treatments. To further investigate ST18 and ST7 in a mixed cultured system terminal restriction fragment length polymorphism (TRFLP) analysis was applied to monitor the change in bacterial community. Through tracking probiont strain specific terminal restriction fragments (TRF) the probionts could be distinguished within the microbial community associated with rotifers and appeared to be readily taken up by rotifers. However, in *Artemia* experiments, uptake of the probionts appeared to be less successful.

See **Book 2 - Chapter 10** for full study details.

10 ADDITION AND TRACKING OF PROBIONTS

Publication Title: Addition and tracking of probionts to yolk-sac and first-feeding Striped Trumpeter (*Latris lineata*) larvae

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Publication status: unpublished thesis

10.1 Abstract

The protective capacity and most effective delivery mode of putative probionts *Pseudoalteromonas agarivorans* ST18 and *Aliivibrio fischeri* ST7 was investigated in the rearing of yolk sac and first-feeding Striped Trumpeter (*Latris lineata*) larvae. In these experiments 4500 larvae were randomly stocked into 24, 300 l black hemispherical fiberglass tanks at 1 dph and held under static conditions for 5 days after which 300% daily water changes was applied. Terminal restriction fragment length polymorphism (TRFLP) was used to monitor the changes in bacterial community. The addition of strain ST18 to yolk sac larvae showed no significant reduction in survival (70 ± 6%) versus a control group (83 ± 5%) reared without potential probionts being added. The addition of strain ST7 with and without strain ST18 was found to be more disadvantageous (58 ± 7% and 55 ± 8% survival respectively). By tracking distinct 16S rRNA-derived TRFs, strain ST18 was specifically detected in treatments where it was added by both bioencapulsation and by direct addition. When strain ST18 was added directly to the water it resulted in decreased survival, due to the high bacterial load and possibly potential oxygen demand. The introduction of ST18 to the larvae bioencapsulated in rotifers resulted in the introduction of comparatively smaller numbers of bacteria that did not compromise the growth of the developing larvae.

See **Book 2 - Chapter 11** for full study details.

11 EFFECT OF ALGAL-INDUCED TURBIDITY AND TANK COLOUR

Publication Title: Feeding by Striped Trumpeter, *Latris lineata*, larvae, in response to changes in prey and larval density, algal-induced turbidity and tank colour

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Publication status: Aquaculture Research (in review)

11.1 Abstract

Maximising the contrast between potential prey items and the background against which they are viewed is particularly important for larvae that are reliant upon vision for feeding. This study investigated the effect of background environment on prey intake by Striped Trumpeter larvae that had either experience, or no experience, of feeding in a specific visual environment. Initial experiments investigated the effect of prey and larval density on rotifer prey intake and feeding rates over time. Rotifer intake increased linearly during the first 1.5 h post-feeding when fed at a rotifer density of 10 ml^{-1}. All subsequent experiments were therefore completed using prey densities ≤ 5 ml^{-1} and feeding periods of not greater than 2 h in order to ensure that prey consumption was not constrained by digestive tract capacity. Background colour significantly influenced prey intake with larvae consuming 5 times more rotifers in blue compared with black aquaria. However, larvae used in this experiment came from a blue culture tank and therefore had experience of feeding in a blue tank. A second experiment investigated the hypothesis that the change in tank colour resulted in the poor feeding in black tanks. Results showed that larvae fed best in the visual environment of which they had prior experience, and poorly in a new unfamiliar environment. The influence of changing visual environment was tested further using larvae with and without experience of feeding in an algal-induced green water environment. Larvae with experience of feeding in green water consumed significantly more rotifers in green water than larvae from a clear water environment. Larvae from a clear water environment fed equally well when shifted to either a clear or green water environment. However, larvae from a green water environment had significantly reduced feeding rates once shifted to a clear water environment. Striped Trumpeter larvae appear to develop a “search image” which is highly specific to the environment in which they are feeding. Quickly changing the contrast between prey and background, and the subsequent visual image available to the larva, resulted in reduced prey intake. The ability to strongly influence feeding rates via changes to the visual environment of larvae in culture highlights the importance of optimising factors that contribute to visual discrimination of potential prey items. In particular, larval rearing systems should be designed such that transfer of larvae between systems minimises any change to the visual environment.

See **Book 2 - Chapter 12** for full study details.

12 EFFECTS OF TURBULENCE AND TURBIDITY

Publication Title: Effects of turbulence and turbidity on growth, survival and rotifer intake of Striped Trumpeter, *Latris lineata*, larvae

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Publication status: Aquaculture (in review)

12.1 Abstract

Both turbulence and turbidity can influence predator-prey interactions and therefore have longer-term effects on growth and survival of larvae. Much research has concentrated on the effects of turbulence and turbidity on short-term processes such as foraging behaviour, or long-term effects on growth and survival. Few studies have investigated simultaneously short and long-term effects or the interaction of turbulence and turbidity on larval performance in culture. Thus, the effects of turbulence induced by aeration and turbidity induced by algal cell addition, on growth, survival and rotifer intake of Striped Trumpeter, *Latris lineata*, larvae were investigated. Experiments were conducted in replicate 300 l black hemispherical tanks with turbulence created via aeration from the centre bottom of each tank. Turbulence levels higher than 200 ml min^{-1} aeration resulted in significantly reduced growth, survival and rotifer intake of larvae from first feeding to 14 days-post hatching (dph), and no larvae survived under static conditions after 12 days. Prey intake increased in larvae 10 - 14 dph in comparison with larvae 6 and 8 dph and prey intake in older larvae 14 dph increased at the higher turbulence level of 800 ml min^{-1} in comparison to younger larvae. Furthermore, larvae 10 dph were more capable of coping with an increased aeration level of 400 ml min^{-1} than larvae receiving aeration at 400 ml min^{-1} from 6 dph. There was no interaction between turbulence and turbidity on growth, survival or prey intake. Larvae reared in turbid (green water) out performed those in clear water. Improvement in growth, survival and feed intake was evident for larvae reared in green water provided by either live algal cells, or concentrated algal paste of *Nannochloropsis oculata*. Green water reared larvae, despite being of the same size 10 dph as clear water reared larvae, were better equipped to cope with an increase in aeration from 200 to 400 ml min^{-1} than larvae reared in clear water. Combining the best level of aeration, 200 ml min^{-1}, with green water, resulted in the highest growth and survival of Striped Trumpeter achieved to date. It remains to be tested whether a turbulence level below 200 ml min^{-1} would provide for further improvement to growth and survival. Further investigation into increasing turbulence with age to gain maximal growth rates requires more attention primarily in regard to timing and strength of turbulent increases.

See **Book 2 - Chapter 13** for full study details.

13 WEANING STRATEGIES FOR POST-LARVAE

Publication Title: Weaning strategies for Striped Trumpeter (*Latris lineata*) post-larvae culture

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Publication status: Journal of the World Aquaculture Society (2010 in press)

13.1 Abstract

The Striped Trumpeter (*Latris lineata*) is a native fish being developed for aquaculture in Tasmania, Australia. Striped Trumpeter have a long post-larval stage and rearing has been reliant on the long-term use of *Artemia*. Two experiments were conducted to investigate weaning strategies. The first experiment used 52 days post hatch (dph) post-larvae previously reared on *Artemia* from 16 to 52 dph enriched with either low or high ascorbic acid. The treatments were *Artemia*, a microdiet or co-feeding. The post-larvae were reared until 67 dph. The *Artemia* treatment yielded significantly higher mean survival, followed by co-feeding and the microdiet. Feeding *Artemia* yielded significantly heavier post-larvae and the microdiet produced significantly smaller post-larvae. A second experiment used 41 dph post-larvae and investigated the effect of co-feeding duration prior to feeding with *Artemia* and microdiet as controls. Co-feeding periods of 5, 10 and 15 days were tested. Co-feeding for 5 days and microdiet feeding yielded significantly poorer survival compared to *Artemia*. *Artemia* feeding yielded significantly heavier post-larvae. In both experiments, the diets did not have a significant effect on jaw morphology. These experiments are the first to examine weaning strategies for Striped Trumpeter post-larvae and suggest co-feeding post-larvae from 40 dph and feeding microdiet exclusively at 50 dph.

See **Book 2 - Chapter 14** for full study details.

14 EFFECT OF TEMPERATURE ON POST-LARVAE

Publication Title: Effects of temperature regime on growth and development of post-larval Striped Trumpeter (*Latris lineata*)

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Publication status: Aquaculture (2010) 305:95-101

14.1 Abstract

The Striped Trumpeter (*Latris lineata*) is a promising new candidate for diversification of aquaculture in temperate regions of Australasia. Striped Trumpeter is also of scientific interest due to an unusually prolonged post-larval phase. The research was aimed at identifying the optimal temperature for rearing post-larval Striped Trumpeter approaching metamorphosis. Three-hundred-day-old post hatch post-larvae (12.1 ± 0.2 g, 114.0 ± 0.5 mm, mean ± SE) were reared at 12, 14, 16 and 18 ^{o}C, over 84 d. Survival, growth and metamorphosis into juveniles were recorded every 21 d. Fish were fed to apparent satiation and reared in oxygen saturated water (95.9 ± 2.6 %). At 14 ^{o}C, fish exhibited the best growth, had significantly higher lipid content and the majority (> 90 %) of the population metamorphosed into juveniles. The performance of fish reared at 16 ^{o}C was similar to those at 14 ^{o}C but the carcass had a significantly higher protein content and a significantly smaller proportion of the population (66.2 ± 3.0 %) metamorphosed into juveniles. Growth rate models predicted that growth was maximised between 12.9 ^{o}C (thermal growth coefficient) and 14.4 ^{o}C (specific growth rate). At 12 ^{o}C, fish showed the highest food conversion efficiency and all of the fish metamorphosed into juveniles. At 18 ^{o}C, fish showed the poorest growth, metamorphosis, and protein and energy retention. This is the first study on the effects of temperature on growth and development of Striped Trumpeter post-larvae. The results have important implications for aquaculture and fisheries management of Striped Trumpeter, in particular the rearing of post-larvae in hatcheries, timing of stocking into sea cages especially prior to metamorphosis, and for wild stock recruitment models.

See **Book 2 - Chapter 15** for full study details.

15 FEED RATION AND POST-LARVAE

Publication Title: The effects of ration and dietary lipid on growth of post-larval Striped Trumpeter (*Latris lineata*)

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Publication status: unpublished

15.1 Abstract

Striped Trumpeter (*Latris lineata*) is a new candidate species for adoption by the Tasmanian aquaculture industry. In preparation for future trials in sea cages, an experiment was conducted to investigate feeding strategies and the effects of dietary lipid inclusion level. Post-larval Striped Trumpeter (8.1 ± 0.1 g fish^{-1}) were reared using a combination of 33 %, 67 % or 100 % satiation rations and low (18 %) or high lipid (24 %) diets at a constant temperature of 15 ^{oC}. The diets were formulated to only vary nutritionally in the fish oil component. The 33 % and 67 % rations were determined by adjusting proportionally to the feed intake of fish fed to satiation (100 % ration) at the start of each week. Fish were reared for 63 d and at the end of the experiment three fish representing post-larvae (incomplete metamorphosis) and three fish representing juveniles (complete metamorphosis) were taken from each replicate tank (n = 4) and measured for whole body chemical composition. The 100 % (22.7 ± 1.0 g) and 67 % ration (21.1 ± 1.1 g) produced similar weight gain; the 33 % ration (15.4 ± 0.7 g) produced significantly smaller fish at the end of the experiment. Food conversion efficiency was highest at the 33 % ration (40.9 ± 3.2 %) compared to the 67 % (37.6 ± 3.0 %) and 100 % (30.1 ± 2.7 %) rations, which were similar. The 67 % (22.6 ± 0.0) and 100 % (22.7 ± 0.0) ration showed significantly higher proportions of fully metamorphosed individuals compared to the 33 % ration (8.6 ± 0.0). Dietary lipid did not have a significant effect on growth nor on food conversion efficiency. Data from starvation trials on similar sized fish reared at similar temperatures were incorporated to estimate optimum rations. The optimum ration for late stage post-larvae was found to be 4 % biomass $^{d-1}$. A dietary lipid content of 24 % of dry matter produced post-larvae with significantly higher carcass lipid content (5.8 ± 0.3 % of wet weight). Post-larvae were shown to have significantly higher carcass ash content and significantly lower carcass lipid content compared to juveniles. Metamorphosis into juveniles required fish to reach a minimum weight of 23 g and a carcass lipid content of at least 4 %. Metamorphosis of the majority of post-larvae (> 50 %) was predicted to occur at weights above 20 g and carcass lipid content of 7 %. All post-larvae were predicted to have metamorphosed by 40 g. This is the first experiment to investigate the effects of ration and different dietary lipid levels on post-larval Striped Trumpeter metamorphosis and growth. The results emphasise the need for proper feed management to increase growth and feed efficiencies to shorten the post-larval rearing period.

See **Book 2 - Chapter 16** for full study details.

16 JAW MALFORMATION LINKED TO WALLING

Publication Title: Jaw malformation in Striped Trumpeter *Latris lineata* larvae linked to walling behaviour and tank colour

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Publication status: Aquaculture (2009) 289:274-282

16.1 Abstract

Jaw malformations are a recurrent obstacle in the hatchery production of high quality juveniles of many marine finfish species. Whilst nutrition and temperature are often cited as the most likely causes, this study investigated manipulation of the physical culture environment and larval behaviour to reduce jaw malformations. The onset of jaw malformation after metamorphosis in Striped Trumpeter *Latris lineata* follows changes in larval behaviour from an even distribution throughout the water column to close association with the tank walls, often with vigorous swimming into the walls known as 'walling' behaviour. Larvae were reared through metamorphosis, 16 to 44 days post-hatching (dph), in twenty four 300 L hemispherical tanks with six different wall colours, black, blue, green, marble (a black, grey and white mottled pattern), red and white. Walling behaviour, jaw malformation and swim bladder hyperinflation were assessed. The highest proportion of severely malformed jaws at 44 dph occurred in red tanks, followed by green, white, blue, black and marble. More fish walled in coloured tanks (25-44%) than in black and marble tanks (9.6 and 3.4%, respectively). The proportion of fish with jaw malformations at 44 dph was positively correlated with fish walling behaviour. Both black and marble tanks had more than 50% of fish with normal jaws at 44 dph, and close to 80% with no or very minor malformations. Growth and survival to 44 dph were highest in the black (15.7 ± 1.3 mm fork length, 7.9 ± 0.9 mg dry weight, 71 ± 6%) and marble (15.6 ± 1.2 mm, 7.6 ± 0.5 mg, 58 ± 17%) tanks, compared with the lowest values in red tanks (14.2 ± 1.1 mm, 6.4 ± 0.4 mg, 11 ± 6%). Swim bladder hyperinflation, an apparent stress response, was greatest in red tanks and surface mortality was positively correlated with the proportion of fish with hyperinflated swim bladders. Potential mechanisms for the influence of walling behaviour on jaw malformation are mechanical damage and poor nutrition, via reduced feed intake and increased energy expenditure. The study highlights the often overlooked importance of hard-surface interactions in the growth and survival of cultured marine fish and demonstrates a cheap and effective technique for assessing tank background colour as a means of reducing malformations in cultured fish.

See **Book 2 - Chapter 17** for full study details.

17 JAW MALFORMATION INDUCED BY WHITE TANKS

Publication Title: Jaw malformation in Striped Trumpeter larvae is correlated with culture conditions, white tanks and clearwater, walling behaviour and live feed enrichment

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Publication status: unpublished.

17.1 Abstract

A high incidence of jaw malformation has hindered the production of quality Striped Trumpeter *Latris lineata* juveniles and has been correlated with walling behaviour in *Artemia*-fed larvae. In this study, Striped Trumpeter were reared from first-feeding in different coloured tanks (black or white), culture conditions (clear or greenwater) and fed different diets (enriched or non-enriched rotifers and *Artemia*), to examine the effects on behaviour and jaw malformation to 29 days post-hatching (dph). The highest incidence and severity of jaw malformations occurred in clearwater and enriched diet treatments, being significantly more common in white (70 ± 15%) than in black tanks (26 ± 15%). In black tanks, jaw malformation was significantly more common in larvae fed enriched diets (18 ± 14%) than in those fed non-enriched diets (8 ± 8%) and in clearwater (19 ± 14%) versus greenwater (7 ± 6%). At the end of the rotifer feeding phase, larvae were significantly larger in black (8.44 ± 0.42 mm, 0.79 ± 0.09 mg) than white tanks (7.51 ± 0.52 mm, 0.55 ± 0.06 mg), and longer in greenwater (8.74 ± 0.23 mm) than in clearwater (8.44 ± 0.42 mm). In the *Artemia* feeding phase, larval growth was slower and mortality was higher in non-enriched treatments compared with those fed enriched diet. Larval length and survival were higher in black (9.4 ± 0.9 mm, 26 ± 13%) than in white tanks (8.0 ± 1.0 mm, 10 ± 10%) and higher in enriched (9.3 ± 1.0 mm, 24 ± 16%) than in non-enriched (8.1 ± 1.0 mm, 12 ± 10%) diet treatments, whilst survival was higher in greenwater (46 ± 18%) versus clearwater (26 ± 13%). Swimbladder inflation was significantly higher in fish reared in black (50 ± 20%) than in white (25 ± 11%) tanks and in greenwater (95 ± 5%) versus clearwater (25 ± 11%). The average proportion of larvae walling over the duration of the experiment was higher in white than black tanks, and higher in enriched than non-enriched treatments, with lowest walling in greenwater. There was no consistent relationship between feed intake and jaw malformation. However, there was a significant positive correlation between walling and the incidence of jaw malformation at 29 dph in larvae fed enriched diets, but not in larvae fed non-enriched diets. The use of greenwater, black tanks and enriched live feeds are required for good growth, development and survival of Striped Trumpeter. The study emphasises the importance of reducing walling in the culture of oceanic larvae and may have direct application in the rearing of other marine fish with similar malformations such as yellowtail kingfish.

See **Book 2 - Chapter 18** for full study details.

18 CHARACTERISATION OF IMMUNOGLOBULIN

Publication Title: Purification and partial characterisation of Striped Trumpeter (*Latris lineata*) systemic immunoglobulin for the purpose of polyclonal anti-serum production

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Publication status: Aquaculture (2009) 287:11-17

18.1 Abstract

The Striped Trumpeter (*Latris lineata*) has been identified as a new species for diversification of the Tasmanian finfish culture industry. It is a deep water species with an unusually long, oceanic post-larval developmental stage and therefore, has not been easy to culture. Recent break throughs in the hatchery phase of culture have enabled the first sea cage grow-out trials. As the culture of Striped Trumpeter moves towards commercial-scale grow-out, knowledge of the immune system as it relates to disease resistance and vaccination is becoming more important. This study began at the basic level of immunoglobulin (Ig) characterisation and then moved onto the creation of anti-serum, which was used as an immunological tool to investigate the onset of the antibody response in the Striped Trumpeter. Similar to many other teleost species, Striped Trumpeter Ig is composed of a light chain of *Mr* 28 ± 3 kDa and a dominant heavy chain of *Mr* 86 ± 7 kDa. As seen in many other species of teleosts, these heavy and light chains form a tetrameric molecule weighing approximately 926 kDa. Purified Striped Trumpeter Ig was used to create polyclonal anti-serum directed against the light chain. The anti-serum was then used to investigate the ontogeny of the antibody response. Using Western blot analysis, Ig could not be detected until larvae were 225 days post-hatch (dph). This is later in terms of days post-hatch than other fish examined and could affect future husbandry and vaccination practices for this species.

See **Book 2 - Chapter 19** for full study details.

19 CLONING AND EXPRESSION ANALYSIS OF PRO-INFLAMMATORY CYTOKINES

Publication Title: Cloning and expression analysis of three Striped Trumpeter (*Latris lineata*) pro-inflammatory cytokines, TNF-α, IL-1β and IL-8 in response to infection by the ectoparasitic, *Chondracanthus goldsmidi*

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Publication status: Fish and Shellfish Immunology (2009) 26:773-786

19.1 Abstract

This study reports the cloning and sequencing of three Striped Trumpeter (*Latris lineata* Forster) pro-inflammatory cytokines, TNF-α, IL-1β and IL-8, as well as their differential expression in response to an infection by the ectoparasite *Chondracanthus goldsmidi*. The Striped Trumpeter TNF-α transcript consisted of 1093 bp, including a 759 bp ORF which translated into a 253 aa transmembrane peptide. The sequence contained a TACE cut site, that would produce a 167 aa soluble peptide containing the TNF ligand family signature. The IL-1β sequence consisted of 963 bp, including a 774 bp ORF which translated into a 258 aa protein. The protein lacked both a signal peptide and an ICE cleavage site, but did contain the IL-1 family signature. The sequence for the chemokine IL-8 contained 906 bp, with an ORF of 297 bp, which translated into a 99 aa protein. The protein lacked an ELR motif as is common with many teleost IL-8 sequences. The differential expression of the three cytokine genes in parasitised fish was investigated via quantitative real-time PCR. A significant up-regulation of all three pro-inflammatory cytokines was found in the gills, which were the site of parasite attachment. Examination of head kidney cells revealed a significant up-regulation of TNF-α, but not IL-1β or IL-8. Conversely, the spleen cells showed significant up-regulation of both IL-1β and IL-8, but not TNF-α. These findings allow for more detailed investigations of the Striped Trumpeter immune response.

See **Book 2 - Chapter 20** for full study details.

20 IDENTIFICATION AND CONTROL OF PARASITES

Publication Title: Identification and control of parasites in a new species for aquaculture: A case study with Striped Trumpeter.

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Publication Status: World Aquaculture (2009) 40(1):30-32

20.1 Abstract

This story takes place in Tasmania, but where exactly is Tasmania? It is Australia's southern most island state situated directly on the 'roaring forties', which has a very changeable weather pattern throughout the year with snowfalls not uncommon in summer. This cool to mild climate is ideal for temperate agriculture (stone- and deciduous fruit) and aquaculture. The Tasmanian aquaculture industry began in the mid 1940's with the cultivation of Pacific oysters. It has since expanded to include other shellfish, such as abalone, scallops and mussels. Today the largest Tasmanian aquaculture industry involves the culture of over 18,000 t of Atlantic salmon per year, which are farmed in sea cages. Since the beginning, the salmon industry has experienced various health challenges, including amoebic gill disease (AGD), which is caused by amoebic protozoans. Treatment of this disease using freshwater is relatively simple but expensive and progress is being made in the development of a vaccine. Atlantic salmon are also reared in sea temperatures close to their thermal maxima in late summer and it makes sense for the industry to diversify into other marine species. The industry is particularly interested in developing an alternative native white-fleshed fish species. Striped Trumpeter, *Latris lineata*, was identified in the 1980's as a possible aquaculture species because of its tolerance to handling, high stocking density as well as the superior flesh quality, which is high in omega fatty acids and highly regarded as sashimi.

See **Book 2 - Chapter 21** for full study details.

21 A NEW SPECIES OF COPEPOD *CALIGUS NUENONNAE*

Publication Title: A new species of copepod (Siphonostomatoida: Caligidae) parasitic on the Striped Trumpeter, *Latris lineata* (Forster), from Tasmania

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Publication status: Zootaxa (2009) 1971:59-68

21.1 Abstract

A new species of caligid copepod (Siphonostomatoida), *Caligus nuenonnae* n. sp., is described based on material collected from the body surface of Striped Trumpeter [*Latris lineata* (Forster)] reared at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories, Australia. *Caligus. nuenonnae* n. sp**.** is characterised by the following combination of features: 1) female genital complex with a mid-lateral indentation and highly concave posterior margin; 2) 1-segmented abdomen in the female that is about one-third the length of the genital complex; 3) distinctly broader first abdominal somite relative to the second abdominal somite in the male; 4) antenna with a spatulate process on the proximal segment; 5) recurved postantennal process without a basal accessory process; 6) female maxilliped with a proximal ridged protrusion on the corpus; 7) sternal furca with widely separated, apically truncate tines; 8) distal exopodal segment of leg 1 with a lateral flange on each apical spine and an accessory process on apical spines two and three; 9) leg 3 protopod with two adhesion pads on the dorsolateral surface; 10) leg 4 exopod 2-segmented, with I-0; I, III armature; 11) terminal exopodal segment of leg 4 with the outer apical spine being slightly shorter than the middle apical spine; 12) male maxillulary dentiform process with numerous small points embossed on the surface; and 13) male legs 5 and 6 represented by distinct lobate projections.

See **Book 2 - Chapter 22** for full study details.

22 DESCRIPTION OF *CHONDRACANTHUS GOLDSMIDI* N. SP

Publication Title: The first chondracanthid (Copepoda: cyclopoida) reported from cultured finfish, with a revised key to the species of *Chondracanthus*

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Publication status: Journal of Parasitology (2007) 93(4):788-795

22.1 Abstract

A new species of the Chondracanthidae (Copepoda: Cyclopoida), *Chondracanthus goldsmidi*, is described based on material collected from the naso-branchial region of Striped Trumpeter (*Latris lineata* [Forster]) cultured at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories, Australia. This represents the first report of a chondracanthid copepod infecting cultured finfish and the first metazoan parasite from cultured Striped Trumpeter. *Chondracanthus goldsmidi* n. sp. can be distinguished from its female congeners by the absence of lateral processes on the head and presence of 3 pairs of lateral trunk outgrowths, 3 mid-dorsal body outgrowths (of which the first 2 are rounded), a small and subcylindrical antennule, and unornamented legs 1 and 2. A revised key to the 39 valid species of *Chondracanthus* is provided.

See **Book 2 - Chapter 23** for full study details.

23 HOST RESPONSE TO GILL PARASITE

Publication Title: Host response to the chondracanthid copepod *Chondracanthus goldsmidi*, a gill parasite of the Striped Trumpeter, *Latris lineata* (Forster), in Tasmania

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Publication status: Journal of Fish Diseases (2010) 33(3):211-220

23.1 Abstract

The chondracanthid copepod, *Chondracanthus goldsmidi* is an ectoparasite of gills, inner opercula and nasal cavities of cultured Striped Trumpeter, *Latris lineata* (Forster). Whilst often present in high numbers (up to 60 parasites per host), little is known about its effect on Striped Trumpeter. In this study *C. goldsmidi* was associated with extensive epithelial hyperplasia and necrosis. Pathological changes were most pronounced near the parasite's attachment site, with papilloma-like growths surrounding the entire parasite resulting in deformation of the filament. The number of mucous cells increased near the parasite attachment sites on both the opercula and gills. Mast cells were absent in healthy gills; in contrast numerous mast cells were identified in the papilloma-like growths. Immunostaining identified piscidin-positive mast cells in the papilloma-like growths, presenting the first evidence of piscidin in the family Latridae.

See **Book 2 - Chapter 24** for full study details.

24 FARM TRIALS

Publication Title: Performance of hatchery reared Striped Trumpeter (*Latris lineata*) on-grown in sea cages

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Publication status: unpublished

24.1 Abstract

Four groups of high quality hatchery produced Striped Trumpeter juveniles were successfully transported and stocked into sea cages in the Huon River region between October 2006 and October 2007. A total of 2,483 fish were stocked from three hatchery cohorts ranging in mean size from 64 to 193 g. The fish were successfully on-grown for 427 to 793 days. The fish were fed a selection of formulated diets through automated Aquasmart feeders in three 20 m sea cages within separate predator exclusion nets. The trials provided the first hatchery reared Striped Trumpeter grown to market size in sea cages. Overall survival was 66, 93 and 94% for the three cohorts. Males from all three cohorts were precociously spermiating during spring 2008. Females remained sexually immature. The water salinity was often below full salinity during the winter. Sea temperature at the cage sites throughout the period at sea ranged from 9.4^{o}C to 18.9^{o}C and averaged 13.9^{o}C. Three copepod parasites *Chondracanthus goldsmidi, Caligus nuenonnae* and *Ceratothoa imbricata* were detected on farmed fish but none proved problematic and fish were not treated. The data collected were used for growth model predictions. The results suggest that southern Tasmanian conditions especially cage culture in a site dominated by freshwater inflow are not optimal for growth. The model predicted that Striped Trumpeter stocked at 100 g would attain a weight of 1.6 kg within three years at sea. The model suggests that the variation of growth with temperature was not significant. Future trials would be best carried out at higher temperatures in a marine dominated site. The average weight of harvested fish was 1430 ± 9g. HOG (head-on, gilled, gutted) yield was excellent at 94%, while fillets were 61% total recovery for a basic trim and 55% for a full trim. The product can be hot and cold-smoked, with hot-smoked product testing most favourably. Over 2 t of fresh farmed fish were sold and market feedback was highly positive. A basic economic analysis of Striped Trumpeter farming suggested it would take 14 years to reach 5000 t production at which point cumulative profit could eclipse cumulative spend.

See **Book 2 - Chapter 25** for full study details.

25 BENEFITS AND ADOPTION

25.1 Industry benefits

The benefits and beneficiaries of the research closely match those identified in the original application. The industry to benefit most from the planned outcomes is the Atlantic Salmon aquaculture industry in Tasmania because it is now closer to having a viable alternative cage culture species endemic to Tasmania. This is a very important Australian seafood industry estimated to produce 26,000 tonnes of Atlantic Salmon in 2007/08 at an estimated farm gate value of $270 million. Fish are farmed towards the upper thermal limit and growth rates are extremely fast, with production taking around 30 months (Battaglene et al. 2008). The importance of the salmonid industry diversifying into alternative species is further highlighted by concerns over climate change and one of the possible mitigation strategies will be to move into alternative species tolerant of higher water temperatures (Battaglene et al. 2008). Further impetus is given to finding a suitable alternate fish to farm by the need for the Tasmanian salmonid industry to move further offshore. This move is driven by most inshore and sheltered sites being close to their maximum carrying capacity. Striped Trumpeter are ideally suited to more oceanic conditions and do not require freshwater bathing for the control of Amoebic Gill Disease (AGD), the major health challenge for the salmon industry. However, a number of new challenges have been identified with Striped Trumpeter culture including the lack of a commercial marine fish hatchery, development of new markets, control of new diseases and possible integration problems with the culture of salmonids (Battaglene et al. 2008).

The emerging marine finfish and rock lobster culture industries throughout Australia also benefit from the research and technological advances. While most of the benefits have been derived by Tasmania, they have also flowed to New South Wales, Queensland, South Australia, Northern Territory and Western Australia through the transfer of technology and knowledge to the new marine fish species being developed in each state. In particular, the development of novel techniques for water treatment, enrichment of live feeds, ozone disinfection of eggs, and the use of coloured and marbled wall patterns has been of direct relevance to Barramundi, Mulloway, Kingfish, Snapper, Whiting and Grouper culture. The research on ozone disinfection has been extended to the Yellowtail Kingfish and prawn industries. The identification of malformations, their onset and solutions to reduce them have also been directly extended to the yellowtail kingfish industry.

Collaborative projects with international institutions have extended the benefits and adoption to New Zealand through joint studies of malformations of cultured Kingfish, and to Scotland (Atlantic Cod) and Spain (Senegal Sole) through joint research on lipid requirements.

The specific benefits of the project are as follows:

1. Provision of good quality hatchery produced Striped Trumpeter juveniles for assessment of performance in semi-commercial cage conditions.
2. A better understanding of the bacterial flora of larval culture tanks and methods to manage bacterial communities through ozonation and probionts.
3. Improved culture environment (turbidity, turbulence and tank colour) for the production of high quality Striped Trumpeter larvae.

4. A better understanding of weaning and the lipid, vitamin and ration requirements of Striped Trumpeter post-larvae.
5. Characterisation of parasites of Striped Trumpeter with potential to impact sea cage culture.
6. Description of the development of the immune system with a view to vaccine development.
7. Transfer of Striped Trumpeter hatchery technology to other finfish hatcheries in Australia, particularly the use of ozone and identification and reductions in jaw malformations.
8. Increased training of skilled hatchery technicians and graduate and post-graduate students.

One area where research was less successful than planned was in the identification and use of probionts, partially due to the late start of the PhD candidate and the refocussing of research efforts in other areas as per the working group recommendations prior to the commencement of the project.

25.2 Adoption and communication

The information from the project has been diligently and extensively communicated as outlined in the communication plan. In brief, the communication has been as follows:

1. Detailed and timely research progress reports as defined in contracts with AQUAFIN.
2. The production of a hatchery manual where the hatchery protocol information has been captured but not widely distributed due to Intellectual Property (IP) caveats.
3. Attendance at some 22 conference and workshops with over 28 presentations to industry and research groups (see Table 25.1).
4. Popular press releases, including TV, radio and print media and grey literature articles in Aquasplash and other trade journals (see Table 25.1 and references in Appendix 3).
5. Some 16 refereed scientific articles have been published in international peer reviewed journals (See Appendix 3).

Table 25.1

Date	Where	Participants	Affiliation	Outcome	Output
A. Conferences and workshops					
Jul 04	Launceston	Bransden Shaw	TAFI	Attendance at the IP and Commercialisation Workshop sponsored by the Aquafin CRC	IP register updated
Oct-04	Sydney	Battaglene Bransden Cobcroft Shaw	TAFI,	Attendance at the Australasian Aquaculture Conference, Sydney, 26-29 th September 2004	Co-chair session, 8 abstracts
Oct-04	Sydney	Battaglene Bransden Cobcroft Shaw	TAFI, +	Attendance Second Hatchery Feeds and Technology Workshop run by Dr Kolkovski (WA Fisheries) and Battaglene (TAFI), Rimmer (QDPI), comparison of methods	Proceedings, paper and Strategic Plan

May-05	Bali, Indonesia	Battaglene	TAFI	World Aquaculture Society Conference international collaboration	Chair session 1 abstract
Jul 05	Hobart	Battaglene Cobcroft Overweter Shaw	TAFI	Attendance Aquafin CRC Conference, national collaboration	Chair session 2 abstracts and presentations
Oct-05	Ghent Belgium	Battaglene Bransden Cobcroft Shaw	TAFI ARC	Attendance LARVI 05 conference International collaboration	Invited presentations, 4 abstracts, 2 posters, 3 papers
Oct-05	Ghent Belgium	Battaglene Cobcroft Shaw	TAFI ARC	Attendance Rotogen (Rotifer) workshop	Invited, type id for rotifers at TAFI
Feb-06	MRL Hobart	Battaglene Cobcroft Andrews Nowak Heyward Aiken	TAFI, CSIRO TASSAL	Attendance Fish Parasitology Workshop, greater knowledge of parasites	Collection and id of parasites
Aug-06	Adelaide	Battaglene Cobcroft Andrews	TAFI	Attendance at the Australasian Aquaculture Conference, Adelaide, 27-31 st August 2006	3 abstracts, 3 presentations, 2 posters
Aug-06	Adelaide	Cobcroft Battaglene	TAFI	Attendance at the Skretting Spectrum Marine Fish Hatchery Session, Adelaide, 30 th August 2006	1 abstract, 1 presentation
Nov-06	Canberra	Cobcroft	TAFI	Attendance FEAST forum, French contacts established	Aquasplash article
Feb-07	MRL Hobart	Battaglene Cobcroft Andrews Nowak Dykova Gardner Covello	TAFI, CSIRO	Attendance Fish Protozoan Parasitology Workshop, greater knowledge of parasites	Collection and id of parasites
Feb-07	MRL Hobart	Battaglene Andrews Covello Cameron	TAFI,	Attendance Epidemiology Workshop	Epidemiology literature
Apr-07	Wageningen Netherlands	Covello	TAFI	Attendance Fish immunology and vaccine workshop	Aquasplash article?
May-07	Barossa Valley, South Australia,	Andrews Choa De'Pannone	TAFI	4th Aquafin CRC conference, 15th to 17th May.	3 abstracts
Sep-07	Melbourne	Battaglene Nowak	TAFI +	Attendance at the FRDC Nodavirus Workshop	1 abstract, strategic plan
Sep-07	Grado Italy	Andrews Nowak	TAFI	13th EAFP International Conference on Diseases of Finfish and Shellfish	1 abstract
Sep 07	Viterbo Italy	Andrews Nowak	TAFI	7th International Symposium on Fish Parasites, 24th to 28th September.	1 abstract

Jun-07	Stirling Scotland	Covello	TAFI	7th International Symposium on Fish Immunology	1 abstract
Jul-2008	Adelaide	Andrews	TAFI	ASP & ARC/NHMRC Research Network for Parasitology Annual Conference	1 abstract
Sep-2008	Reykjavik, Iceland	Covello	TAFI	International Conference on Fish Diseases and Fish Immunology,	1 abstract
Aug-2008	Brisbane	Battaglene Cobcroft	TAFI	Attendance & session chairs at the Skretting Australasian Aquaculture Conference, 3-6 August.	Session chairs 1 abstract
Aug-2008	Brisbane	Battaglene Cobcroft	TAFI	Advances in marine fish hatchery technology in Australia	Workshop facilitators, 2 abstracts
Aug-2008	Cairns QLD	De'Pannone John Bowman	TAFI TIAR	12th International Symposium on Microbial Ecology – ISME- Microbial Diversity – Sustaining the Blue Planet	1 abstract
Jun-2009	Prague Czech Republic	Covello	TAFI	11th Congress of the International Society of Developmental and Comparative Immunology	1 abstract
Sep-2009	Prague Czech Republic	Covello	TAFI	Diseases of Fish and Shellfish, 14th European Association of Fish Pathologists Conference, 14-19, Prague, (F3).	1 abstract

B. Promotion

May 05	Hobart	Battaglene Bransden	TAFI	Presentation to stake holders at Aquaculture Review and RAG	Promotion
May 06	Hobart	Battaglene	TAFI	Presentation to stake holders at Aquaculture Review and RAG	Promotion
Aug-06	Hobart	Battaglene	TAFI	ABC interview for science week and to promote Striped Trumpeter project	Promotion
May 07	Hobart	Battaglene	TAFI	Presentation to stake holders at Aquaculture Review and RAG	Promotion
Apr 08	Hobart	Cobcroft	TAFI	Presentation on hatchery malformation research to stake holders at Aquaculture Review	Promotion
Apr 08	Hobart	Battaglene Buxton Montague	TAFI Aquafin CRC	Media promotion of project by Hon Minister Llewellyn, include TV and newspaper articles	Promotion
Jun 08	Hobart	Cobcroft	TAFI	Media promotion of project through Hook Line and Sinker TV program	Promotion

C. Visits and training

May 05	Launceston, TAS	Battaglene Cobcroft	TAFI, UTAS	Lectures on swim bladder and eye development in fish	Student recruitment
Nov 05	Cairns, QLD	Goldsmid	TAFI, QDPI	Collaborative talks and visits to institutions and aquaculture facilities holding broodstock	Trip report
Oct-05	Trondhiem, Norway	Battaglene Cobcroft Shaw	TAFI UST SINTEF	Understanding of Norwegian research and industry development	Farm visits, Presentations, research facility tours, Aquasplash article
Jun 06	Port Lincoln, South Aust	Andrews	TAFI SARDI	Field trip to identify parasites in SBT	Exchange of information
Feb-06	Hobart	Battaglene Shaw Overweter	TAFI, NDA	Ability to manipulate and build Databases	Internal report
May 06	Launceston, TAS	Cobcroft Shaw	TAFI, UTAS	Lectures on swim bladder and eye development in fish	Student recruitment
Aug-06	MRL,Hobart	Midtlyng	VESO Norway	Better understanding of Norwegian health research especially vaccine development	Presentation
Feb 07	MRL, Hobart	Buxton Battaglene Bender Morehead Evans Ford	TAFI, HAG, DPIW	Collaborative research project proposal plan	Draft project proposal
Jun 07	Port Lincoln Arno Bay	Battaglene Cobcroft	Clean Seas TAFI	Exchange of information on malformations in larval fish.	Draft SfCRC project proposal
Jun-07	Port Lincoln	Andrews	TBOA	Parasites collected off SBT	Training for student
May 07	Launceston, TAS	Battaglene Cobcroft	TAFI, UTAS	Lectures on swim bladder and eye development in fish	Student recruitment
Aug-Oct 2007	Norway Italy	Andrews	TAFI	Six week study tour, experimental design of parasite trials learnt, attendance at two conferences (see above)	Training for student
Apr-Jun 2007	Aberdeen Scotland	Covello	TAFI SFIRC	Ten week study visit at the Scottish Fish Immunology Research Centre	Training for student
Sep-Nov 2007	Port Lincoln Port Augusta South Australia	Battaglene Cobcroft Overweter Goldsmid	TAFI Clean Seas	Three trips of two staff each trip for one week to train Clean Seas staff in ozonation and identification of malformations in kingfish	See SfCRC project for outputs

D. Reviews and working groups

Jul-04	Hobart	Battaglene, Buxton, Hutchinson, Fielder, Jungalwalla	TAFI, SARDI, NSW Fisheries, TSGA	Working group discussions and planning for Striped Trumpeter trials in other institutions and for proposal review	New project endorsed

E. Major Collaborations

2005	Hobart	Bartels	Abtec	Grow-out of Striped Trumpeter in recirculation systems	Grow-out for 2 years
2005	Hobart	Bartels	Abtec	Collaboration on breeding of banded morwong	Larval rearing of banded morwong to metamorphosis
2006	Sydney		Ecotox	Supplied eggs and rotifers to undertake toxicity tests for Gunns Pulp Mill proposal	Demonstrated transport of eggs and larvae, external funds received
Jul-2006	MRL, Hobart	Chong	Universiti Sains Malaysia	International collaboration on gene expression through AMI Australia Malaysia Fellowship	Paper
Feb-Mar 2006	France	Cobcroft	TAFI, IFREMER	Isolated Striped Trumpeter genes and learnt new molecular techniques	Paper Trip report, Aquasplash article, ARC Linkage proposal
2007	Sydney		Ecotox	Supplied eggs and rotifers to undertake toxicity tests for water purification project	Demonstrated transport of eggs and larvae, external funds received
Mar-2007	Malaysia	Cobcroft	Universiti Sains Malaysia TAFI	International collaboration on gene expression	Paper
Sep- Nov 2007	Arno Bay Port Augusta	Battaglene Cobcroft	Clean Seas TAFI	Malformation study funded through the Seafood CRC	Reported through Seafood CRC

25.3 Educational benefits

An important component of a successful CRC project involves post-graduate training. The Striped Trumpeter project has been particularly successful in this area having supported five PhD students. A brief summary of the students, their project title and progress to date follows.

Dr Gavin Shaw became a formally adopted PhD candidate within the Aquafin CRC in 2002 and was supervised by Dr Tish Pankhurst and A/Prof Stephen Battaglene. His research thesis was entitled “Feeding behaviour of Greenback Flounder and Striped Trumpeter larvae in greenwater culture”. The early component of his research was conducted at the School of Aquaculture in Launceston on Greenback Flounder. He started research at MRL on Striped Trumpeter in March 2003. He completed his studies in March 2006 and has presented results at the Aquafin CRC Conference and 2nd Year CRC Review in 2003, at the Australasian Aquaculture Conference in 2004, and at Larvi 05 in Belgium in 2005 and has published one paper with another three submitted for publication. His research has provided the project with a better understanding of feeding behaviour in relation to prey density and highlighted the benefits of greenwater culture. He is currently employed by Skretting Australia Pty Ltd as a Technical Services Manager.

Mr Mark De' Pannone an Australian PhD student on an Aquafin CRC scholarship started in August 2005 and was supervised by A/Prof John Bowman and A/Prof Stephen Battaglene. His research thesis was entitled "The identification and development of probionts for use in marine fish hatcheries" is currently being evaluated. He has presented his results at the 4th Aquafin CRC conference, in May 2007 in the Barossa Valley, South Australia and the 12th International Symposium on Microbial Ecology in Cairns, Australia, in August 2008. His thesis is currently being reviewed.

Dr Bryan Choa an international PhD student from the Philippines was formally adopted into the Aquafin CRC in October 2005 and was supervised by Prof Chris Carter and A/Prof Stephen Battaglene. His research thesis was entitled "Culture of Striped Trumpeter (*Latris lineata*) post-larvae". He started research at MRL in April 2005. He has presented results at two Aquafin CRC conferences and had presentations accepted for the 13th International Symposium on Fish Nutrition and Feeding in Brazil in 2008. His research has provided the first weaning and feeding experiments with post-larvae and critical information on optimal temperatures, body composition and feeding ration. He completed his degree in May 2010.

Dr Melanie Andrews an international PhD student from South Africa was adopted into the Aquafin CRC in March 2006 and was supervised by A/Prof Barbara Nowak, A/Prof Stephen Battaglene and Dr Jennifer Cobcroft. Her research thesis was entitled "Arthropod parasites of cultured Striped Trumpeter *Latris lineata* (Forster 1801), and potential treatments". She has presented her results at two Aquafin CRC conferences, the Australasian Aquaculture Conference in 2006 and two international conferences in Italy in 2007 and published four scientific papers. Her research has described and identified two new copepod parasites and examined control and treatment methods. She completed her degree in January 2010.

Dr Jennifer Covello an international PhD student from Canada was adopted into the Aquafin CRC in February 2006 and was supervised by A/Prof Barbara Nowak, Dr Richard Morrison and A/Prof Stephen Battaglene. Her research thesis was entitled "Aspects of the Striped Trumpeter (*Latris lineata*) Immune System". She presented her results at three international conferences and spent ten weeks on an overseas study tour at the Scottish Fish Immunology Research Centre learning new techniques. She has published two scientific publications describing the development of the immune system in Striped Trumpeter. She completed her degree in March 2010.

Several students spent time with the Striped Trumpeter research team on short-term (2-3 weeks) work experience placements including: two from Friends (July 2006), one from Rosny College (Oct 2007), two from Hobart College, one from UTas Zoology Honours, 2 from TAFE (Tomoko Chida and Nan Kang, Nov 2004), Mark Jensen, Kwan Tzu N in Sept 2006 from UTas School of Aquaculture. The students provided general assistance in many areas of our work, including handling broodstock, larval rearing, bacteriology, larval sampling, histology to assess grey gut syndrome, larval quality tests, and water quality monitoring. They in return received valuable work place experience and training.

26 FURTHER DEVELOPMENT

The research undertaken through the Aquafin CRC was further developed in three projects.

1. A four year project developed with FRDC to conduct larger sea cage trials using cultured Striped Trumpeter entitled "Optimising size of fish and time of release of cultured Striped Trumpeter (*Latris lineata*) into sea cages in Tasmania" (FRDC:

2008-201 total cash funding of $1.674 million, $837,470 from FRDC and $560,00 from Huon Aquaculture Company (HAC) and $277,470 from Tasmanian DPIW).

2. A three year approved Australian Research Council (ARC) Linkage grant entitled "Reducing skeletal malformations in cultured marine fish using gene expression, improved nutrition and advanced system operation" to extend the expertise on larval rearing to other marine fish hatcheries and to reduce malformations in cultured juveniles. (ARC: LP0882042 total cash funding of $619,307, $450,000 from ARC and $169,307 from Tasmanian DPIW, HAC, Clean Seas, Skretting Australia and Nutrakol).
3. A one year scoping study funded by the Seafood CRC entitled "Yellowtail Kingfish juvenile quality: Identify timing and nature of jaw deformities in Yellowtail Kingfish and scope the likely causes of this condition". (Seafood CRC 2007-718 total cash funding of $97,157 with support from Clean Seas).

The successful development of hatchery protocols to produce healthy juvenile Striped Trumpeter and the results of preliminary sea cage trials at HAC generated considerable interest in Striped Trumpeter culture. A proposal for a new project was developed with the full support of the Aquafin CRC, TSGA (Tasmanian Salmon Growers Association) and Tasmanian Government. A Technology Transfer Business Plan for Striped Trumpeter commercialisation was developed and contained a background intellectual property register, IP management provisions to license the Centre IP to the University of Tasmania for the remainder of the Aquafin CRC Grant Period, with the assignment of IP to UTAS taking effect from 1st July 2008. These arrangements for transfer of rights to UTAS following the windup of the CRC in June 2008 provided the security necessary for industry to participate in a new project. The Huon Aquaculture Company (HAC) had an MOU with TAFI and collaborated on cage trials since December 2006 (see Chapter 25). HAC was endorsed by the CRC and TSGA as the best placed and most committed company to progress Striped Trumpeter aquaculture following a formal call for expressions of interest. The new FRDC project proposal was ranked a high priority by the Tasmanian FRAB and Tasmanian Aquaculture and Fisheries RAG (Research Advisory Group). The objectives of the new research project were to:

1. To establish if Striped Trumpeter post-larvae can be grown faster in tanks or sea cages.
2. To determine the optimal size and condition of Striped Trumpeter for stocking in commercial sea cages.
3. To evaluate the best season to stock Striped Trumpeter into sea cages to obtain maximum growth and survival.
4. To examine health risks and treatment options for parasitic, viral and bacterial diseases encountered during farming trials.

The project proposal to continue research on Striped Trumpeter was submitted to FRDC on the 1st November 2007. A positive response from the FRDC was provided in March 2008. It built on 20 years of research through the FRDC and two consecutive CRC's which has enabled excellent control of reproduction and the pilot-scale production of high quality Striped Trumpeter juveniles. This was a project that had been well backed with both research and industry resources and had been extraordinarily productive and innovative in outputs. If successful it would have allowed more efficient production of juveniles and established cost-

effective methods for transfer to sea cages, facilitating capacity building and collaboration among the key institutions developing new marine species and provide an excellent training opportunity for hatchery technicians and farmers. The cost of producing Striped Trumpeter juveniles is potentially higher than that of other farmed marine fish because they do not metamorphose till 50 g in weight. If a way could be found to stock post-larvae of 5 g into sea cages or to rear them more cost-effectively in land based systems it will greatly improve the economic potential of large-scale culture. The new project aimed to identify any major impediments to commercial farming and allow a fuller economic assessment of the potential farming benefits allowing informed decisions to be made on large-scale capital investments in marine fish hatcheries and grow-out facilities. The projected value to Tasmania of a potential Striped Trumpeter industry was estimated at $40 million per annum in 1994 (Searle and Zacharin 1994). A revised economic assessment was to have been undertaken which would have taken into account higher fish consumption, hatchery production and grow-out information. Unfortunately, after gaining FRDC approval the industry partner withdrew from the project during the world economic difficulties in February 2009. The Striped Trumpeter project is currently under review by the Tasmanian Government and likely to be scaled back or terminated by the end of 2010.

The ARC and Seafood CRC projects have supported the transformation of existing marine fish aquaculture industries, promoted the sustainable use of Australian species in aquaculture, and responded to climate change by enabling the salmon industry that is challenged by global warming to have the ability to diversify to alternative temperate species. Despite the growing understanding of factors that contribute to malformation, they remain a chronic issue for the international finfish hatchery industry. Without the production of healthy seedstock, the development of Australian sea cage culture, intensive shore-based recirculation grow-out systems and expanding saline water culture are threatened. The costs of hatchery production are proportionally very high in fish farming and currently limiting expansion. Malformations add greatly to the cost of farming by increasing grow-out time, reducing survival, adding infrastructure and labour costs, reducing marketability and fish quality. Variability among batches of fish is common, requiring complete culling or expensive grading. Accurate estimates are difficult to obtain but >40% malformed fish have been suggested for some Australian hatcheries and even in the more established European industry ~17% of all juveniles are malformed (Sweetman 2004). Improved hatchery production will increase the capacity of the industry to work on more difficult, but potentially economically rewarding tropical, deep sea and oceanic species. The projects' aim to break down the barriers between hatcheries while preserving confidentiality, and document the occurrence and describe skeletal malformations in larvae reared in hatcheries using different water sources, rearing protocols and personnel. Importantly, the research had agreed industry commitment, which allowed the information accessed to underpin the research program. Protection of IP will be carefully managed through patents on products/systems and release of information only with the full consent of all parties. Community perceptions of the aquaculture industry are adversely affected by the production and sale of malformed fish. The information from the projects will be a positive marketing tool for those industries where malformations are reduced, and a starting point for improvement of larval quality and monitoring for others. The studies will allow the Australian industry to move closer to the standardised assessment of larval quality, as a routine element of quality control, as is currently practised by the Mediterranean aquaculture industry (Sweetman 2004).

27 PLANNED OUTCOMES

The three main planned outcomes from the project were in large measure achieved:

1. Australian aquaculture has been provided with more systematic ways to control microbial communities, and to evaluate, identify and produce probionts for use in improving hatchery survival rates in finfish. Microbial control has been achieved in culture through the innovative use of ozonated seawater in both live feeds and larval rearing.

2. There will ultimately 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. Research and/or production hatcheries in SA, WA and NZ have requested information and advice from the Striped Trumpeter project team. Greater communication among hatcheries has been achieved through development of a successful ARC Linkage project, research with yellowtail kingfish through the Seafood CRC, and a key role in the planning and running of the third marine fish hatchery workshop held in Brisbane in August 2008.

3. The Atlantic salmon industry has moved significantly closer to diversification into new species, particularly Striped Trumpeter through the first sea cage trials using cultured fish. In time this should improve their profitability while reducing their risks, hence ensuring their long-term sustainability.

28 CONCLUSION

The project was reviewed by a Striped Trumpeter Working Group in 2004 and modified to reflect a more holistic approach with less emphasis on probiotics and more on the use of ozonation, disease control and improved weaning. A Charter for the project and all the necessary approvals were obtained on the 9th of October 2004.

1. *Investigate the control of microbial communities in intensive larval fish culture using ozonation and probiotics.*

A large-scale replicated experiment investigated the effects of two types of greenwater (fresh or instant algae), as well as clearwater on growth, survival and bacterial proliferation during rotifer feeding. Fresh algae produced the largest larvae and survival was not significantly different to clearwater. Instant algae produced fewer fish and without ozonation is not a good algae substitute for fresh algae in greenwater rearing. Bacterial loads were highest in instant algae. Libraries of bacteria were developed and assessed for potential probionts along with a number of other candidates from the Fish Health Unit collection. Research was refocused into better understanding the microbial dynamics of culture environments and the development of probiotics progressed through the development of clone libraries for bacterial 16S genes. A new method that allows greater data mining of bacterial community fingerprint patterns called automated ribosomal intergenic spacer analysis (ARISA) was adopted. Another technique Terminal Restriction Fragment-length Polymorphism (T-RFLP), similar to ARISA was also used to distinguish closely related bacterial species. Clone libraries and T-RFLP of the six key water treatments were successfully completed and 16s similarity trees compiled. Two isolates ST18 and ST7 inhibited *Vibrio* sp in plate tests and were further evaluated for probiotic potential in live feeds (rotifers and *Artemia*). Pilot testing of ST18 and ST7 in isolation and combination showed no detrimental effects on live

feeds. T-RFLP was used to trace the probionts in live feeds and RT-PCR was used to quantify the probiont populations that the rotifers and *Artemia* had taken up.

2. *Determine the optimal environmental parameters, and water quality systems and tank design for reducing hatchery mortality and malformations in finfish larvae.*

The proportion of Striped Trumpeter post-larvae with jaw malformations improved with consecutive cohorts of fish produced in 2005. In 2006 we achieved the highest level of normal and minor malformations to Day 100 (76%) of any production batch to date, with consistently high levels at Day 200. This was repeated with another batch in 2007 that had a similar result to Day 100 (75%). These results are promising, particularly in light of an overall increased survival, such that many more normally formed fish were produced. Three large-scale replicated experiments were conducted to improve survival and reduce malformations. They also provided large numbers of fish samples for jaw structure assessment. The first experiment completed in 2005 investigated the effect of vitamin C addition and *Artemia* density on larval morphology and jaw development during metamorphosis. The addition of Vitamin C to the *Artemia* enrichment diet reduced the severity and incidence of malformation in older post-larvae after weaning. Tissue concentrations of Vitamin C in larvae were achieved up to 1600 μg AsA g^{-1} DW. Optimal feeding rates during the *Artemia* phase were determined and this information has reduced the cost and labour associated with production trials. The second experiment completed in June 2006 tested the effects of background tank colour on Striped Trumpeter larvae and post-larvae during the *Artemia* feeding period. The experiment demonstrated that tank wall colour had a significant effect on growth, survival, distribution and walling behaviour. Fish reared in black and marble tanks exhibited a lower proportion of walling behaviour, they grew longer, had higher survival and had a lower incidence of malformations than fish in other tank colours. The incidence and severity of malformation remained relatively constant from Day 30 in the black and marble tanks with ~80% of fish with no or minor malformations (scores 0 + 0.5), but became progressively worse in tanks with high levels of walling behaviour, especially red, green and white tanks. The absence of other significant skeletal malformations, besides that of the jaw elements, is strong evidence that physical contact with the walls is the primary cause of malformations in Striped Trumpeter, although compromised nutritional status and infections appear to exacerbate the problem. The third experiment examined the effect of culture conditions (tank wall colour and greenwater) and live feed enrichment on growth, survival, behaviour, and jaw malformation of Striped Trumpeter larvae.

3. *To better understand "grey gut syndrome" and the ontogeny of the immune system, including linkages to developments with probionts and immunostimulants.*

This objective relates to the health of larvae and post-larvae. No experiments were conducted specifically on “grey gut syndrome”. The syndrome is associated with bacterial infection or accumulated lipid in enterocysts. It was recorded for all larvae samples taken during large-scale experiments and production trials and commonly occurred in fish with high intake of enriched live feeds, typical of the lipid accumulation type and not directly linked to mortality events. Health research investigated the metazoan parasites of Striped Trumpeter post-larvae and juveniles. A range of previously undescribed parasites are known to cause problems in rearing juvenile Striped Trumpeter and will need to be controlled during farming operations. The research first described and identified the main parasites and then investigated control and treatment methods. Two copepod parasite species have been found commonly on cultured Striped Trumpeter (*Chondracanthus goldsmidi* n. sp. and *Caligus nuenonnae* n.

sp.). Both species have been carefully described and their taxonomy established with the assistance of recognised experts in the field. Bathing treatments for controlling *C. goldsmidi* have been investigated. The use of freshwater and hydrogen peroxide bathing is effective at reducing but not eliminating *C. goldsmidi*. The tolerance of Striped Trumpeter to freshwater is short at around 30 minutes. In-feed treatments with emamectin benzoate (25 to 100 mg kg^{-1}) are currently being trialled in infected juvenile fish.

The specific immune response of the Striped Trumpeter was investigated with particular focus on the development of the antibody response and vaccination against bacterial disease. The research was divided into three main areas: characterization of the antibody molecule (Ig), ontogeny and vaccination. Similar to many other teleost species, Striped Trumpeter Ig is composed of a light chain and a dominant heavy chain. The heavy and light chains form a tetrameric molecule weighing approximately 926 kDa. Purified Striped Trumpeter Ig was used to create polyclonal anti-serum directed against the light chain. The anti-serum was then used to investigate the ontogeny of the antibody response. Using Western blot analysis, Ig could not be detected until larvae were 225 days post-hatch (dph). This preliminary investigation has shown that the ontogeny of the antibody response in larval Striped Trumpeter may occur later than in other marine teleosts. It is hypothesised that the unusually long larval stage of this species may play a part in the delayed onset, as the production of antibodies may be linked to developmental changes as post-larvae become striped juveniles. Further work developed sensitive RT-PCR primers and *in situ* hybridisation probes targeted to recombination activating gene (RAG) and Ig molecules. These studies further examined the timing of a functional antibody response in the Striped Trumpeter.

4. Evaluate formulated diets and their use in early weaning

Two experiments in 2005 demonstrated that post-larvae can be weaned around Day 40. Experiments in 2006 demonstrated that weaning can be achieved as early as Day 30 using new improved diets from Europe. The weaning of post-larvae in production trials is now routinely done at 40 days of age. However, the research into larger post-larvae was constrained by the design of the larval rearing tanks in the fish hatchery. A new experimental room was designed and established specifically to conduct research on post-larvae, complete with a 32 tank replicated system with temperature control and computer operated automated feeders and lights. During 2006 a growth experiment was conducted on post-larvae using a range of temperatures (12 to 18°C). The optimal temperature for culture was found to be from 14 to 16°C. Once fish metamorphosed into juveniles temperature effects on growth rates were less apparent. This suggests that to achieve optimal growth, juveniles should be stocked in sea cages after they have undergone final metamorphosis. Research on feed ration found no significant difference between a 67% and 100% satiation ration on growth performance of post larvae, indicating that the optimum feeding regime is to slightly underfeed. Lipid content (9 and 14% fish oil) had no effect on growth and analysis of flesh quality is ongoing. Experimental research results have been incorporated into production trials and fish are fed Gemma Micro from Day 30 to Day 50 and weaned off *Artemia* by Day 50.

5. Evaluate the growth and survival of Striped Trumpeter post-larvae and juveniles reared under semi-commercial conditions

Production trials were run concurrently with large-scale experiments. In 2004 a production run produced 3,400 fish to Day 75 a survival rate of 11% with fish fully weaned off live feeds by Day 50. All of the advances made in 2005 culminated in the most successful production run ever, resulting in 23.4% survival to Day 100 well above the Day 50 target of >20%. Total production of Day 100 post-larvae in 2005 was 5,415, up from the 2,157 produced in 2004. In 2006 we produced 8,149 post-larvae to Day 100 in two trials. A trial in a 25,000 L tank started in 2006 produced ~3,000 fish to Day 275 in June 2007. In 2007, one production trial produced > 5,000 fish to Day 50 and ~12,000 post-larvae were produced to Day 100. Growth of post-larvae was monitored in all trials and the benchmark for Day 80 post-larvae is 30 mm FL and at Day 100 it is 43 mm TL. The production of high quality juveniles generated increased interest from industry and the first transfer of a trial shipment of hatchery-produced post-larvae was undertaken in December 2006, followed by another three shipments in 2007. Growth and survival of fish in sea cages were good. Fish were harvested after two years and over 2 t of fresh product sold with good consumer feedback.

6. *Evaluate the possibility for the culture of Striped Trumpeter using alternative systems and/or sites*

Considerable progress was made in obtaining the necessary approvals to translocate eggs to NSW DPI and SARDI. Appropriate risk assessments have been undertaken. The working group recommended that this work be continued to the point where eggs were transferred to determine whether they survive transport and hatch. Several shipments of eggs and larvae have successfully taken place to Ecotox Pty Ltd in Sydney in 2006 and 2007. However, the working group reached the conclusion that any attempt to culture Striped Trumpeter at alternative hatchery sites, particularly on the mainland, had a high risk of failure and was not pursued further.

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References for research papers are provided at the end of Chapters 7 to 24 in Book 2. All other references in the report are provided here or in Appendix 3.

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APPENDIX 1: INTELLECTUAL PROPERTY

The Intellectual Property from the project 2004-221, 1B.4(b) is documented in the AQUAFIN CRC Project Intellectual Property Register. It contains sections on Centre, Background and Third party IP, confidentiality agreements with consultants, staff and students. An agreement for the transfer of all intellectual property and data from the project 2004-221, 1B.4(b) was made by all AQUAFIN CRC participants to the University of Tasmania taking effect from 1st July 2008 to allow development of new projects.

APPENDIX 2: STAFF LIST

Staff engaged in the project were:

Name	**Position**	**Qualifications**	**Project funded/ In-kind**	**Time**
Dr Stephen Battaglene (TAFI)	Associate Professor, Aquaculture Section Leader, Principal Investigator	PhD, 20 years experience rearing marine fish larvae	In-kind	50
Mr Alan Beech (TAFI)	Senior Technical Officer (Aquaculture facilities)	Diploma of Aquaculture, over 13 years aquaculture research	In-kind	50
Dr John Bowman (AgSc)	Associate Professor Agricultural Sciences	PhD, 15 years bacterial research	Project	5
Dr Matthew Bransden (TAFI) Till Aug 05	Postdoctoral Scientist	PhD, 10 years aquaculture research, salmonid & larval fish nutrition	Project	100
Dr Jeremy Carson (TAFI)	Senior Scientist	PhD, > 20 years microbiology experience	Project In-kind	12.5 12.5
Dr Chris Carter (TAFI)	Professor, Head of Aquaculture School	PhD, fish nutritionist	In-kind	5
Dr Jennifer Cobcroft (TAFI)	Hatchery Manager / Postdoctoral Scientist	PhD, 12 years aquaculture research, broodstock and larval rearing	Project	100
Ms Debrah Gardner (TAFI) 2007 only	Hatchery Manager	B.Sc., M.Sc., 13 years aquaculture experience	Project	100
Mr Ross Goldsmid (TAFI)	Technical Officer (Broodstock)	Aquaculture Diploma, 12 years maintaining broodstock and larval rearing	Project In-kind	50 50

Ms Anna Overweter (TAFI)	Technical Officer (Live feeds and hatchery)	B.Sc.App.Sci. (Hons) 7 years aquaculture experience	Project In-kind	50 50
Dr Gavin Shaw (TAFI) 2006 only	Hatchery Manager	PhD, 5 years aquaculture experience	Project	100
Technical Officers (TAFI)	Technical Officers, Weekend staff	Associate Diploma or greater	Project	20
Ms Young (FHU)	Technical Officer	B.Sc. Degree	Project	50
Mr Bill Wilkinson (TAFI)	Senior Technical Officer (Algae)	M.Sc. Aquaculture, over 13 years culturing algae	In-kind	50

APPENDIX 3: PROJECT PUBLICATIONS

Publications from research started during the previous Aquaculture CRC and completed during the Aquafin CRC are marked by **
Student publications not directly funded by the CRC are marked by ***

Scientific papers published or accepted for publication

Andrews, M., Bott, N., Battaglene, S., Nowak, B. 2009. A new species of copepod (Siphonostomatoida: Caligidae) parasitic on the Striped Trumpeter *Latris lineata* (Forster, 1801) from Tasmania. Zootaxa. 1971:59-68.

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ENHANCED HATCHERY PRODUCTION OF STRIPED TRUMPETER, *Latris lineata*, IN TASMANIA THROUGH SYSTEM DESIGN, MICROBIAL CONTROL AND EARLY WEANING

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7 ADVANCES IN THE CULTURE OF STRIPED TRUMPETER LARVAE: A REVIEW

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7.1 Abstract

Striped trumpeter, *Latris lineata*, was chosen as the best new candidate for sea cage culture in Tasmania in the late 1980s. It has a complex and extended post-larval or 'paperfish' stage lasting up to 9 months and has historically proven difficult to culture. Excellent progress has been made in understanding and controlling reproduction and broodstock are spawned year-round through photothermal control. Problems with early larval rearing have been overcome and egg incubation and early larval rearing protocols have been established. A mortality peak associated with first-feeding has been reduced using better live feed production techniques and improved water quality. Using antibiotics showed that high bacterial loads were an important factor in larval mortality. A new water filtration and ozonation system has removed the need for antibiotics. Larval nutrition research focused on the link between potential deficiencies or imbalances in the three essential PUFA in live feeds: docosahexaenoic acid, eicosapentaenoic acid and arachidonic acid. Novel experimental emulsions were applied with dose response experimental designs to identify the dietary requirement for selected PUFA and vitamins. Despite the advances in live feed enrichments, the live feeds, particularly *Artemia*, were found to have sub-optimal lipid profiles. Copepods were cultured, as a supplement to traditional live feeds, and improved larval rearing success. Costs to scale up production and to control extensive cultures presently restrict the usefulness of copepods. Important breakthroughs have occurred in health with the detection and control of nodavirus, myxozoan and bacterial disease. Ozone disinfection of eggs and sterilisation of hatchery seawater have been important control measures. Another bottleneck to production has been mortality of larvae from notochord flexion to metamorphosis. System changes to reduce nocturnal movements and a better understanding of optimal live feed densities, and weaning onto formulated diets, have improved survival and growth. High rates of jaw malformation remain a challenge and no definitive cause has been established. Reduced rates of malformations have been associated with one or a combination of high feed rates, lower larval densities and temperatures, and reductions in 'walling' behaviour. Future research is aimed at finding ways to reduce malformations, develop probiotics and early weaning strategies, control parasites and scale-up production to assess performance of juveniles in sea cages.

Keywords: *Latris lineata*, marine fish larvae, development, PUFA, jaw malformations, wall-nosing, bacteria, ozone, betanodavirus, myxozoan

7.2 Introduction

A major bottleneck in the development of many new marine fish species for aquaculture in Australia has been the production of high quality juveniles (Battaglene and Fielder, 1997). There are currently only two species cultivated from eggs and reared in sea-cages in large numbers in Australia. The largest production is for Atlantic salmon at 14,828 m t per annum in Tasmania with much smaller but growing production of barramundi at 1,567 m t in Queensland, Northern Territory, South Australia, and New South Wales (ABARE, 2004). In line with world trends there has been some diversification into new species, including yellowtail kingfish and mulloway in South Australia and snapper in NSW. There is currently no aquaculture species suited to colder water to help diversify the Atlantic salmon industry, whose rapid growth has recently slowed and is under threat from global competition, rising seawater temperatures and the high cost of managing diseases (Battaglene and Cobcroft, 2003).

Striped trumpeter, *Latris lineata* (Bloch and Schneider, 1801), was chosen as the best candidate for diversifying salmonid sea cage culture in Tasmania in the late 1980's following a review of aquaculture research in Tasmania (Searle and Zacharin, 1994). The species is widely distributed in the temperate latitudes of southern Australia, around Tasmania and New Zealand, and isolated island groups in the Indian and Atlantic Oceans (Last et al., 1983; Tracey and Lyle, 2005). Once plentiful in Tasmania, the fishery for striped trumpeter has declined to typically less than 100 m t per annum. The limited information on wild fish indicates that they are long-lived (>45 years), rapidly growing in the first five years of their life, and are opportunistic carnivores found over rocky reefs at depths from 5 to 300 m (Tracey and Lyle, 2005). Described as the "most excellent of all Tasmanian fishes" in 1882 they have long been prized as one of the best eating fishes in Australia and have firm white flesh, which is both tasty and fatty (Morehead, 1997; Nichols et al., 2005). This contention is backed by recent studies into the flesh qualities of 'farmed striped trumpeter' (wild-caught juveniles reared in captivity to adulthood), which ranked them alongside Australia's best white-fleshed fish in controlled taste tests (Pakes Research, 2000). They have also been recorded as having the highest omega-3 polyunsaturated fatty acids (PUFA) concentrations in the flesh of any fish in Australia (Nichols et al., 2005). In addition, striped trumpeter were selected for their docile nature, lack of cannibalism, ability to take formulated feeds and tolerance to being held in captivity at high densities.

Research on striped trumpeter culture started in the late 1980s with a production approach based on the successful development of Atlantic salmon farming in Tasmania. At the same time other Australian states were developing new species like barramundi, snapper, and mulloway (Battaglene and Talbot, 1992,1994; Rimmer et al., 1994). It quickly became apparent that striped trumpeter, a deeper water more oceanic species, were not easy to culture and had a complex life cycle with an extended larval phase, including a 9-month neustonic 'paperfish' stage (Furlani and Ruwald, 1999). Early research examined morphological development of striped trumpeter larvae including the use of histology and histochemical techniques (Ruwald et al., 1991; Goodsell et al., 1996). Rearing of striped trumpeter larvae was characterised by low and highly variable survival from first feeding through to metamorphosis and a high incidence of jaw and spinal malformations in post-larvae (Ruwald et al., 1991; Pankhurst and Hilder, 1998; Trotter et al., 2001; Cobcroft et al., 2001a). These problems compromised the early research and proved difficult to solve, shaping the research agenda for the next ten years.

Despite a paucity of information on their biology in the wild, striped trumpeter are one of the most highly studied of the new aquaculture candidate species in Australia with over 30 scientific publications over the last ten years and five doctoral theses (Morehead, 1997; Cobcroft, 2002; Trotter, 2003; Grossel, 2005; Shaw, 2006). Research has been based at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories, Hobart and has been focused in four key areas starting with the control of reproduction, then early larval development and more recently larval nutrition and health. In this paper we synthesise the outcomes within the four key areas with some final comments on the direction for future research.

7.3 Control of reproduction

Control of reproduction in striped trumpeter is among the best for any species being developed for aquaculture in Australia (Morehead, 1997). Striped trumpeter are multiple spawners with group synchronous oocyte development, producing eggs on a four-day cycle over 3 months (Hutchinson, 1994; Morehead et al., 1998). Mature fish caught during the spawning season, that typically extends from August to November, were implanted with LHRHa pellets and provided hormone-induced ovulations for early larval rearing trials (Morehead, 1997). Striped trumpeter are easily acclimated and held in captivity in 25,000 l tanks under artificial light. Broodstock of 3 to 6 kg have been held since 1997 under controlled photoperiod and temperature and entrained to spawn out of season (Morehead et al., 2000). Spawning usually occurs within 6 months of capture at ambient temperatures of 12 to 16^{o}C. Compression of the spawning cycle can be achieved within six months and three different broodstock groups are now maintained to provide a steady supply of eggs at regular intervals during the year. The stress and ovarian response of captive striped trumpeter is unlike that of most other marine species under investigation in Australia, such as snapper or barramundi (Morehead, 1997; Pankhurst, 1997; Cleary et al., 2000). Striped trumpeter can be hand-stripped daily for several weeks without inducing ovarian atresia. Methods have also been developed to cryopreserve sperm and transport eggs and larvae (Ritar, 1999; Ritar and Campet, 2000; Jones, 2003). Natural spawning and total egg production has increased in recent years with better husbandry practises and changes in sex ratios to 1 female to 2 males, partially because the length of the spawning season increases the longer fish are held in captivity. Egg production from ambient held stock has increased to around 2 million eggs per female in 2003 (Figure 7.1).

The life cycle for striped trumpeter has been partially closed and F1 generation broodstock spawn viable gametes in their fourth year (Morehead et al., 1999). To date there has been no genetic selection. Broodstock have traditionally been fed pelleted, formulated feeds and fresh fish with a change in 2004 to a composite moist diet with vitamin supplementation. Assessment of the lipid content of eggs from wild and captive fish identified no differences in total lipid, although individual PUFA content reflected the diet (Morehead et al., 2001). The amino acid composition of striped trumpeter eggs is similar to that of other pelagic marine fish eggs, having high concentrations of free amino acids and being rich in leucine, lysine, glutamine, alanine and valine (Brown et al., 2005a). There has been no evidence that eggs from different groups or times during the spawning season produce superior larvae. Individual females and males show annual spawning traits including, timing of spawning (early or late), fecundity, egg fertilisation and larval hatch histories (Goldsmid, personal communication, 2005). Fertilisation and hatch rates in rearing trials are now typically >90%.

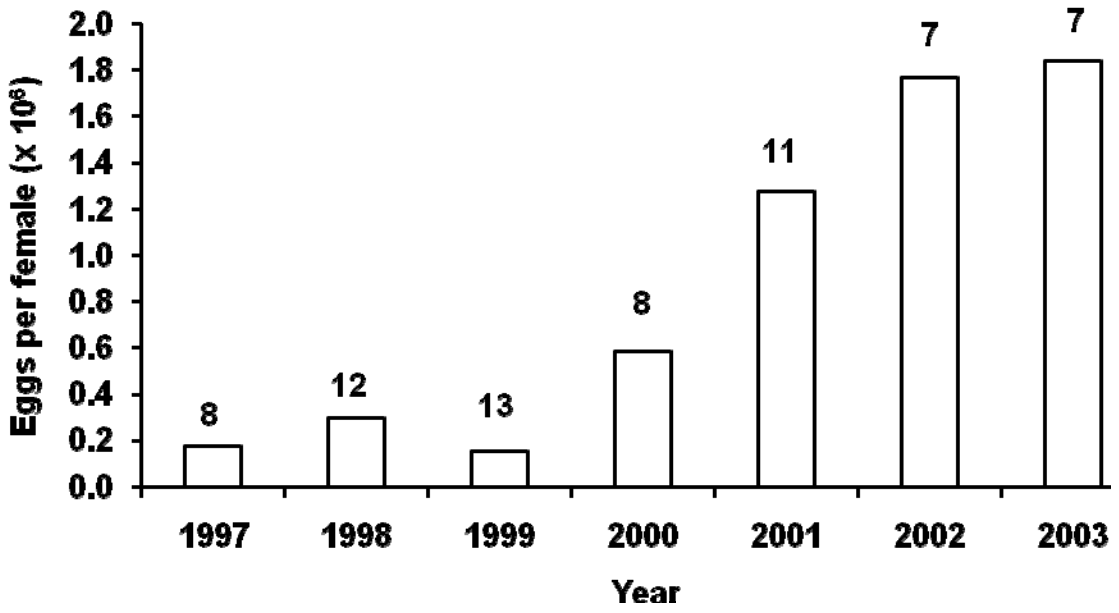

Figure 7.1 Mean seasonal egg production per female striped trumpeter held under ambient conditions. Numbers are females spawning per season in populations of 16 females and 8 males until 2001 and out of 8 females and 16 males from 2002 onwards.

Striped trumpeter have pelagic eggs with a single oil droplet (Figure 7.2, Table 7.1). Time to hatch decreases from 9.6 to 4.2 days as temperature increases from 10 to 16 ^{0}C (Morehead and Hart, 2003a). Prior to 1999 eggs were incubated at 12 ^{0}C (Bermudes and Ritar, 1999). However, 14 ^{0}C is now considered optimal (Morehead and Hart, 2003a). Changes in optimal egg incubation temperatures may be due to changes in the thermal history of broodstock groups. Eggs are incubated in 250 l conical tanks on recirculating and flow-through seawater. Disinfection of eggs, originally using glutaraldehyde, has now become routine using 1 mg l^{-1} ozone for 1 min soon after fertilisation (Morehead and Hart, 2003b; Battaglene and Morehead, in press).

7.4 Early larval rearing

During the late 1990s there was a concerted effort to improve early larval survival using a more experimental approach similar to that adopted by IFREMER in the development of sea bass and sea bream rearing protocols. The first comprehensive studies were conducted on early morphological development, particularly of the eye and swim bladder, including assessment of visual acuity and the effects of light and temperature, primarily in clear water intensive rearing systems (Cobcroft, 2002; Cobcroft and Pankhurst, 2003; Trotter, 2003; Trotter et al., 2004). Subsequent studies focused on the effects of other abiotic factors including turbidity and turbulence, and biotic factors including the effect of prey and larval density, on the feeding behaviour, growth, development and survival (Battaglene and Brown, 2006; Battaglene et al., 2006; Shaw, 2006).

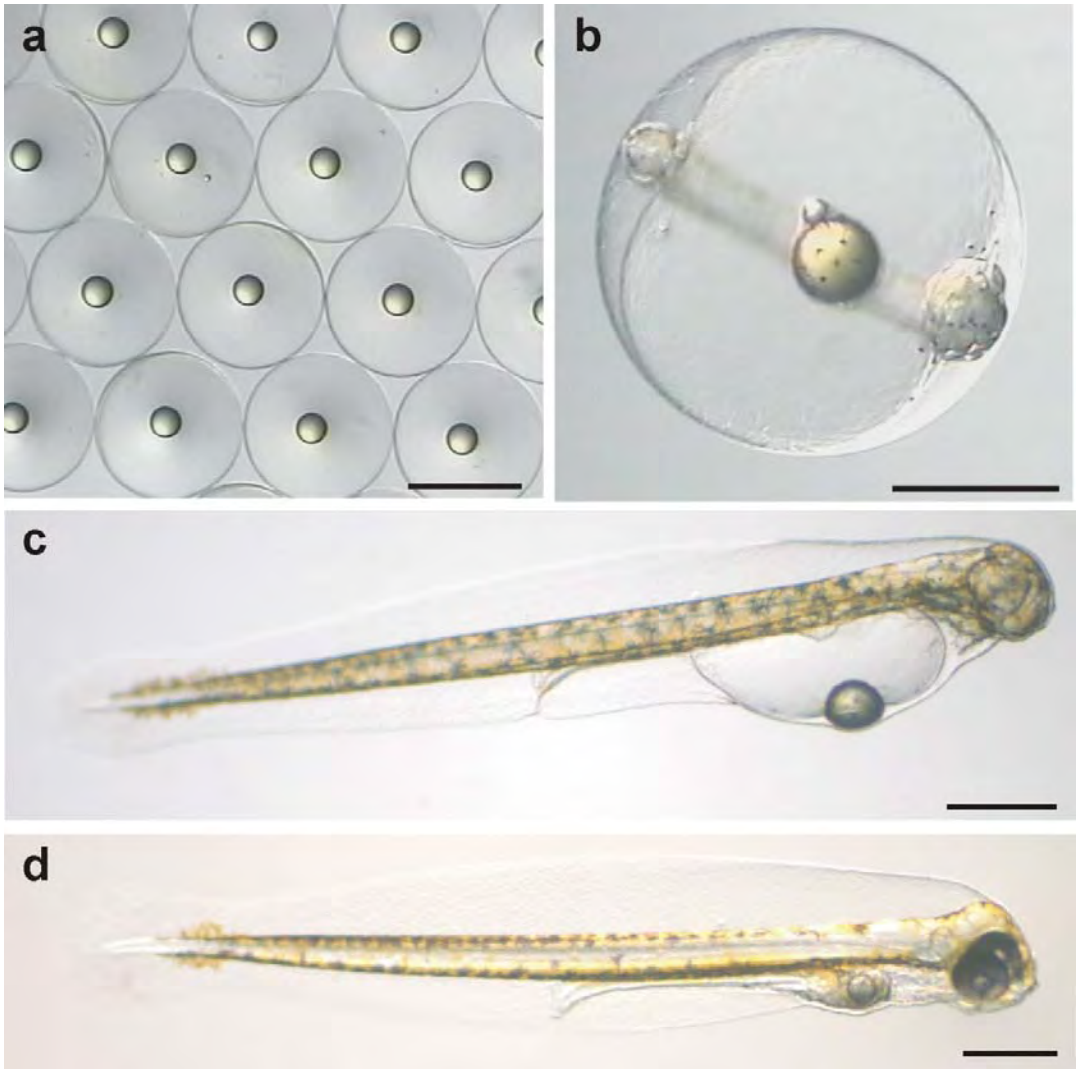

Figure 7.2 Striped trumpeter eggs (a) unfertilised and (b) 2 days after fertilization, and larvae (c) 1 day-old and (d) 4 days-old. Scale bars are (a) 1 mm and (b-d) 0.5 mm.

The morphological development of striped trumpeter larvae is similar to that of other broadcast spawning marine teleosts (Cobcroft, 2002). Yolk-sac larvae are reared in static tanks with oily surface films to avoid fragile larvae sticking to the surface. Initial swim bladder inflation starts a few days after feeding and low amplitude changes in swim bladder volume occur on a nocturnal cycle (Trotter et al., 2005a). Larvae are negatively buoyant before first feeding and positively buoyant during the dark phase following inflation (Trotter et al., 2005a). There is a finite inflation window when larvae require surface access to gulp air (Table 7.1; Trotter et al., 2005b). Overlapping optimal temperature ranges for survival and swim bladder inflation narrow the thermal optima for post-inflation viability (Table 7.1; Trotter et al., 2003a). Both photoperiod and light intensity also affect initial swim bladder inflation (Trotter et al., 2003b). Trials prior to 1999 often produced larvae with non-inflated swim bladders and the few post-larvae produced had high incidences of malformed or non-functional swim bladders in which a gaseous lumen was absent (Goodsell et al., 1996; Trotter et al., 2001). Improved swim bladder inflation rates, >80%, are now achieved through a better understanding of larval buoyancy and diel changes in swim bladder volume resulting in timely deployment of surface cleaners, and the use of optimal temperatures and light conditions.

Table 7.1 Characteristics of striped trumpeter larvae at different stages of development, showing standard temperatures, aeration and light conditions during larval rearing (see Cobcroft, 2002 and Trotter, 2003 for further details).

	Larvae					Light	
Stage of development	Age days	Size mm	Density no. l^{-1}	Temperature ^{o}C	Aeration	Photoperiod[2] light:dark h	Intensity $\mu mol\ s^{-1}\ m^{-2}$
Egg	0 - 5[1]	1.25	400	14	Light/Mod	14:10	20 - 30
Oil droplet diameter		0.28					
Yolk-sac larvae	0	3.2 - 3.4	5	14 - 16	None/Light	16:8	10
Mouth opening	5	5.1					
Oil droplet absorbed	8	5.5					
Rotifer feeding larvae	6 - 16	5.2 - 7.3	5	16	Light	16:8	10
Yolk-sac absorbed	7	5.4					
Swim bladder inflation	8 - 12	5.7 - 6.1					
Artemia feeding larvae	16 - 50	7 - 18	2	16	Mod	24:0	10 - 35
Caudal flexion	22 - 28	9 - 11					
Metamorphosis	36 - 44	13 - 15					
Post-larvae	40 - 270	14 - 150	0.5	16 - 18	Strong	16:8	5 - 10
weaned	30 - 50	12 - 18					

Mod = Moderate, [1]Age of embryos post fertilisation, [2]Lights fade on and off

Striped trumpeter larvae are primarily visual feeders with a small visual field relative to body size. However, prey searching and ingestion are probably also mediated by chemical stimuli with olfactory organs present from an early age (Pankhurst and Hilder, 1998; Cobcroft and Pankhurst, 2003). Larvae feed well in a wide range of light intensities and there is a shift to increased capabilities at lower intensities with age (Cobcroft et al., 2001b). Early feeding larvae have small reactive distances, just under larval length(Cobcroft, 2003). The first-feeding larvae are fed L-strain rotifers *Brachionus plicatilis* biotype Austria, although a small percentage of larvae can start feeding on newly-hatched *Artemia* (Figure 7.3). Rotifer density has a marked effect on growth and survival of striped trumpeter larvae and is optimal around 10 rotifers ml^{-1} . Rotifers are generally fed twice a day with rapid removal of uneaten food via water exchange. Rotifers are intensively cultured at densities up to 1000 ml^{-1} on a diet of microalgae *Nannochloropsis* sp. and bakers yeast *Saccharomyces cerevisiae* in recirculation systems using ozonated seawater, which has reduced bacterial loads and contamination from ciliates and harpacticoid copepods (Battaglene et al., 2004). Striped trumpeter fed a mixture of rotifers and *Artemia* rapidly switch to feeding on *Artemia* within 24 h. *Artemia* nauplii are reared from decapsulated cysts hatched in ozonated seawater.

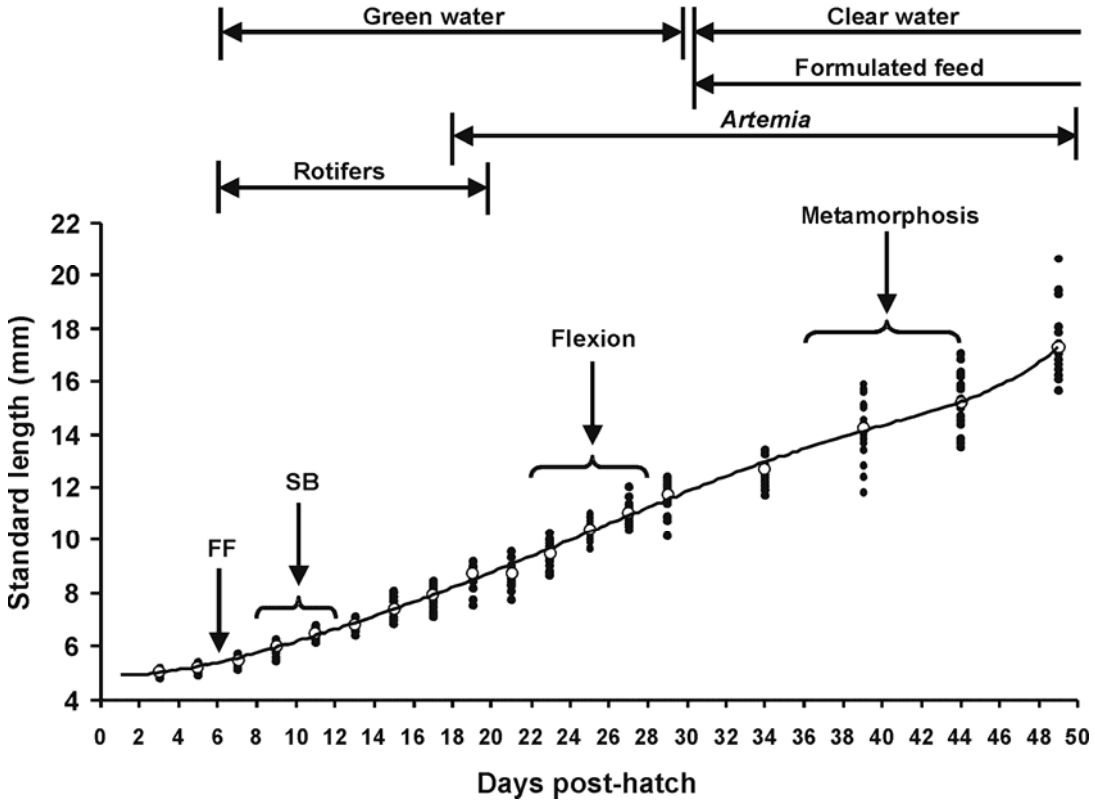

Figure 7.3 Generalised culture technique and development of striped trumpeter larvae showing growth in length with age. FF, first feeding; SB, swim bladder inflation. (After Morehead et al., 2005).

Most of the early rearing trials, prior to 2000, that produced juvenile striped trumpeter were conducted in green water and involved the supplementary feeding of copepods and or antibiotic addition (Morehead et al., 2005). Green water rearing has enhanced feeding, growth and survival of larvae of many different marine species, although the exact

mechanisms are complex and not completely understood (Cobcroft et al., 2001b). Initial studies suggested that striped trumpeter larvae reared in clear water had equivalent growth and survival to those raised in green water (Cobcroft et al., 2001b). Early green water trials used *Tetraselmis suecica* that tended to drop out in culture due to its large cell size. From 1999, green water culture switched to *Nannochloropsis oculata* which has a smaller cell size and stays in suspension. It is important to carefully manage changes in rearing conditions, in relation to light, tank colour and addition of algae, because changes can affect larval feeding responses (Cobcroft et al., 2001b). Recent studies have demonstrated that larvae reared in green water have outperformed those in clear water (Shaw, 2006). Green water increases the feeding rate of larvae and alters larval distribution within tanks, reducing the proportion of larvae swimming constantly into the sides of the tank (Shaw, 2006). This behaviour, 'walling', is referred to in other species as 'wall-nosing' or 'clinging' (Malison and Held, 1991; Bristow et al., 1996). Reducing walling increases feeding and may also protect larvae from physical damage and bacterial disease associated with constant touching of hard surfaces. However, green water has less effect on distribution of larvae as they go through flexion.

7.5 Larval nutrition and feeding

Following improvement in early survival and growth of larvae, efforts were refocused on solving the mortality and malformations that commonly occurred around notochord flexion and metamorphosis. The general hypothesis was that the mortalities in older larvae were due to a primary metabolic disorder with a nutritional and/or health basis. Nutrition research started in 2001 and focused on investigating the link between possible deficiencies or imbalances in the three essential PUFAs - docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) - found in live feeds (Sargent et al., 2002). Striped trumpeter have relatively low proportions of EPA and a high ratio of DHA to EPA (12:1) in their flesh (Nichols et al., 1994). The PUFA content of the eggs and yolk-sac larvae, while different, also have a relatively high ratio of DHA to EPA (up to 4:1) and n3 to n6 (9:1) and low ARA (Table 7.2; Morehead et al., 2001). With no information on the diet of striped trumpeter larvae in the wild, the research initially aimed to match the lipid profile of the live feeds to the striped trumpeter eggs (Sargent et al., 1999). In addition, other potentially limiting nutrient fractions, vitamins (ascorbic acid and α-tocopherol) and free amino acids, were also investigated (Brown et al., 2005a,b).

In general, a dose response approach was used to investigate requirements for fatty acids and vitamins during the rotifer and *Artemia* feeding stages. Graded levels of key long chain polyunsaturated fatty acids (LC-PUFA i.e., $\geq C_{20}$) and vitamins were obtained in live feeds by feeding novel emulsions incorporating specialty oils and vitamins. There are practical difficulties in this approach with live feeds, particularly with *Artemia*, as it is not easy to increase the DHA to EPA ratio above 1, due to retroconversion of DHA (Bell, 1998; Izquierdo et al., 2000). The background levels of vitamins in live feeds also make it difficult to design treatments with known deficiencies (Brown et al., 2005b). To partially address this challenge control treatments were used where live feeds were enriched on the commercial enrichment products AlgaMac 2000 for rotifers and AlgaMac 3050 for *Artemia* (Aquafauna Biomarine, CA) (Table 7.2). The difficulty of determining survival during trials prior to termination, because dead larvae rapidly decompose, was overcome with the use of digital photography to estimate relative survival and define mortality events. Another technical constraint experienced during the studies was the variability in the chemical composition of

the live feeds, which was generally similar within experiments, but in some cases varied markedly between experiments. The reasons for the variation included batch differences in: enrichment products; pre-enrichment dietary history of the live feeds; the vitality of the live feed cultures; extraction and processing methods for analysing biochemical samples (Merchie et al., 1997; Brown et al., 2005b; Battaglene et al., 2006). Live feed culture techniques and standardisation of biochemical methodologies both improved as the research progressed. In general, the live feed biochemical profiles in relation to fatty acids, amino acids and vitamins are similar to those reported in other studies (Øie and Olsen, 1997; Mourente et al., 1999; Kolkovski et al., 2000).

Table 7.2 Typical amounts of essential fatty acids, and group totals in live feeds and striped trumpeter larvae fed rotifers enriched on AlgaMac 2000 and *Artemia* enriched on AlgaMac 3050.

	Live Feeds		Striped trumpeter			
	Rotifers[1]	*Artemia*[2]	Egg[1]	Yolk-sac[1,3] larvae	Larvae fed[1,4] Rotifers	Larvae fed[2,5] *Artemia*
Fatty acids (mg g^{-1} DM)						
DHA	10.5	18.2	15.6	27.1	23.3	18.4
EPA	2.5	9.9	7.9	9.8	4.0	5.0
ARA	1.6	1.0	1.6	2.9	4.9	0.7
Total saturates	35.4	40.8	34.5	38.1	28.0	25.6
Total monounsaturates	20.2	88.1	38.7	36.9	29.6	22.5
Total polyunsaturates	24.4	54.5	36.8	53.1	46.4	44.5
n- 3 polyunsaturates	14.1	29.9	28.4	42.9	30.3	29.6
n- 6 polyunsaturates	8.2	23.6	8.1	10.0	16.0	13.4
DHA/EPA	4.2	1.8	2.0	2.7	5.8	3.7
DHA/ARA	6.7	18.2	9.8	9.3	4.8	26.3
ARA/EPA	0.1	0.1	0.2	0.3	1.2	0.1
Total fatty acids	80.3	183.4	109.5	128.0	104.1	92.7

[1] Brown et al., 2005b, [2] Bransden et al., 2005b ,[3] 5 days-old pre feeding yolk-sac larvae ,[4] 23 day-old larvae, [5] 36 day-old larvae

The dietary requirements for n-3 PUFA in striped trumpeter larvae were found to be akin to those for other marine fish (Izquierdo, 1996; Sargent et al., 1999, 2002). Increasing the absolute amount of DHA and EPA in rotifers did not influence growth, survival or the ability to tolerate temperature or hypersaline challenges in 18 day-old larvae (Bransden et al., 2004b, 2005a). The accumulation of docosapentaenoic acid (DPA) in the tissue of larvae on lower DHA and EPA lipid diets suggested a possible DHA deficiency and on this basis a requirement of 12.7 mg g^{-1} DHA during rotifer feeding was proposed (Bransden et al., 2004b). Below the requirement, larvae were found to be less efficient in capturing prey, reacted negatively to varying light conditions and exhibited abnormal changes to the gut and

liver structure (Bransden et al., 2005a). Increasing dietary ARA in rotifers from 1.3 to 11.2 mg g^{-1}, with near constant EPA and DHA, had no significant effect on survival or growth of 23 day-old larvae, although increasing dietary ARA influenced eicosanoid production (Bransden et al., 2004a). Extending the dose response approach to the *Artemia* feeding phase showed that increasing dietary DHA did improve growth in larvae but not survival (Bransden et al., 2005b). A requirement for near maximal growth during *Artemia* feeding was determined to be higher than for rotifers at 20.8 mg DHA g^{-1} DM (Bransden et al., 2005b). There are two possible reasons for the higher requirement for DHA during *Artemia* feeding. First, the larvae may require more DHA to meet increased physiological demands during notochord flexion and metamorphosis as has been shown for other species (Dhert et al., 1990; Kjørsvik et al., 2004). Second, larval requirements for fatty acids tend to increase with increasing dietary lipid (Izquierdo, 1996). While rotifers and *Artemia* provide similar absolute amounts of dietary DHA, *Artemia* contain more total lipid than rotifers and therefore lower relative proportions of DHA (Bransden et al., 2005b).

So, if increasing dietary DHA increased growth during metamorphosis why not survival in striped trumpeter larvae? The answer was in the behaviour of the larvae, not their dietary requirements. Larvae during the rotifer feeding stage and up to 23 days-old increase the volume of the swim bladder at night and float just below the surface (Trotter et al., 2005a). During the *Artemia* experiment, larger fish approaching metamorphosis were massing at the bottom of the tanks during the dark phase and dieing (Bransden et al., 2005b). The switch in nocturnal vertical migration patterns occurred as larvae reached 9.5 mm in total length and were undergoing flexion. The problem of nocturnal downward migration and mortality at flexion was overcome through a combination of lighting and system adjustments from day 20 post-hatch.

Nutritional studies suggested that *Artemia* have a substandard balance of fatty acids for striped trumpeter larvae (Bransden et al., 2005b). Subsequently, marine copepods, the natural diet of many marine fish species, were evaluated as a supplementary live feed (Morehead et al., 2005). Both harpacticoid and calanoid copepod species were collected from the field and added directly or cultured prior to feeding larvae. Their inclusion in the diet improved the success of early production trials (Morehead et al., 2005). The advantages of copepods may be their high polar lipid fraction, which has been suggested to increase digestion, retention of fatty acids and improve lipid transport (Coutteau et al., 1997; Shields et al., 1999). However, their use has been discontinued because copepods proved difficult to mass culture or to reliably collect from the wild, allowed the introduction of undesirable organisms, and fluctuated in nutritional valve (Morehead et al., 2005).

The high requirement for LC-PUFA in striped trumpeter larvae could result in the generation of detrimental lipid peroxides and auto-oxidation in live feeds and larvae (Mourente et al., 1999; Brown et al., 2005a). Vitamins, ascorbic acid (AsA) and α-tocopherol (α-T) play an important role as antioxidants and larval requirements change during ontogeny, in particular the need for α-T increases with dietary PUFA (Merchie et al., 1997; Stéphan et al., 1995). The first studies of vitamin levels in striped trumpeter larvae indicated that concentrations of α-T decreased from 258 μg α-T g^{-1} dry weight (DW) in larvae at first feeding to 120 μg α-T g^{-1} 14 days post-hatch, suggesting α-T levels in enriched rotifers of 60 μg α-T g^{-1} may be suboptimal and below those reported to provide good growth for other species (Rønnestad et al., 1999; Kolkovski et al., 2000; Brown et al., 2005a). Further dose response research supported the view that increased dietary α-T between 437 and 1040 μg α-T g^{-1} improves growth and survival of larvae on high PUFA diets (Brown et al., 2005b). However, the survival results were highly variable and the control diet of rotifers fed AlgaMac 2000 with a

lower level of 110 μg α-T g^{-1} provided better growth. Unlike α-T concentrations in larvae, AsA remained constant during exogenous feeding between 510 to 590 μg AsA g^{-1} DM in larvae up to 14 days-old and, based on the maintenance of tissue concentrations, 307 μg AsA g^{-1} DW in rotifers appears sufficient to meet requirements for good growth and survival (Brown et al., 2005a). The potential value of feeding *Artemia* enriched on mega doses of AsA is currently being investigated as a potential solution to the high incidence of jaw malformations in post-larvae.

The detailed nutritional studies undertaken over recent years, 2001 - 2005, have provided valuable insights into the nutritional requirements of striped trumpeter larvae during the period they are feeding on live prey. The research focus has now changed to the development of early weaning strategies to reduce the cost of live food production and to provide better nutrition in the belief that it may assist in reducing jaw and spinal malformations. New commercial particulate diets from Europe have shown promise as weaning diets for post-larvae. Weaning is now possible when post-larvae are 50 days-old but more research is required to establish the earliest age at which larvae can be weaned.

7.6 Health

The effective management of striped trumpeter larval health has been central to recent improvements in survival, growth and larval quality. The main health issues affecting striped trumpeter larvae, with the potential to constrain juvenile production, are betanodavirus (Munday et al., 2002), bacterial enteritis and intestinal dysfunction (Cobcroft et al., 2004a; Bransden et al., 2005a), a myxozoan parasite (Grossel et al., 2003), and jaw and skeletal malformations (Cobcroft et al., 2001a; Trotter et al., 2001). Several techniques have been adopted to successfully manage viral and bacterial pathogens and myxozoan parasites in larval production. Progress in reducing malformations has been slower.

7.6.1 *Nodavirus*

Piscine betanodavirus was diagnosed in striped trumpeter larvae and broodstock in 2001 (Munday et al., 2002; Morehead et al., 2006). Prior to 2001, sudden and catastrophic mortalities of larvae and post-larvae occurred, which while not apparently related to stage of development, were linked to stressors such as poor water quality, movement of fish, and bottom cleaning of tanks. Fish exhibited a range of symptoms including abnormal swimming behaviour (spiral swimming and darting), swim bladder hyperinflation and anorexia, all characteristic of nodaviruses in other species (Munday and Nakai, 1997; Munday et al., 2002). Histopathology of moribund 30 day-old striped trumpeter larvae revealed vacuoles in the brain, spinal cord and retina (Morehead et al., 2006). Transmission electron microscopy identified icosahedral particles with a diameter of 30±2 nm associated with these lesions. Larvae tested strongly positive for antibodies against striped jack nervous necrosis virus using an immuno-fluorescent antibody test and sera from captive-held striped trumpeter broodstock tested positive to nodavirus using a standard antibody response ELISA (Morehead et al., 2006). A sensitive and specific molecular detection test (nested RT-PCR) has been developed which successfully detects Australian nodavirus infections in larvae and broodstock samples, including striped trumpeter (Moody et al., 2005). Transmission of the nodavirus to larvae appears to occur vertically from broodstock via eggs and spawning fluids as reported for other species (Grotmol and Totland, 2000; Mundy et al., 2002). Ozone (O_3) disinfection of eggs, while not totally effective, does reduce the risk of transmission of betanodavirus in other species (Grotmol and Totland, 2000; Buchan et al., 2004). The

tolerance of striped trumpeter eggs at different stages of development to ozone has been established and the standard practice is to disinfect with 1 mg O_3 l^{-1} for 1 min shortly after fertilisation and sometimes again three days later when the embryo is two-thirds developed around the yolk (Battaglene and Morehead, in press). In addition, strict hygiene protocols (hand-sprays, protective clothing, foot baths etc) have been adopted and all incoming seawater supplying the hatchery and live feeds is ozonated for 10 min with an ORP > 800 mV. Lowering fish stocking density from 30 to 5 l^{-1} has also improved growth but not early survival of larvae (Battaglene and Brown, 2006). Since adopting the practice of ozone disinfection of eggs, and other control measures including disinfection of equipment between trials, there has been no detection of betanodavirus.

7.6.2 *Bacterial enteritis*

Poor gut and intestinal health of larvae was a regularly observed pathology during rearing trials prior to 2002. Pathology included decay of the posterior intestine, grossly associated with swelling of the anterior region of the mid gut and blockage of the hind gut. Possible causative factors included live feeds with high bacterial loads, poor digestion of *Artemia* and inadequate nutrition. Histological examination of larvae identified bacterial enteritis characterised by sloughing of gut epithelial cells associated with bacteria. At early stages of infection the larvae had an opaque grey colouration of the mid gut, referred to as 'grey gut' syndrome. Experiments using the antibiotic oxytetracycline demonstrated that bacterial control had a greater influence than lipid nutrition on the survival and growth of larvae during the rotifer feeding stage (Battaglene et al., 2006). Treating larvae with antibiotics improved survival, increased digestion, and reduced the incidence of grey gut syndrome and the presence of urinary calculii, resulting in more viable larvae (Battaglene et al., 2006). The use of antibiotics is not a sustainable option for commercial hatcheries and the new water filtration and ozonation system, originally designed to control myxozoans, has also improved water quality and allowed greater microbial control, without antibiotics. Improved water quality has led to improvements in production trials and has enabled better interpretation of experimental results by limiting confounding influences associated with a highly variable supply of seawater from the Derwent Estuary. Grey gut syndrome has persisted in larvae reared in ozone-disinfected water, although it is no longer associated with severe degeneration of the gut or high mortality. Recent investigations suggest that a dietary imbalance in fatty acids may be interfering with lipid transport in the gut and liver (Bransden et al., 2005a). Larval density has also been an important factor in controlling bacterial disease and was examined experimentally within the range of 1 to 40 larvae l^{-1} and significantly effected growth but not survival (Battaglene and Brown, 2006). Recommended stocking densities in ozonated clear water intensive culture should not exceed 5 larvae l^{-1}.

7.6.3 *Malformations*

Malformations of cultured striped trumpeter include spinal and jaw malformations (Cobcroft et al., 2001a; Trotter at al., 2001). Spinal malformations have been linked to non-inflation of the swim bladder and may be associated with myxozoan and nodavirus infections. A high proportion of the post-larvae produced to date, >85%, exhibit malformation of the jaw, which while not lethal reduces feeding capacity and probably contributes to high attrition rates in post-larval rearing (Morehead et al., 1999). Jaw malformations are detectable in post-flexion larvae around 10 mm in total length. Typically malformed post-larvae exhibit open jaws in which the maxilla and premaxilla are aligned dorso-ventrally, and the anterior hyoid arch elements are in an abnormal ventral position (Cobcroft et al., 2001a

Figure 7.4). Physical damage due to 'walling' probably contributes to breakages at the symphysial joint and may explain the large variation in jaw malformations exhibited. However, no definitive cause of malformations in striped trumpeter has been established despite considerable research into the vitamin and lipid enrichment of live feeds. Experimentation with different coloured and patterned backgrounds has reduced walling in post-larvae but malformations have persisted (Cobcroft et al., unpublished data). Comparative studies with kingfish reared in New Zealand showed a high degree of similarity in malformation (Cobcroft et al., 2004b). The onset and severity of jaw malformation has been delayed from around 30 days-old to after 50 days-old through a combination of better husbandry practices, the use of ozonated seawater, higher feed rates and lower larval densities and temperatures. Jaw malformations remain the major enduring obstacle to the hatchery culture of high quality juvenile striped trumpeter.

7.6.4 *Myxozoans*

In early rearing trials post-larvae over 30 days-old frequently developed nervous conditions, including walling, flashing, whirling, loss of swimming equilibrium and inability to feed. Some post-larvae also developed scoliosis and lordosis of the spine from 60 days-old. Examination of the brains and spinal cords of older postlarvae and juveniles showed severe meningoencephalomyelitis caused by a myxozoan parasite, Kudoa neurophila (formerly Pentacapsula neurophila), although a direct link between the clinical symptoms and the disease has not been established (Grossel et al., 2003). A single-round polymerase chain reaction (PCR) diagnostic assay was developed to detect K. neurophila in striped trumpeter (Grossel et al., 2005). All cultured juvenile and adult fish tested up to 2004 have been positive to K. neurophila with varying degrees of infection. Wild striped trumpeter held in captivity do not test positive, suggesting there is an age, possibly following settlement as juveniles, when they are no longer susceptible to infection. Histopathology and in situ hybridisation (ISH) has been used to study the progression of the disease. The infective stage of the parasite enters the fish host via epithelial cells as early as 25 days-old, develops in muscle tissue before entering the brain and spinal cord where distinctive mature pentacapsular spores are present in larvae around 100 days-old (Grossel, 2005). The disease has been studied in and around the hatchery system and ten species of marine fish within the vicinity of the water intake have tested positive for *K. neurophila* and are the probable source of infection (Grossel, 2005). Post-larvae cultured to 100 days-old in ozonated seawater have all tested negative to the PCR assay and have showed few clinical signs, demonstrating the effectiveness of ozonation as a barrier to the parasite. It is possible that post-larvae will need to be cultured in ozonated seawater for up to 9 months when they undergo final metamorphosis and settlement as juveniles. Holding post-larvae to settlement would greatly add to the costs of seed production and requires further investigation.

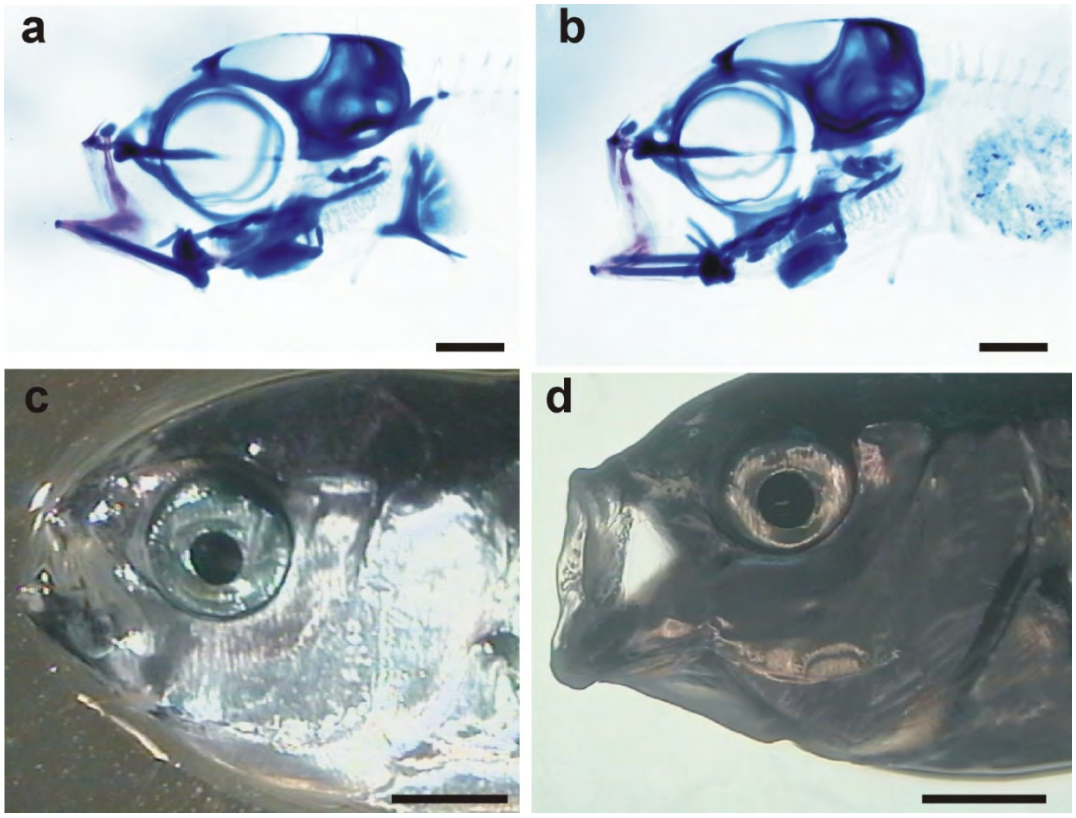

Figure 7.4 Typical jaw malformations in cultured striped trumpeter post-larvae. (a) normal jaw 11 mm SL larvae (b) malformed jaw 11 mm SL larvae (c) normal jaw 20 mm TL post-larvae (d) malformed jaw 30 mm TL post-larvae. Sale bars are (a-b) 0.5 mm and (c-d) 2 mm.

7.7 Production and future directions

The challenges with striped trumpeter culture appear similar to those of other difficult to rear cold temperate species, particularly, Atlantic halibut which also have a long pelagic larval stage (Kjørsvik et al., 2004; Verner-Jeffreys et al., 2004). Australia does not have the same resources to develop new species as those available to research in Europe and one of the limitations of the striped trumpeter research effort has been the restriction to one hatchery. It is possible that some of the health challenges may be less severe at other locations with more oceanic water supplies. However, the limited geographical distribution of the species, and strict translocation regulations within Australia, have not favoured research outside Tasmania. Viable methods for the transportation of eggs and embryos have been developed with a view to conducting research at other locations in the future (Jones, 2003).

An integral component of the research into striped trumpeter culture has been the concurrent seasonal attempts to produce striped trumpeter juveniles at a semi-commercial scale. The trials have incorporated the latest improvements and findings from the experiment program combined with innovative changes to the hatchery systems including water treatment, tank design and live feed production. Wherever possible trials have been conducted in replicate tanks and in association with experiments so that results could be compared under different conditions and at different scales (Morehead et al., 2005; Battaglene and Brown, 2006). A convenient historical benchmark for production trials was survival to 100 days-old. In general terms over the past five years there has been a gross 50% improvement in survival during rotifer feeding from a maximum of 30% to 80% when larvae are 20 days-old.

Survival during the *Artemia* feeding period has shown even greater percentage improvement from around 5% when larvae are 50 days-old, to a maximum of 50% today. In addition, weaning of post-larvae onto formulated diets has been reduced from 100 days to 50 days-old. Of 26 production runs over the four years 2002-2005, around 50% successfully produced a total of over 9000 fish to the 100 day-old benchmark (Figure 7.5). With improvements in survival there have also been good improvements in growth. The average weight of 100 day-old post-larvae has increased to 0.5 g in 2005. The improvement in growth performance has been achieved at all stages of development and is due to a combination of many abiotic and biotic factors (Figure 7.6).

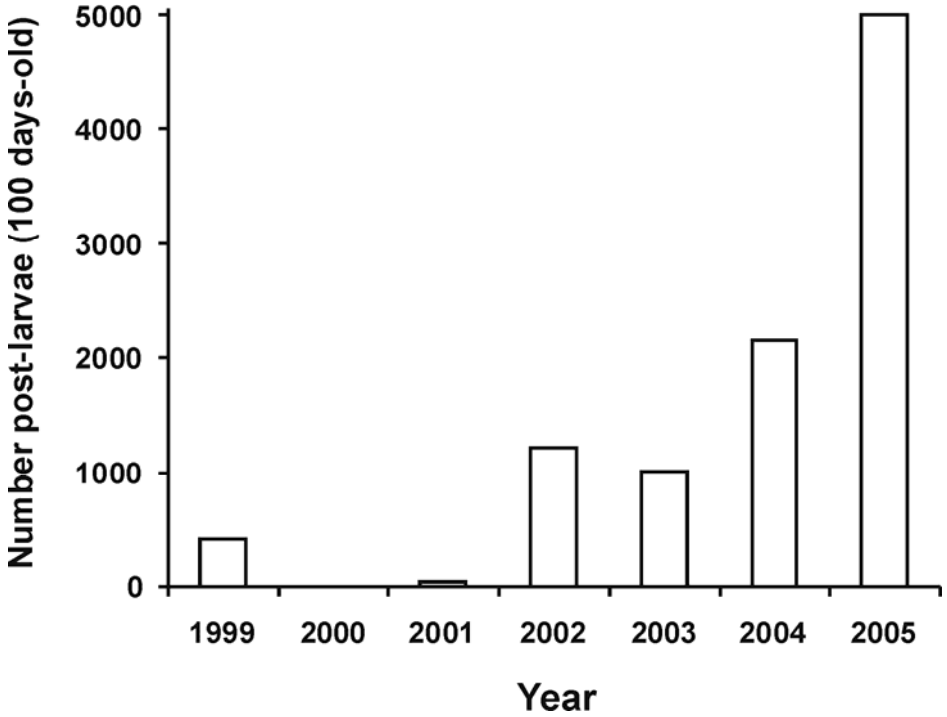

Figure 7.5 Production of striped trumpeter post-larvae to 100 days-old from 1999 to 2005. Production in 2000 was reduced by nodavirus, and in 2001 by the implementation of new hygiene protocols and a reduced focus on production-scale trials.

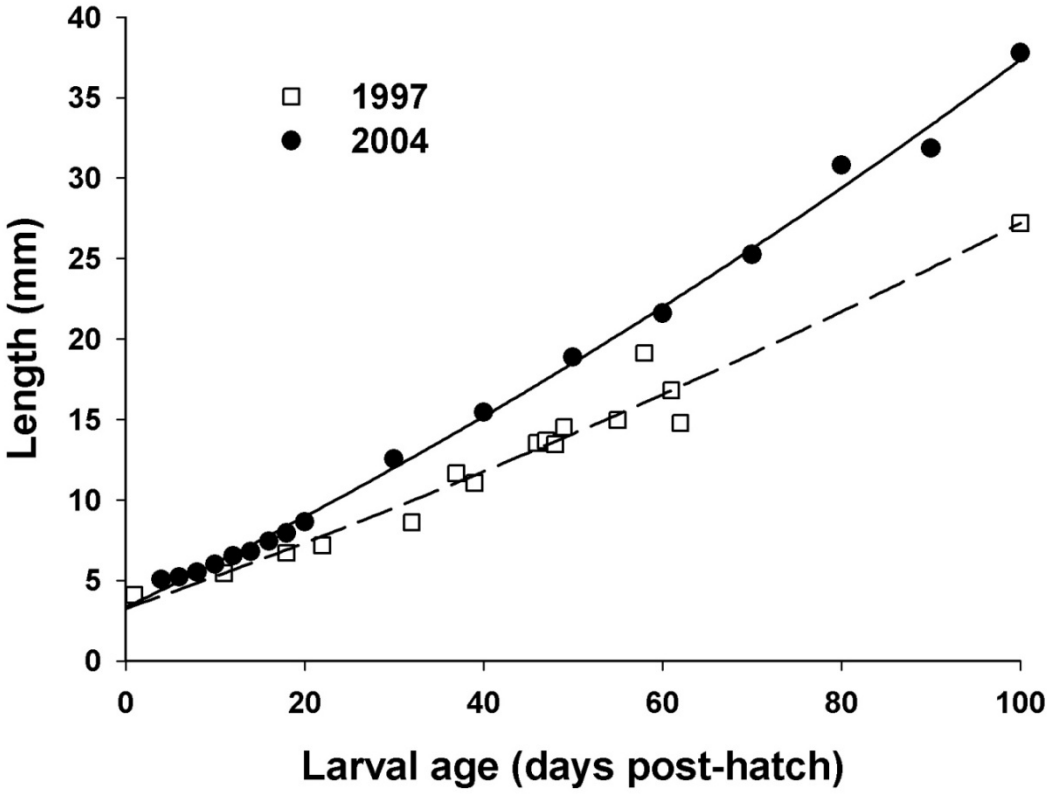

Figure 7.6 Growth of striped trumpeter larvae in production trials in 1997 (open squares, broken line) and in 2004 (closed circles, solid line). Lengths are standard length to 30 days-old, and total length thereafter. Points are mean of 10 until 32 days-old and n=2-6 thereafter in 1997 (20 fish survived to 100 days-old), and mean of 20 larvae in 2004. Lines are second order polynomials.

Research on striped trumpeter is continuing through the Research Program of the CRC for Sustainable Aquaculture of Finfish (Aquafin CRC) in three key areas. First, the health of post-larvae is being further improved through better control of bacterial diseases and parasites and the use of probiotics. Second, the development of live feeds enriched with vitamins continues combined with early weaning strategies, with the aim of reducing malformations. Finally, the larger-scale assessment of survival and growth of post-larvae and juveniles on a semi-commercial scale is planned, leading ultimately to the grow-out of striped trumpeter in sea cages.

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8 MODIFICATION OF BACTERIAL FLORA AND LARVAL PERFORMANCE IN THE CULTURE OF STRIPED TRUMPETER (*LATRIS LINEATA*) LARVAE USING LIVE OR CONCENTRATED ALGAE OR CLEARWATER

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8.1 Abstract

Marine fish larvae are susceptible to mortality caused by bacterial infection during larval rearing. This study investigated performance and cultivable bacteria of striped trumpeter larvae reared to 15 days post-hatch (dph) in clearwater or greenwater based on live microalgae or a concentrated algal paste (instant). BTB-Teepol (BTBT) agar had better recovery of environmental Vibrios than TCBS and was used as the presumptive *Vibrio* selective media. Larval growth was higher in live algae (7.1 ± 0.3 mm standard length, SL, 330 ± 28 µg larva^{-1} dry weight, DW) than in clearwater (6.6 ± 0.3 mm SL, 268 ± 22 µg larva^{-1} DW) or instant algae (6.6 ± 0.3 mm SL and 232 ± 30 µg larva^{-1} DW). However, survival was higher in both live algae and clearwater (35 ± 9 and 38 ± 5%, respectively) than in instant algae (7 ± 4%). Mortality of larvae reared in instant algae was consistently high from 12 dph and coincided with significantly higher cultivable bacteria in the larvae (presumptive *Vibrio* 0.2 colony forming units [cfu] µg^{-1} larva DW at 3 dph to 650 cfu µg^{-1} larva DW at 15 dph) and in larval culture tanks (presumptive *Vibrio* peak at 8 x 10^5 cfu ml^{-1} at 7 dph and decline to 2 x 10^3 cfu ml^{-1} at 15 dph). Bacterial isolates (n = 515) were classified as presumptive Vibrios (39%), with eight well-defined species and eight unspeciated types with distinct phenotypes (75% similarity level), and glucose non-fermenters (61%), with 19 unspeciated types with discrete phenotypes (82% similarity level). Potential pathogenic isolates included *Vibrio anguillarum* and *V. ichthyoenteri*, and a strain with probiotic properties, *V. alginolyticus*, was identified. There was a correlation between bacterial flora of the larvae and the culture environment, demonstrated by a more complex flora associated with larvae held in live algae compared to instant algae or clearwater. Live microalgae is recommended for greenwater culture of striped trumpeter to provide for good growth and survival. Concentrated algal paste products should be used with caution as greenwater for marine fish larval rearing as they may lead to potentially harmful bacterial proliferation and larval mortality.

8.2 Introduction

Marine fish larvae hatch at an early stage in development and are susceptible to mortality associated with bacteria in culture (Ringø and Birkbeck, 1999; Muller-Feuga, et al., 2003). Larvae may be compromised by pathogenic bacteria, often *Vibrio* spp. (Muroga, et al., 1990;

Villamil, et al., 2003; Vadstein, et al., 2004), or by total bacterial load in the culture water or diet (Munro, et al., 1993; Battaglene, et al., 2006). The initial transient intestinal microflora of larvae becomes a persistent community and may influence the digestive ability of larvae, and consequently has an impact on growth and larval quality (Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999). The bacteria present in larval cultures come from a variety of sources, but are predominantly introduced with the incoming seawater, microalgae used for greenwater, and live feeds particularly rotifers and *Artemia* (Ringø and Birkbeck, 1999; Vadstein, et al., 2004).

Striped trumpeter, *Latris lineata*, is a candidate species for aquaculture in Tasmania. Research in Tasmania has addressed the control of spawning, environmental conditions for egg incubation and larval rearing, and larval nutrition, culminating in the reliable hatchery production of post-larvae (Battaglene and Cobcroft, 2007). A significant step in improving larval survival in tank culture was the discovery that a high total bacterial load in larvae (measured by cultivable bacteria, colony forming units, cfu) was associated with high mortality (Battaglene, et al., 2006). In the wild, striped trumpeter larvae are believed to be oceanic and neustonic and may have a lower tolerance to high levels of bacteria in culture conditions than other species from different early life-history habitats (e.g. estuarine species) with a natural resistance/tolerance to bacteria and adaptation to variable water quality conditions (Vadstein, et al., 2004). Since the use of antibiotics is not a sustainable practice for hatchery production, a larval culture method was required for striped trumpeter that would reduce bacterial load to promote higher survival. Live algae is frequently used for greenwater culture, and it is claimed to have beneficial effects on the microflora of the culture water. Algae concentrates are proposed as an alternative to live algae for greenwater culture and as a diet for zooplankton (e.g. rotifers and *Artemia*).

In relation to the approach to microbial assessment of larval culture conditions, the most commonly used medium for isolating viable marine bacteria is ZoBell's 2216E marine agar (Oppenheimer and ZoBell, 1952) or one of its derivatives such as Johnson's marine agar (Johnson, 1968). This medium type is considered non-selective and permits the growth of compliant heterotrophic bacteria such as the marine Pseudoalteromonads and *Vibrio* species, both groups often associated with aquatic animals.

Selective media can be used in addition to non-selective media to estimate viable bacterial numbers of specific types of bacteria. Of the groups of bacteria of interest, the Vibrios represent the most important type as species of this group are widely associated with aquatic animals and are frequently implicated as a primary cause of disease or as opportunist pathogens. The most commonly used selective medium for the Vibrios is TCBS (thiosulphate citrate bile sucrose) agar which was developed for the isolation of human pathogenic species, principally *V. cholerae* and *V. parahaemolyticus* (Kobayashi et al. 1963). TCBS is a versatile medium and available commercially, factors which have led to its rapid uptake and application to many environmental studies (Bolinches et al., 1988).

Despite the widespread use of TCBS, it may not necessarily be the medium best suited to isolate all species of *Vibrio* (Munro et al., 1993). It has been reported that some species do not grow on the medium or growth is poor because of the inhibitory properties of the medium (Nicholls et al., 1976). Other formulations of media selective for *Vibrio* species have been developed such as Monsur's medium (Morris et al. 1979), TCI (thiosulphate chloride iodide) agar (Pfeiffer and Oliver, 2003) or BTB-Teepol (BTBT) (Honda et al. 1982). Of these formulations BTBT appears to be the most suited for general purpose use.

The choice of media for assessing bacterial loads of larvae held under different conditions is important and before choosing a selective medium for the Vibrios, a comparison was made of TCBS and BTBT for ability to support growth. The remaining methodologies adopted for assessing the bacterial floras are well documented and prior experimentation to establish suitability of procedures was not required.

The aim of the research was to determine the effect of greenwater, using live algae or a concentrated algal product, and clearwater rearing methods on the performance of larval striped trumpeter and the bacterial flora determined with bacterial culturing techniques. For other microbial assessment approaches see Chapter 9.

8.3 Materials and Methods

8.3.1 *Larval rearing*

Eggs were collected from a female striped trumpeter broodstock by strip-spawning, and fertilised with the milt of four males. Fertilised eggs were incubated and hatched as previously described (Bransden, et al., 2004b). Larvae were randomly stocked into each of twenty-four, 300 l black hemispherical fibreglass tanks at 1 day post-hatch (dph) at a density of 15 larvae l^{-1} (4500 per tank). Larvae were held under static, clearwater conditions from 1-5 dph. A photoperiod of 16 h L:8 h D was used throughout the experiment and was produced by a computerised halogen light source (~11 µmol s^{-1} m^{-2} at the water surface) with a gradual fade in and fade out. At 0900 h on 6 dph internal screens (390 µm mesh size) were placed into the centre of each tank to allow outflow and removal of rotifers, algae and incoming seawater overnight (see below).

Three treatments were evaluated during the experiment, each with eight replicate tanks. The first treatment ('live') consisted of rearing larvae in a 'greenwater' environment with live *Nannochloropsis oculata*, at a turbidity level of 3 nephelometric turbidity units (NTU) measured with a HACH 2100P portable turbidity meter (Hach Company, Colorado, USA). The second treatment ('instant') was an algal paste of concentrated *N. oculata* (Reed Mariculture Inc., California, USA) that was also used to produce a 'greenwater' environment by re-suspension of the concentrate in seawater to a turbidity level of 3 NTU. The final treatment ('clear') consisted of rearing larvae under 'clearwater' conditions with no additional algae (<0.01 NTU). At 0830 h, either algae (live or instant) or clear filtered seawater was added to each tank according to treatment at a rate of 4.7 l min^{-1} (total 70 l) from reservoirs accompanying each individual tank. The algal suspensions and seawater were acclimated to tank temperature overnight in the aerated reservoirs. Tanks remained static during the light phase (0900-0100 h) with gentle aeration (200 ml min^{-1}). At 0100 h a central screen (mesh size 390 µm) was placed in the tank and filtered (sand filter, 20 µm cartridge filter and foam fractionation) seawater was supplied at 112.5 l h^{-1}. The screen prevented the loss of larvae but allowed the passage of algal cells and live food.

All tanks were supplied with rotifers (*Brachionus plicatilis* Austria) enriched on AlgaMac 2000 (Aquafauna Biomarine, USA) as described by (Bransden, et al., 2004a). Enriched rotifers were supplied at 10 ml^{-1} each morning at 0900 h. Surface skimmers were used from 8-15 dph to remove surface oil and promote swim bladder inflation (Trotter, et al., 2005). Water quality was measured daily and temperature was 15.9 ± 0.2 °C (range 15.5-16.5 °C), salinity 34.5 ± 0.1 (34.1-34.7), pH 8.25 ± 0.03 (8.11-8.30), and dissolved oxygen 100 ± 5% (88 – 117%). Mortalities were spot siphoned and counted daily to calculate cumulative

mortality, up to the conclusion of the experiment (15 dph) when all live larvae were removed and counted to determine final survival.

8.3.2 *Larval sampling*

At 0, 2 and 4 days post-fertilisation, and at 1 dph (prior to stocking), 50-80 eggs or larvae were removed from incubators, anaesthetised in 0.06% 2-phenoxyethanol and used to evaluate dry matter content (n = 30-50) or total bacterial counts (n = 20). From 3 dph, and thereafter at 2 day intervals up to 15 dph, 50 larvae were removed from each of the experimental tanks and, following anaesthesia, used to determine morphometric indices and dry matter content (n = 30 larvae only) or total bacterial counts (n = 20). At the same time 10 ml of water was removed via sterile pipette from each tank and placed into sterile containers. Two ml of algal suspension or seawater was removed from each of the reservoirs, pooled by treatment into a sterile container. Tank and treatment water samples were plated to determine total bacterial numbers. All sampling took place at 0830 h, prior to feeding, with the exception of an additional water and larval sampling for bacterial analysis at 2130 h on 14 dph, 12.5 h after rotifer addition, using the same methods as described above. From 7-15 dph at 2 day intervals ~2000 enriched rotifers were sampled to determine total bacterial counts.

8.3.3 *Larval morphometrics and dry matter*

Following anaesthesia in 0.06% 2-phenoxyethanol, larvae (n = 30 per tank) were individually assessed using an Olympus SZ dissecting microscope with an eyepiece graticule for standard length, the presence or absence of a swim bladder, the presence or absence of urinary calculi, and the severity of 'grey gut' syndrome (scale of 0 – 3, with 0 being no grey gut and 3 being the highest level of grey gut, [Bransden, et al., 2005]). These same larvae were subsequently rinsed onto pre-weighed, glass microfibre filters, washed with 0.5 M ammonium formate to remove salt, frozen and later freeze dried to constant weight to determine dry matter content.

8.3.4 *Total bacterial counts*

Larvae (n = 20 per tank) and rotifers (n = ~2000 per sample day) sampled for bacterial analysis were passed through an autoclaved, 13 mm filter unit containing a 5 µm nitrocellulose filter (Millipore, Massachusetts, USA). Prior to filtration, larvae were anaesthetised in chilled (< 4 °C) seawater. Samples were washed twice by passing 10 ml of sterile seawater through the filter. The filter and sample was removed using axenic procedures and placed into 1.5 ml microfuge tubes. Sterile seawater, 250 µl, was added and the sample homogenised with a plastic pestle and motorised drive. All plasticware was previously sterilised by autoclaving. The sample was pulse centrifuged (15 s at 5000 x g) and the supernatant was diluted in micro-well plates to produce serial dilutions in sterile seawater (for analysis of the pellet, see Chapter 9). Bacterial counts were performed using a contained form of the Miles and Misra method (Miles and Misra, 1938). ZoBell's Marine Agar (ZMA) and a *Vibrio* selective agar (BTBT) agars were loaded into 6-well tissue culture plates (35 mm well diameter), which enabled both types of media (one column of wells for each medium) and sufficient dilutions of the sample to be assessed in one 'plate' unit (Figure 8.1 and Figure 8.2). To ensure the inoculum would spread evenly over the surface of the ZMA medium, Triton-X 100 (Liu et al., 1995) was added to the medium prior to autoclaving to a achieve a final concentration of 0.05%. Twenty µl of three appropriate water sample or supernatant dilutions (range 'neat' to 10^{-5}) were pipetted onto both agar media in triplicate.

The wetted nature of the media ensured that the inoculum was well spread out across the surface of the agar without the need of mechanical spreading of the sample. Plates were incubated at 22 °C for 48 h and the number of colonies counted at a dilution where the number of colonies was in the range of 5-30.

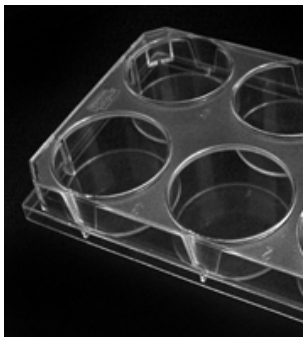


Figure 8.1 6-well tissue culture plate used for bacterial culture from water, fish and rotifer samples.

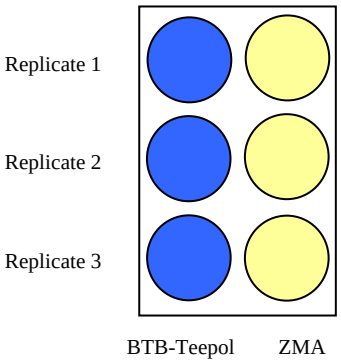


Figure 8.2 Agar layout in microwell plate indicating replicate wells for each dilution of water, fish and rotifer samples.

8.3.5 *Bacterial community identification*

Evaluation of selective media for *Vibrio* species – seawater inoculum

Water samples, 100 ml were collected from Windermere pier (brackish site) and Bell Bay (estuarine site) on the Tamar River and a third sample from Bridport representing a marine sample. At the time of collection, temperature and DO was measured using an Oxyguard Handy Mark III combination meter; salinity was measured using a Hanna H19033 conductivity meter. Samples were kept cool and processed within five hours of collection. Culture plates of ZoBell's marine agar (ZMA), TCBS and BTBT were inoculated with 0.1 ml of seawater sample by spread plate, five replicate plates per medium. Plates were incubated at 25 °C for 72 h. Colonies were counted and expressed as the number of bacteria in a sample as colony forming units ml^{-1}. Both selective media contain the fermentable substrate sucrose and counts were dissected as sucrose positive and negative types.

Evaluation of selective media for *Vibrio* species – named species

The productivity of the two selective media was assessed by the ecometric method (Mossell, 1980). Species of *Vibrionaceae* (Table 8.1) were grown on ZMA for 48 h at 25 °C. A

suspension of cells was prepared in 1% NaCl to a density equivalent to McFarland 0.5 and streaked out across four quadrants. Plates were incubated at 25 °C for 48 h and the extent of growth across the four quadrants, a measure of productivity, was assessed. Growth was scored as 1-4 based on the quadrant on which growth occurred.

Table 8.1 Species used to assess productivity and selectivity of TCBS and BTBT agars

Vibrionaceae	**Strain**	**Other species**	**Strain**
Aliivibrio fischeri	ATCC 7744	*Aeromonas hydrophila*	ATCC 7966
Aliivibrio logei	ATCC 29985	*Aeromonas veronii* bv *sobria*	ATCC 9071
Grimontia hollisae	CIP 101886	*Pseudomonas fluorescens*	NCTC 10038
Moritella viscosa	NCIMB 13584	*Pseudomonas putida*	TCFB 1889
Photobacterium angustum	ATCC 2519	*Yersinia ruckeri*	TCFB 1849
Photobacterium damselae ssp *piscicida*	NCIMB 2058	*Escherichia coli*	ATCC 25922
Photobacterium damselae subsp *damselae*	NCIMB 2184	*Proteus vulgaris*	ATCC 13315
Photobacterium iliopiscarium	ATCC 51760	*Shewanella putrefaciens*	TCFB 0870
Photobacterium leiognathi	ATCC 25521	*Plesiomonas shigelloides*	TCFB 0861
Photobacterium phosphoreum	NCIMB 1282	*Lactococcus garvieae*	ATCC 49156
Vibrio aestuarianus	NCIMB 2236	*Staphylococcus warneri*	TCFB 1880
Vibrio agarivorans	CECT 5085		
Vibrio alginolyticus	ATCC 17749		
Vibrio anguillarum	ATCC 19264		
Vibrio calviensis	DSMZ 14347		
Vibrio campbellii	ATCC 25920		
Vibrio cholerae	TCFB 0186		
Vibrio cincinnatiensis	LMG 7891		
Vibrio cyclitrophicus	CIP 106644		
Vibrio diazotrophicus	ATCC 33166		
Vibrio fluvialis	NCTC 11327		
Vibrio furnissii	ATCC 35016		
Vibrio halioticoli	IAM 14596		
Vibrio harveyi	ATCC 14126		
Vibrio ichthyoenteri	DSMZ 14397		
Vibrio lentus	CECT 5110		
Vibrio mediterranei	CIP 103203		
Vibrio metschnikovii	NCTC 8443		

ATCC: American Type Culture Collection; **CECT**: Colección Española de Cultivos Tipo; **CIP**: Collection de l'Institut Pasteur; **DSMZ**: Deutsche Sammlung von Mikroorganismen und Zellkulturen; **NCIMB**: National Collection of Industrial & Marine Bacteria; **NCTC**: National Collection of Type Cultures; **TCFB**: Tasmanian Collection of Fish Bacteria

Colony selection, bacterial identification and characterisation

For each of the three treatments, clearwater, greenwater instant and greenwater live, one replicate plate was chosen for each of the 24 tanks and sample times and 10 colonies selected at random from the ZMA wells, from plates of fish, water and rotifer samples. Colonies were subcultured onto ZMA plates and incubated at 25 °C for 48 h. Cultures were subcultured once more for purity. Bacteria were stored frozen at –80 °C in glycerol peptone water (Ward and Watt, 1971). A library of 750 isolates were collected and stored frozen. Frozen cultures were revived on ZMA and subcultured twice to ensure phenotypic vigour was restored and to check purity. Cultures were profiled using MicroSys V36 identification panels developed for the *Vibrionaceae* (Carson et al. 2006). All isolates that fermented glucose, were oxidase positive and sensitive to the pteridine compound 0/129 at 150 µg, were presumptively considered to be *Vibrionaceae*. Identification of these types was undertaken by probabilistic computer identification using the VibEx7 database and the software package PIBWin

(Carson et al. 2006). An identification was accepted when the Willcox probability value was ≥ 0.99 and the modal likelihood score was ≥ 0.001.

A species and/or strain identification was not attempted on isolates which did not ferment glucose as no practical identification database exists for bacteria of this type.

8.3.6 *Statistical Analysis*

Larval performance indicators

Separate one way ANOVAs were used to test the effects of water type on larval performance indicators within age; length, dry weight, swim bladder inflation, urinary calculi, grey gut, survival, cumulative mortality and bacterial counts. Percentage data were transformed to achieve homogeneity of variance and normal distribution of residuals by arcsin $\sqrt{p}$, where p is the proportion, and by $\log_{10}$ for grey gut severity, cumulative mortality and bacterial counts. Where significant treatment effects were detected, Tukey's HSD post hoc analysis was used to determine differences between means. Statistical analyses were performed with SPSS 17.0 (SPSS Inc., Illinois, USA). Results are reported as mean ± SD and significance accepted as $P < 0.05$.

Cluster analysis

Bacterial community structure was assessed by allocating unidentified strains into common groups as a means of determining the distribution of major types against the three different tank conditions: clearwater, greenwater instant and greenwater live. Using phenotypic data, strains were grouped using ClustanGraphics ver7.04 (Clustan, Edinburgh). Proximities were calculated using the Jaccard coefficient S_j and clusters determined using the Unweighted Pair-Group Method with arithmetic Average (UPGMA) method. Glucose fermenting and non-fermenting types were treated as two separate groups. For the Vibrios, clusters were defined at the 75% similarity level and for the glucose non-fermenters at 82% similarity.

Probability identification matrix by phenotype for glucose non-fermenters

A probability matrix, General Normal Form (GNF), was developed for the identification of major types of glucose non-fermenters. Identification data was calculated for those clusters containing three or more strains. The number of strains positive for each test within a cluster was calculated; where strains were either all positive or negative for a given test, the probability value was adjusted to 99% or 1% respectively (Willcox et al., 1973). The measure of overlap between cluster groups was determined from the hypothetical median organism (HMO) for each cluster using the IDSC tool in PIBWin. Clusters were considered well separated when the Willcox probability value for the cluster HMOs were ≥ 0.99. Performance of the matrix was assessed by retrospective analysis to determine the ability of the identification matrix to correctly allocate strains to parent clusters and establish the accuracy of identification. Using the phenotypic profile of each strain, an identity was determined using PIBWin and the GluNF matrix.

8.4 Results

8.4.1 *Larval performance*

A significant difference in the standard length of larvae reared in the different treatments was recorded as early as 7 dph ($F_{2, 21} = 0.25$, $P = 0.002$), with the longest larvae in the live (5.14 ± 0.13 mm) and instant (5.15 ± 0.11 mm) treatments compared with larvae from clearwater (5.06 ± 0.14 mm) (Table 8.2). By 15 dph, larvae reared in clearwater (6.63 ± 0.30 mm) and instant algae (6.61 ± 0.33 mm) were comparable in length but significantly smaller than larvae reared in live algae (7.09 ± 0.25 mm) ($F_{2, 21} = 44.11$, $P < 0.001$). Similarly, there was a significant difference in the dry weight of larvae reared in the different treatments at 15 dph ($F_{2, 21} = 27.49$, $P < 0.001$), where larvae reared in live algae (330 ± 28 µg larva^{-1}) weighed significantly more than larvae reared in clearwater (268 ± 22 µg), which were in turn heavier than those from instant algae (232 ± 30 µg).

Table 8.2 Standard length, dry weight and morphometric performance indicators of striped trumpeter larvae reared in clearwater ('clear') or greenwater conditions using 'live' or 'instant' algae to 15 dph.

Treatment	Clear	Live	Instant	Statistical significance *P*
Standard length (mm)				
3 dph	4.70 ± 0.14 [b]	4.75 ± 0.13 [a]	4.73 ± 0.14 [ab]	0.011 *
5 dph	4.95 ± 0.08	4.95 ± 0.09	4.94 ± 0.08	0.781 ns
7 dph	5.06 ± 0.14 [b]	5.14 ± 0.13 [a]	5.15 ± 0.11 [a]	0.002 **
9 dph	5.53 ± 0.17 [c]	5.74 ± 0.15 [a]	5.63 ± 0.16 [b]	<0.001***
11 dph	5.97 ± 0.24 [b]	6.14 ± 0.21 [a]	6.02 ± 0.21 [b]	0.002 **
13 dph	6.22 ± 0.23 [c]	6.62 ± 0.20 [a]	6.32 ± 0.25 [b]	<0.001***
15 dph	6.63 ± 0.30 [b]	7.09 ± 0.25 [a]	6.61 ± 0.33 [b]	<0.001***
Dry weight (µg larva^{-1})				
3 dph	80 ± 19	88 ± 37	91 ± 44	0.801 ns
5 dph	95 ± 36	138 ± 47	128 ± 43	0.131 ns
7 dph	109 ± 47	166 ± 53	137 ± 51	0.102 ns
9 dph	143 ± 29	180 ± 46	165 ± 47	0.218 ns
11 dph	151 ± 10	168 ± 15	177 ± 36	0.103 ns
13 dph	187 ± 12 [b]	221 ± 9 [a]	193 ± 10 [b]	<0.001***
15 dph	268 ± 22 [b]	330 ± 28 [a]	232 ± 30 [c]	<0.001***
Other indices (15 dph)				
Swim bladder inflation (%)	92.5 ± 10.7	96.3 ± 3.5	96.3 ± 3.5	0.877 ns
Grey gut severity	0.03 ± 0.04 [b]	0.21 ± 0.13 [a]	0.04 ± 0.04 [b]	<0.001***
Grey gut incidence (%)	2.5 ± 3.8 [b]	20.0 ± 12.5 [a]	3.8 ± 4.4 [b]	<0.001***
Urinary calculi incidence (%)	18.1 ± 11.6 [a]	10.6 ± 7.3 [ab]	5.0 ± 5.3 [b]	0.047 *
Final survival (%)	37.6 ± 4.9 [a]	35.0 ± 9.0 [a]	7.1 ± 3.9 [b]	<0.001***

Superscripts denote a significant difference between treatments within age. Values are mean ± SD, n = 8 replicate tanks. Asterisks denote significance of one-way ANOVAs * = P < 0.05, ** = P < 0.01, *** = P < 0.001. ns = not significant.

At 15 dph there was no significant effect of treatment on swim bladder inflation ($F_{2, 21}$ = 0.81, P = 0.459), with an overall average of 95 ± 7% (Table 8.2). There was a significant effect of water type on 'grey gut' syndrome (incidence $F_{2, 21}$ = 12.64, P < 0.001; severity $F_{2, 21}$ = 13.31, P < 0.001), where the incidence and severity were significantly higher in larvae reared in live algae (20 ± 13%; 0.21 ± 0.13) compared to those reared in instant algae (4 ± 4%; 0.04 ± 0.04) or clearwater (3 ± 4%; 0.03 ± 0.04). Likewise, there was a significant effect of water type on the incidence of larvae with urinary calculi ($F_{2, 21}$ = 3.54, P = 0.047), which was significantly higher in clearwater-reared larvae (18 ± 12%) compared to those reared in instant algae (5 ± 5%), while those larvae reared in live algae (11 ± 7%) had intermediate values and were not significantly different to any other treatment. Final survival was significantly affected by treatment ($F_{2, 21}$ = 63.35, P < 0.001), and was higher in larvae reared in clearwater (38 ± 5%) and live algae (35 ± 9%) than for larvae cultured in instant algae (7 ± 4%).

Differences in cumulative mortality were recorded as early as 6 dph, with larvae reared in clearwater having greater mortality compared to those reared in live or instant algae treatments until 11 dph (P < 0.05) (Figure 8.3). From 12 dph, larvae reared in instant algae had significantly greater cumulative mortality compared to larvae in clearwater or live algae (P < 0.05). This trend continued for the duration of the experiment.

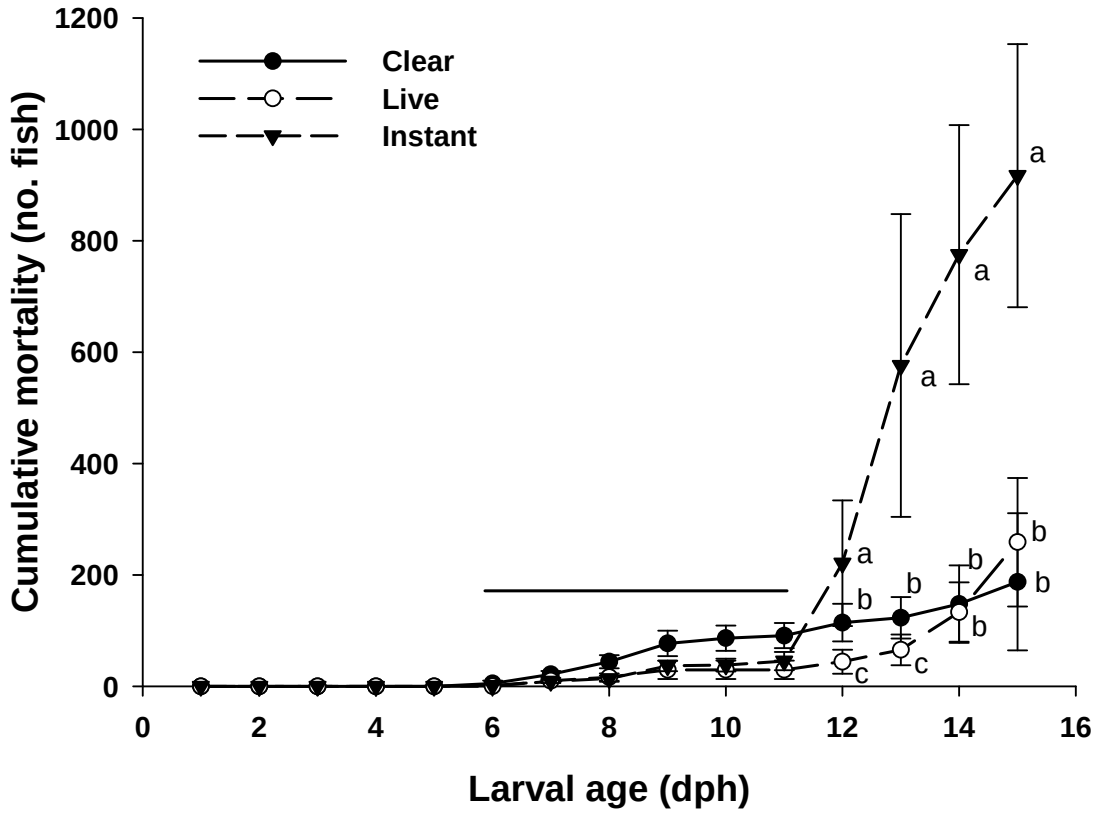


Figure 8.3 Temporal trends in cumulative mortality (number) of larval striped trumpeter reared in clearwater ('clear') or greenwater conditions using 'live' or 'instant' algae to 15 dph. Horizontal bar indicates mortality in clearwater treatment significantly higher than instant and live algae (6 to 11 dph). Superscripts denote a significant difference between treatments within age (P < 0.05). Values are mean ± SD, n = 8 replicate tanks.

8.4.2 *Total bacterial counts*

There was minimal change in the cultivable bacteria on ZMA in larvae from clearwater and live algae treatments with age, ranging from 5 to 48 cfu μg^{-1} larva DW (Figure 8.4a). In contrast counts were significantly higher ($P < 0.01$) in larvae from the instant algae treatment on 5, 11 and 15 dph, demonstrating a 48-fold increase between 3 and 15 dph. A similar pattern was observed in counts of presumptive *Vibrio* from BTBT plates (Figure 8.4 b), where counts from larvae reared in instant algae were significantly higher ($P < 0.001$) than both other treatments on 7, 11 and 15 dph and there was more than a 3000-fold increase in cfu between 3 and 15 dph (0.2 to 650 cfu μg^{-1} larva DW). Presumptive *Vibrio* counts were similar within age in clear and live algae treatments, increasing from 0.3 cfu μg^{-1} larva DW at 3 dph to 6-12 cfu μg^{-1} larva DW at 15 dph. At 14 dph, bacterial counts taken from larvae at 2130 h (12.5 h after rotifer addition), regardless of the growth medium (ZMA or BTBT) were significantly higher in larvae held in instant algae compared with the remaining two treatments ($F_{2, 21} = 29.85$, $P < 0.001$ and $F_{2, 21} = 26.83$, $P < 0.001$, respectively) (Table 8.3), and for each treatment were higher than counts obtained on all days before rotifer feeding (0830 h) (Figure 8.4).

Table 8.3 The number of cultivable bacteria (colony forming units, cfu) in larvae and tank water samples at 2130 h on 14 dph in striped trumpeter reared in clearwater ('clear') or greenwater conditions using 'live' or 'instant' algae to 15 dph, and the number of bacteria (cfu ml^{-1}) in reservoirs containing the different treatments at 0830 h. Colonies were grown on ZMA and BTBT agar.

Treatment	Clear	Live	Instant	*P*
Larvae, 14 dph (cfu μg^{-1} larva DW)				
ZMA	83 ± 56^{b}	197 ± 361^{b}	1989 ± 1968^{a}	<0.001***
BTBT	24 ± 27^{b}	100 ± 253^{b}	1294 ± 1400^{a}	<0.001***
Tank water, 14 dph (cfu ml^{-1})				
ZMA	54583 ± 27223^{b}	106310 ± 14026^{a}	nd	0.001 **
BTBT	3979 ± 1575^{b}	5595 ± 2171^{b}	68458 ± 25452^{a}	<0.001***
Reservoirs (cfu ml^{-1})				
ZMA	39875 ± 53135^{b}	63083 ± 62803^{b}	9500000 ± 666667^{a}	<0.001***
BTBT	128 ± 83^{b}	826 ± 1321^{b}	1477778 ± 559100^{a}	<0.001***
Reservoirs, 14 dph (cfu ml^{-1}), time after setup to acclimate				
ZMA 0.5 h	3333	38333	4667	
10.25 h	15500	58333	11333333	
20.75 h	116667	43333	9500000	
BTBT 0.5 h	17	133	2333	
10.25 h	17	67	3500000	
20.75 h	133	83	833333	

Superscripts denote a significant difference between treatments within rows. Values are mean ± SD, n = 8 replicate tanks for larvae and tank water samples, $n \geq 3$ for reservoirs. Asterisks denote significance of one-way ANOVAs * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. nd = not determined (too many colonies to count at highest dilution).

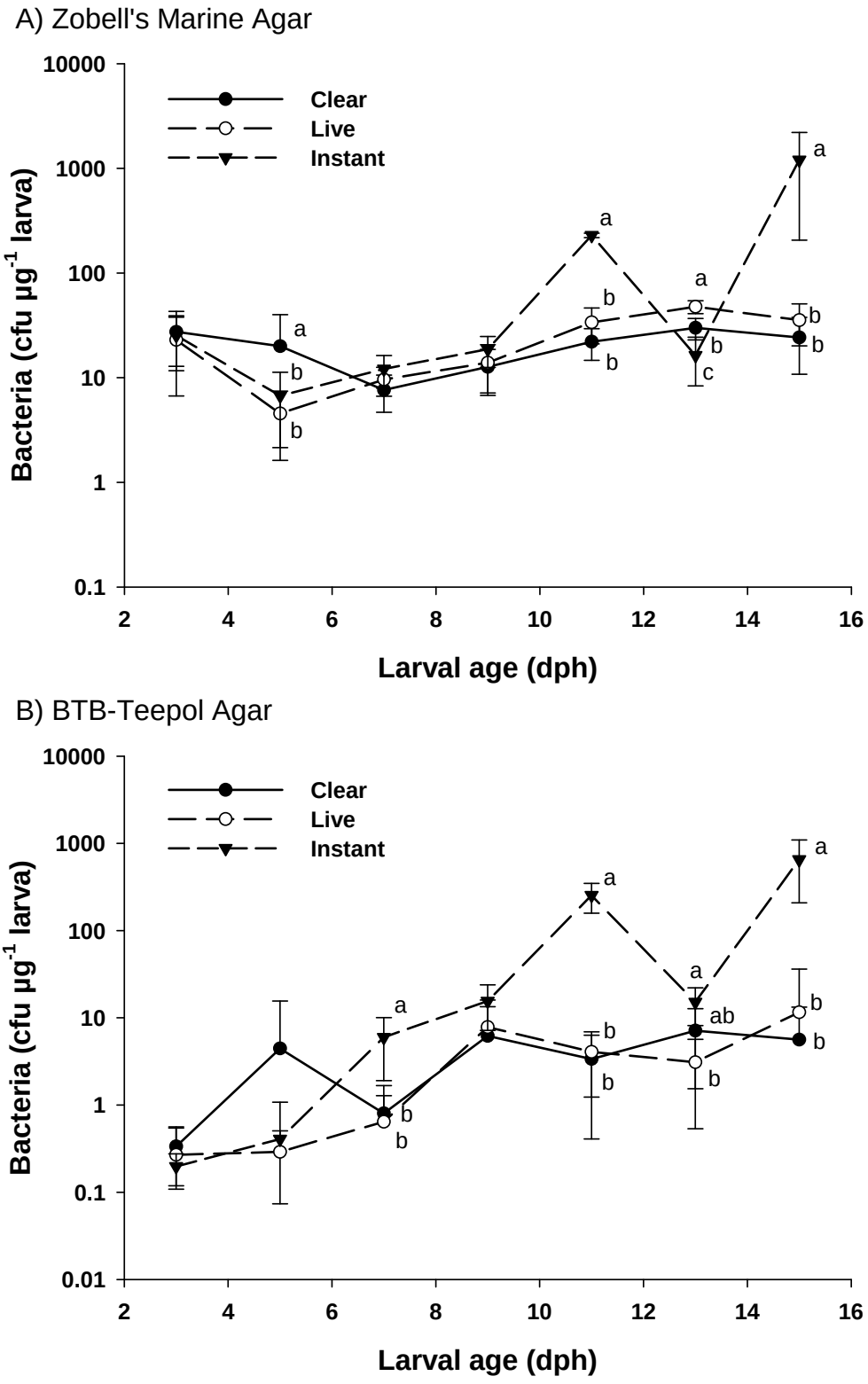


Figure 8.4 Temporal trends in the number of bacteria (colony forming units, cfu) per µg (dry weight) of larva in striped trumpeter reared in clearwater ('clear') or greenwater conditions using 'live' or 'instant' algae to 15 dph. Colonies were grown on (A) Zobell's Marine Agar or (B) BTB-Teepol agar. Superscripts denote a significant difference between treatments within age ($P < 0.05$). Values are mean ± SD, n = 8 replicate tanks. Y-axes are on a logarithmic scale.

Similarly, water samples taken from the reservoirs each morning at 0830 h revealed that total bacterial numbers were significantly higher in the instant treatment compared with clearwater and live algae (for ZMA $F_{2, 10} = 19.69$, $P < 0.001$ and for BTBT $F_{2, 12} = 99.87$, $P < 0.001$) (Table 8.3). Counts from water samples within culture tanks gave a similar result, with significantly higher bacterial numbers on ZMA and BTBT in instant than other treatments from 7 to 15 dph (Figure 8.5a). There was an overall decrease in counts on ZMA with culture age, from 3 x 10^5 cfu ml^{-1} for all treatments at 3 dph decreasing to 4%, 4% and 13% of initial values in clear, live and instant treatments, respectively at 15 dph. Presumptive *Vibrio* on BTBT increased to a peak on 7 dph in the instant treatment (8 x 10^5 cfu ml^{-1}) and then declined to 15 dph (2 x 10^3 cfu ml^{-1}, 62% of count at 3 dph) (Figure 8.5b). There was a greater reduction in presumptive *Vibrio* in culture water to 15 dph in clearwater (4% of count at 3 dph) than in live algae (45% of count at 3 dph).

Samples from the reservoirs on 14-15 dph at 0.5, 10.25 and 20.75 h from setting up the water to acclimate revealed a rapid proliferation of cultivable bacteria from as early as 30 min in the instant treatment (Table 8.3). There was a peak in bacteria in the instant reservoir after 10.25 h to 1 x 10^7 on ZMA and 4 x 10^6 on BTBT, then a decline before addition to larval tanks at 20.75 h. In rotifers, the counts on ZMA and BTBT were 192 ± 7 cfu $rotifer^{-1}$ and 53 ± 52 cfu $rotifer^{-1}$, respectively (n = 5 sample days).

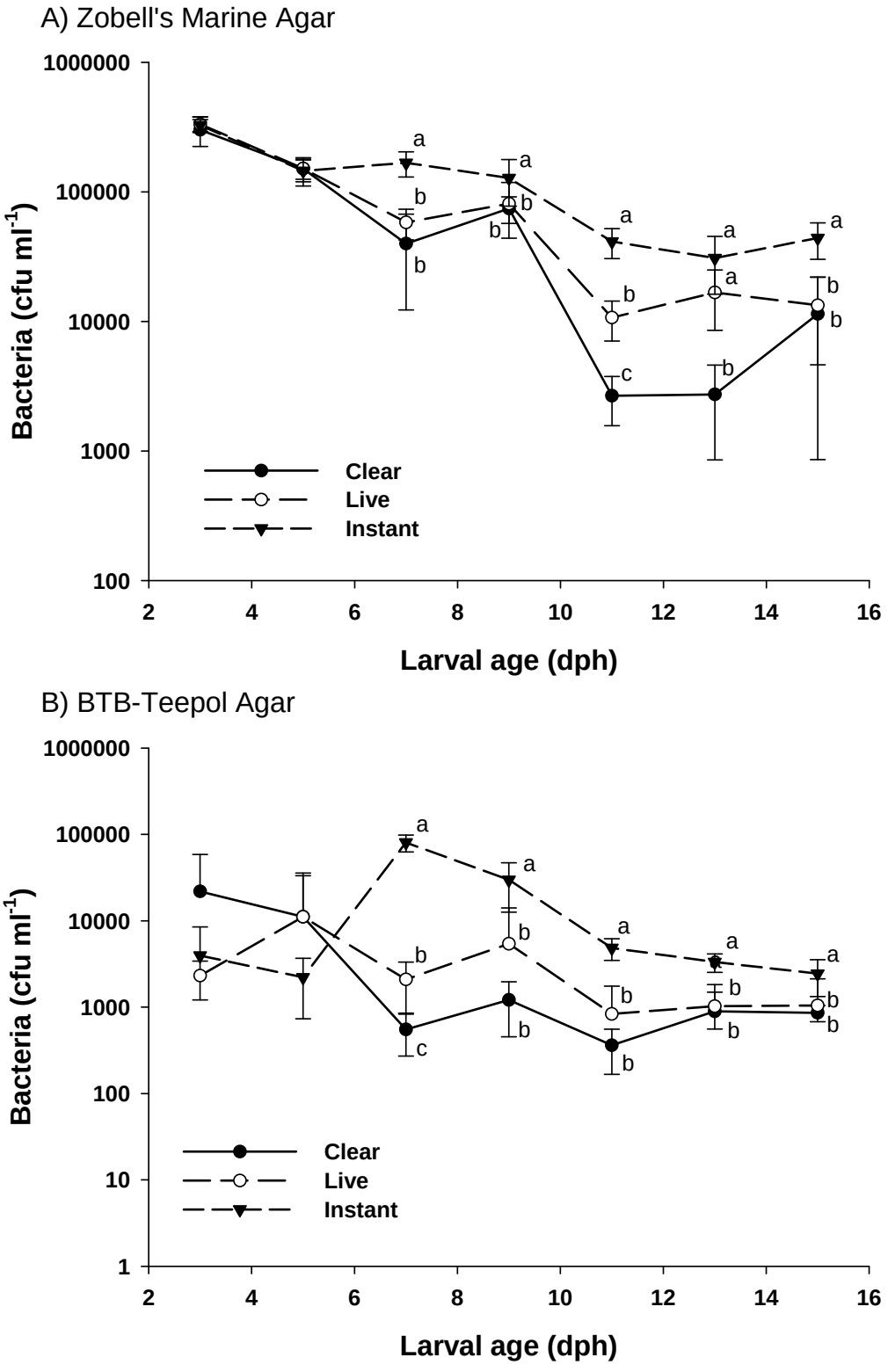


Figure 8.5 Temporal trends in the number of bacteria (colony forming units, cfu) per ml of tank water from in striped trumpeter reared in clearwater ('clear') or greenwater conditions using 'live' or 'instant' algae to 15 dph. Colonies were grown on (A) Zobell's Marine Agar or (B) BTB-Teepol agar. Superscripts denote a significant difference between treatments within age ($P < 0.05$). Values are mean ± SD, n = 8 replicate tanks. Y-axes are on a logarithmic scale.

8.4.3 *Bacterial community identification*

Evaluation of selective media for *Vibrio* species - seawater inoculum

The difference in salinity between the three sample sites was marginal between Bell Bay and Bridport suggesting that at the time of collection the Bell Bay site was almost truly marine (Table 8.4). While salinity level at the two sites was similar it was evident on the basis of presumptive *Vibrio* counts that higher levels of *Vibrio* were present at Bridport, the marine site. At the estuarine and brackish sites, *Vibrio* counts were consistently lower irrespective of medium used. Using ZMA as a reference, the recovery of presumptive Vibrios was consistently higher using BTBT compared to TCBS. Differences in performance between the two media were less pronounced at the marine site. Differences in the ratio of sucrose positive and negative colony types were pronounced between the types of media and the sample sites. For the marine site, the ratios were similar between media and indicate that there were almost twice as many sucrose positive types compared to sucrose negative types in the sample. At the other two sites there were marked differences in recovery rates between sucrose positive and negative types of *Vibrio*. Differences may reflect the degree of selectivity between the media which might distort the apparent differences in the types of bacteria which were recovered. Overall, the data suggests that BTBT is more suitable for recovering environmental Vibrios compared to TCBS.

Table 8.4 Selective *Vibrio* media performance based on water samples

Site	Salinity ‰	Medium	cfu/ml	BTBT/ZMA	TCBS/ZMA	TCBS/BTBT	Su^+/Su^- ratio
Windermere	20	ZMA	386				
		BTBT	56	15%	2%	14%	65%
		TCBS	8				33%
Bell Bay	30	ZMA	470				
		BTBT	222	47%	20%	42%	85%
		TCBS	93				167%
Bridport	34	ZMA	306				
		BTBT	258	84%	60%	71%	207%
		TCBS	184				188%

Evaluation of selective media for *Vibrio* species - named species

An objective assessment of growth on TCBS and BTBT was made with a range of named *Vibrionaceae* (Table 8.5). All the species grew on BTBT but *Ph. phosphoreum* and *V. metschnikovii* both failed to grow on TCBS. The growth index was consistently higher on BTBT suggesting that for the range of species tested, better growth was achieved on this medium than on TCBS. The only exception was the wild type strain of *V. cholerae* which grew better on TCBS than on BTBT; this reflects the intended purpose of TCBS which is to select for *V. cholerae*.

Growth of the non-*Vibrionaceae* varied on both media (Table 8.6). TCBS was better than BTBT at inhibiting growth of these non-target species (based on total growth index score); neither medium suppressed growth of all the species tested though both appeared effective at suppressing growth of the two Gram-positive species, *L. garvieae* and *St. warneri*.

Table 8.5 Productivity and selectivity of BTBT and TCBS selective agars of *Vibrionaceae*

Species	Growth index		Species	Growth index	
	BTBT	TCBS		BTBT	TCBS
Ph. angustum	3	3	*V. aestuarianus*	2	1
Ph. damselae subsp *damselae*	3	3	*V. cincinnatiensis*	2	1
Ph. iliopiscarium	3	3	*A. fischeri*	1	1
V. alginolyticus	3	3	*A. logei*	1	1
V. diazotrophicus	3	3	*G. hollisae*	1	1
V. fluvialis	3	3	*M. viscosa*	1	1
V. furnissii	3	3	*Ph. damselae* ssp *piscicida*	1	1
V. mediterranei	3	3	*V. agarivorans*	1	1
V. harveyi	3	2	*V. anguillarum*	1	1
V. campbellii	3	1	*V. ichthyoenteri*	1	1
V. cholerae	2	3	*V. lentus*	1	1
V. calviensis	2	2	*Ph. phosphoreum*	1	0
V. cyclitrophicus	2	2	*V. metschnikovii*	1	0
V. halioticoli	2	2			
Ph. leiognathi	2	1	**Total score**	**55**	**48**

Figure 8.6 Productivity and selectivity of BTBT and TCBS selective agars of non-*Vibrionaceae*

Species	Growth index	
	BTBT	TCBS
L. garvieae	0	0
St. warneri	0	1
Ps. fluorescens	1	1
Pl. shigelloides	1	1
Y. ruckeri	1	3
Pr. vulgaris	2	2
A. veronii bv *sobria*	2	2
Sh. putrefaciens	2	3
E. coli	3	0
Ps. putida	3	0
A. hydrophila	3	2
Total score	18	15

From these assessments of the two media using named species as well as water samples, it was concluded that BTBT would likely recover more Vibrios than TCBS. Accordingly, BTBT was used as the selective *Vibrio* medium for assessing bacterial floras of larvae held under different culture conditions.

Bacterial identification and characterisation

Of the 750 isolates held at –80 °C, 515 were recovered viable, representing 69% of the collection. The cause of storage failure in this number of strains is not known but is unexpectedly high.

The recovered isolates could be placed into two broad groups: the Vibrios and the glucose non-fermenters. Isolates were considered presumptive Vibrios if they fermented glucose, were oxidase positive and were sensitive to the pteridine compound 0/129 at 150µg. Using

these criteria, 39% of the total flora were considered to be Vibrios. The remaining 61% of isolates did not ferment glucose although of these 312 isolates, 266 or 85% of strains were able to use glucose as a sole carbon source.

Of the 203 isolates considered to be Vibrios, 48 strains could be confidently identified to species level (Table 8.6); these strains all had Willcox probability scores of $P \geq 0.99$ and modal likelihood scores ≥ 0.001. A second group of 52 isolates could be identified presumptively on the basis of a Willcox probability score $P \geq 0.99$ but with modal likelihood scores ≤ 0.001. Isolates in this category have all the major characteristics of the nominated species but have at least one phenotypic outlier that places the isolate outside the established boundaries for the species. Species to which isolates are allocated on this basis are listed in Table 8.7. A third group of 102 isolates could not be allocated to a species within the VibEx7 database on the basis of probabilistic identification.

Table 8.6 High confidence identification of *Vibrionaceae* from all striped trumpeter larvae, irrespective of source

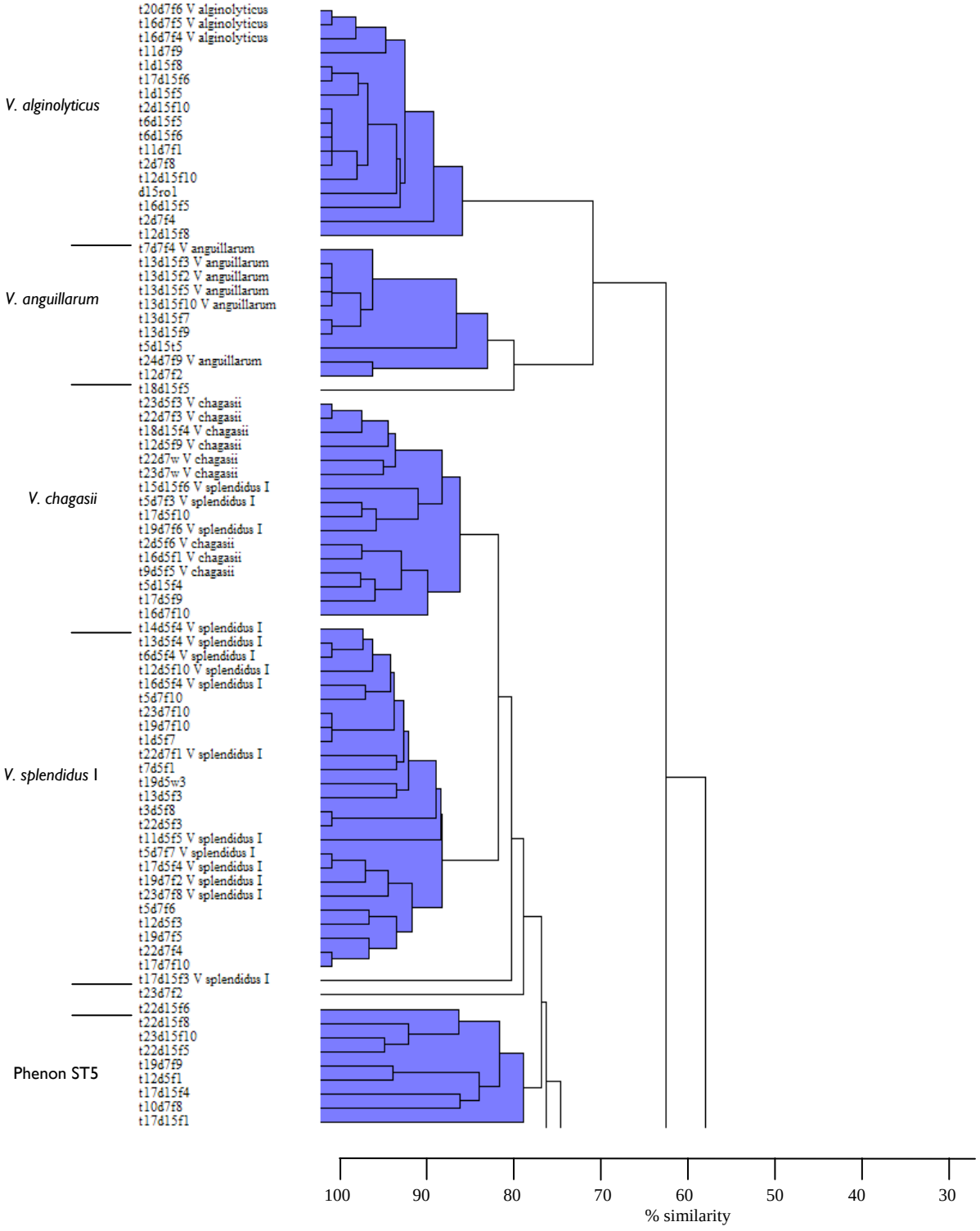
Species	**Species**
Aliivibrio fischeri biovar I	*Vibrio chagasii*
Aliivibrio fischeri biovar II	*Vibrio ichthyoenteri* biovar II
Vibrio alginolyticus	*Vibrio penaeicida*
Vibrio anguillarum	*Vibrio splendidus* biovar I

Table 8.7 Species containing isolates with outlier characteristics

Species
Aliivibrio fischeri biovar I
Aliivibrio fischeri biovar II
Vibrio anguillarum
Vibrio chagasii
Vibrio splendidus biovar I
Vibrio Phenon V36

Cluster analysis

By cluster analysis of the presumptive *Vibrio* group, 16 groups could be defined at the 75% similarity level, comprising the species listed in Table 8.6, together with a further 8 unique but un-named groups arbitrarily designated Phenon ST5, ST6, ST8, ST9, ST10, ST11, ST13 and ST16 (Figure 8.7). Of the 203 isolates designated presumptive Vibrios, 166 could be allocated to one of the 16 clusters, representing 81% of strains; 38 strains could not be allocated to a cluster.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V. anguillarum
V. chagasii
V. splendidus I
Phenon ST5
t20d7f6 V alginolyticus
t16d7f5 V alginolyticus
t16d7f4 V alginolyticus
t11d7f9
t1d15f8
t17d15f6
t1d15f5
t2d15f10
t6d15f5
t6d15f6
t11d7f1
t2d7f8
t12d15f10
d15ro1
t16d15f5
t2d7f4
t12d15f8
t7d7f4 V anguillarum
t13d15f3 V anguillarum
t13d15f2 V anguillarum
t13d15f5 V anguillarum
t13d15f10 V anguillarum
t13d15f7
t13d15f9
t5d15t5
t24d7f9 V anguillarum
t12d7f2
t18d15f5
t23d5f3 V chagasii
t22d7f3 V chagasii
t18d15f4 V chagasii
t12d5f9 V chagasii
t22d7w V chagasii
t23d7w V chagasii
t15d15f6 V splendidus I
t5d7f3 V splendidus I
t17d5f10
t19d7f6 V splendidus I
t2d5f6 V chagasii
t16d5f1 V chagasii
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t5d15f4
t17d5f9
t16d7f10
t14d5f4 V splendidus I
t13d5f4 V splendidus I
t6d5f4 V splendidus I
t12d5f10 V splendidus I
t16d5f4 V splendidus I
t5d7f10
t23d7f10
t19d7f10
t1d5f7
t22d7f1 V splendidus I
t7d5f1
t19d5w3
t13d5f3
t3d5f8
t22d5f3
t11d5f5 V splendidus I
t5d7f7 V splendidus I
t17d5f4 V splendidus I
t19d7f2 V splendidus I
t23d7f8 V splendidus I
t5d7f6
t12d5f3
t19d7f5
t22d7f4
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80
70
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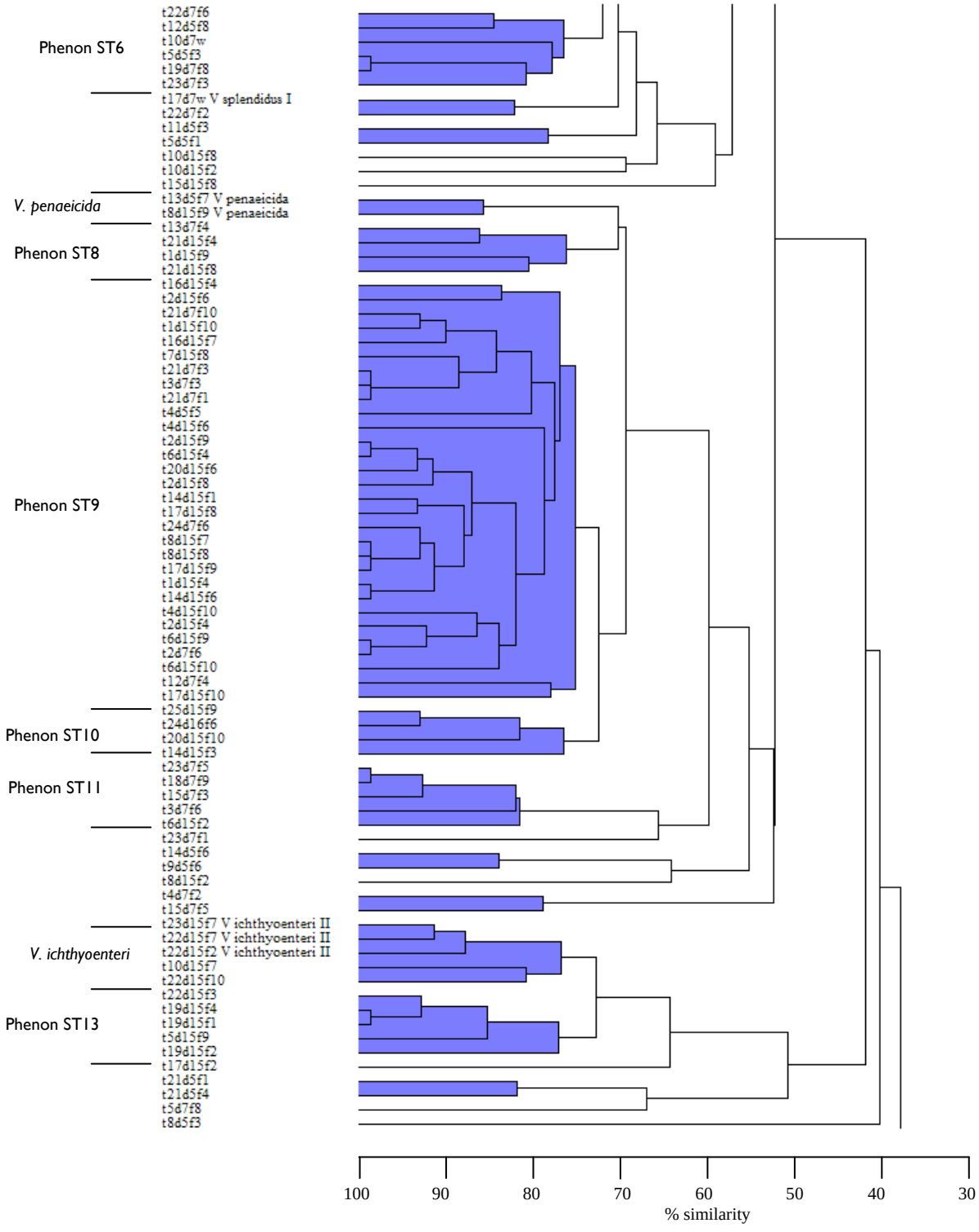
50
40
30
% similarity

.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V. penaeicida
Phenon ST8
Phenon ST9
Phenon ST10
Phenon ST11
V. ichthyoenteri
Phenon ST13
t22d7f6
t12d5f8
t10d7w
t5d5f3
t19d7f8
t23d7f3
t17d7w V splendidus I
t22d7f2
t11d5f3
t5d5f1
t10d15f8
t10d15f2
t15d15f8
t13d5f7 V penaeicida
t8d15f9 V penaeicida
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t1d15f9
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t16d15f4
t2d15f6
t21d7f10
t1d15f10
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t2d15f4
t6d15f9
t2d7f6
t6d15f10
t12d7f4
t17d15f10
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t6d15f2
t23d7f1
t14d5f6
t9d5f6
t8d15f2
t4d7f2
t15d7f5
t23d15f7 V ichthyoenteri II
t22d15f7 V ichthyoenteri II
t22d15f2 V ichthyoenteri II
t10d15f7
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t22d15f3
t19d15f4
t19d15f1
t5d15f9
t19d15f2
t17d15f2
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100
90
80
70
60
50
40
30
% similarity

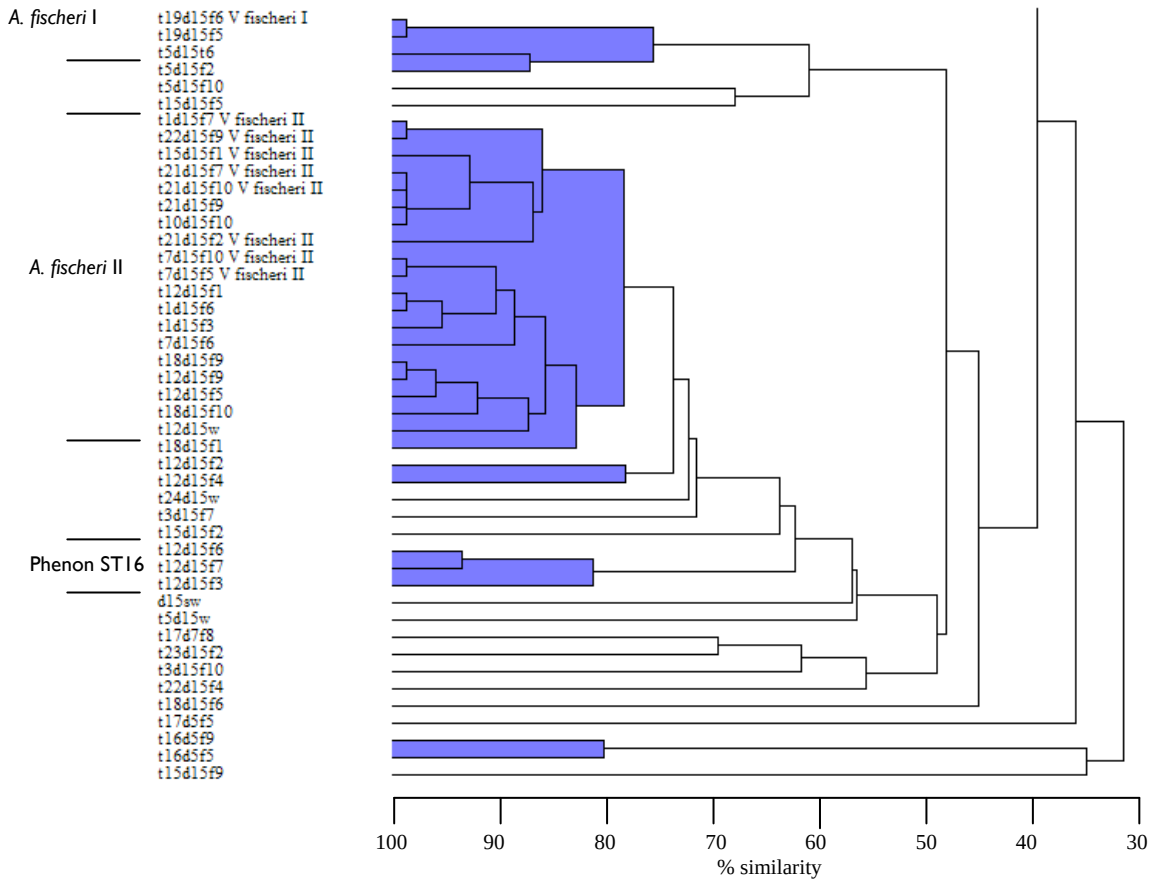
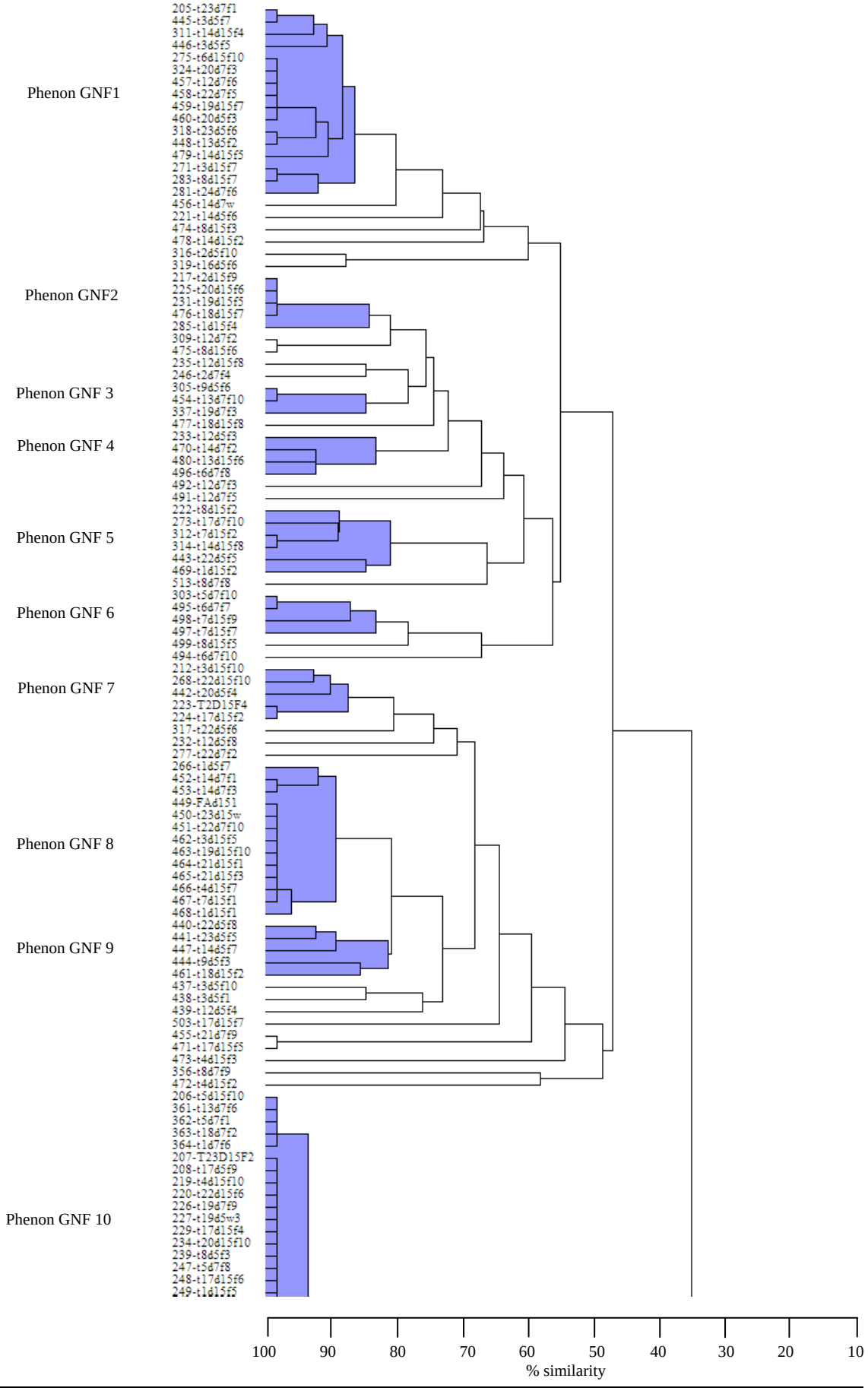


Figure 8.7 Phenogram of *Vibrio* isolates from striped trumpeter larvae; proximities calculated by Jaccard's coefficient and clustered by the UPGMA method. Strains from all tank treatments and times. Clusters defined at the 75% similarity level.

The glucose non-fermenters could be partitioned into 19 clusters at an 82% similarity level (Figure 8.8). This similarity level is more stringent than that used for the *Vibrio* data set because no defined markers were available which could be used to set cluster boundaries. In the absence of such data a more conservative approach was used for defining clusters. The 19 clusters accommodate 80% of the isolates; 64 strains could not be allocated to a cluster. The distribution of strains across the 19 different clusters is summarised in Table 8.8, together with the hypothetical median organism for each cluster. The largest group is Phenon GNF10 which contains 41% of the allocated isolates.

Table 8.8 Distribution of strains across defined clusters of glucose non-fermenters. The Hypothetical Median Organism (HMO) is the exemplar of the specified cluster

Phenon	HMO strain	No. in cluster
Phenon GNF 1	t6d15f10	16
Phenon GNF 2	t19d15f5	5
Phenon GNF 3	t13d7f10	3
Phenon GNF 4	t14d7f2	4
Phenon GNF 5	t7d15f2	6
Phenon GNF 6	t5d7f10	4
Phenon GNF 7	t22d15f10	5
Phenon GNF 8	t3d15f5	13
Phenon GNF 9	t22d5f8	5
Phenon GNF 10	t6d7f3	102
Phenon GNF 11	t20d7f5	29
Phenon GNF 12	t18d15f1	6
Phenon GNF 13	t15d7f5	3
Phenon GNF 14	t1d15f3	7
Phenon GNF 15	t4d15f6	17
Phenon GNF 16	t11d7f1	9
Phenon GNF 17	t16d7f8	3
Phenon GNF 18	t8d5w3	4
Phenon GNF 19	t15d15f5	8


Phenon GNF1
Phenon GNF2
Phenon GNF 3
Phenon GNF 4
Phenon GNF 5
Phenon GNF 6
Phenon GNF 7
Phenon GNF 8
Phenon GNF 9
Phenon GNF 10
205-t23d7f1
445-t3d5f7
311-t14d15f4
446-t3d5f5
275-t6d15f10
324-t20d7f3
457-t12d7f6
458-t22d7f5
459-t19d15f7
460-t20d5f3
318-t23d5f6
448-t13d5f2
479-t14d15f5
271-t3d15f7
283-t8d15f7
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232-t12d5f8
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266-t1d5f7
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472-t4d15f2
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207-T23D15F2
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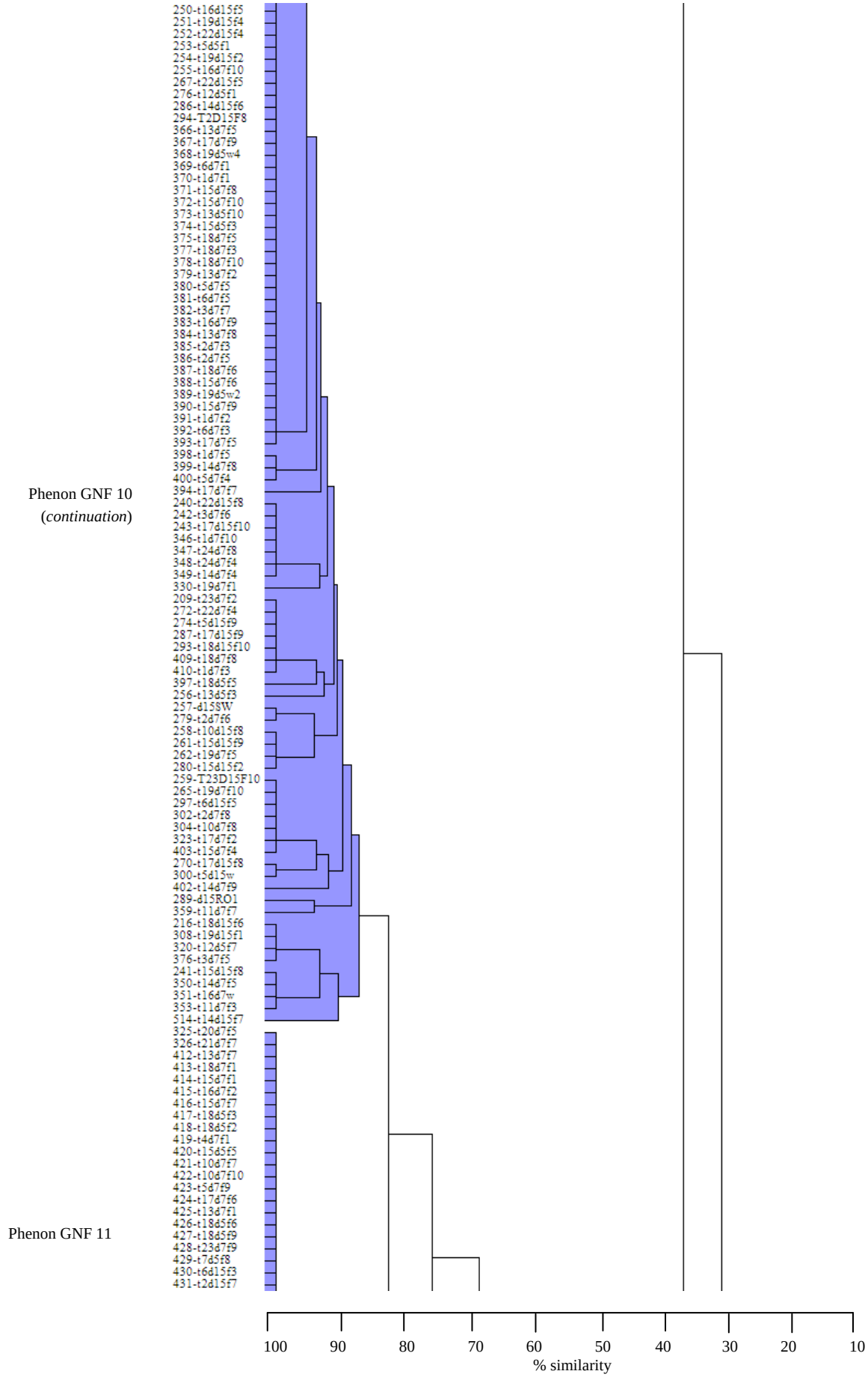
226-t19d7f9
227-t19d5w3
229-t17d15f4
234-t20d15f10
239-t8d5f3
247-t5d7f8
248-t17d15f6
249-t1d15f5
100
90
80
70
60
50
40
30
20
10
% similarity


Phenon GNF 10
(*continuation*)
Phenon GNF 11
250-t16d15f5
251-t19d15f4
252-t22d15f4
253-t5d5f1
254-t19d15f2
255-t16d7f10
267-t22d15f5
276-t12d5f1
286-t14d15f6
294-T2D15F8
366-t13d7f5
367-t17d7f9
368-t19d5w4
369-t6d7f1
370-t1d7f1
371-t15d7f8
372-t15d7f10
373-t13d5f10
374-t15d5f3
375-t18d7f5
377-t18d7f3
378-t18d7f10
379-t13d7f2
380-t5d7f5
381-t6d7f5
382-t3d7f7
383-t16d7f9
384-t13d7f8
385-t2d7f3
386-t2d7f5
387-t18d7f6
388-t15d7f6
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390-t15d7f9
391-t1d7f2
392-t6d7f3
393-t17d7f5
398-t1d7f5
399-t14d7f8
400-t5d7f4
394-t17d7f7
240-t22d15f8
242-t3d7f6
243-t17d15f10
346-t1d7f10
347-t24d7f8
348-t24d7f4
349-t14d7f4
330-t19d7f1
209-t23d7f2
272-t22d7f4
274-t5d15f9
287-t17d15f9
293-t18d15f10
409-t18d7f8
410-t1d7f3
397-t18d5f5
256-t13d5f3
257-d15SW
279-t2d7f6
258-t10d15f8
261-t15d15f9
262-t19d7f5
280-t15d15f2
259-T23D15F10
265-t19d7f10
297-t6d15f5
302-t2d7f8
304-t10d7f8
323-t17d7f2
403-t15d7f4
270-t17d15f8
300-t5d15w
402-t14d7f9
289-d15RO1
359-t11d7f7
216-t18d15f6
308-t19d15f1
320-t12d5f7
376-t3d7f5
241-t15d15f8
350-t14d7f5
351-t16d7w
353-t11d7f3
514-t14d15f7
325-t20d7f5
326-t21d7f7
412-t13d7f7
413-t18d7f1
414-t15d7f1
415-t16d7f2
416-t15d7f7
417-t18d5f3
418-t18d5f2
419-t4d7f1
420-t15d5f5
421-t10d7f7
422-t10d7f10
423-t5d7f9
424-t17d7f6
425-t13d7f1
426-t18d5f6
427-t18d5f9
428-t23d7f9
429-t7d5f8
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% similarity

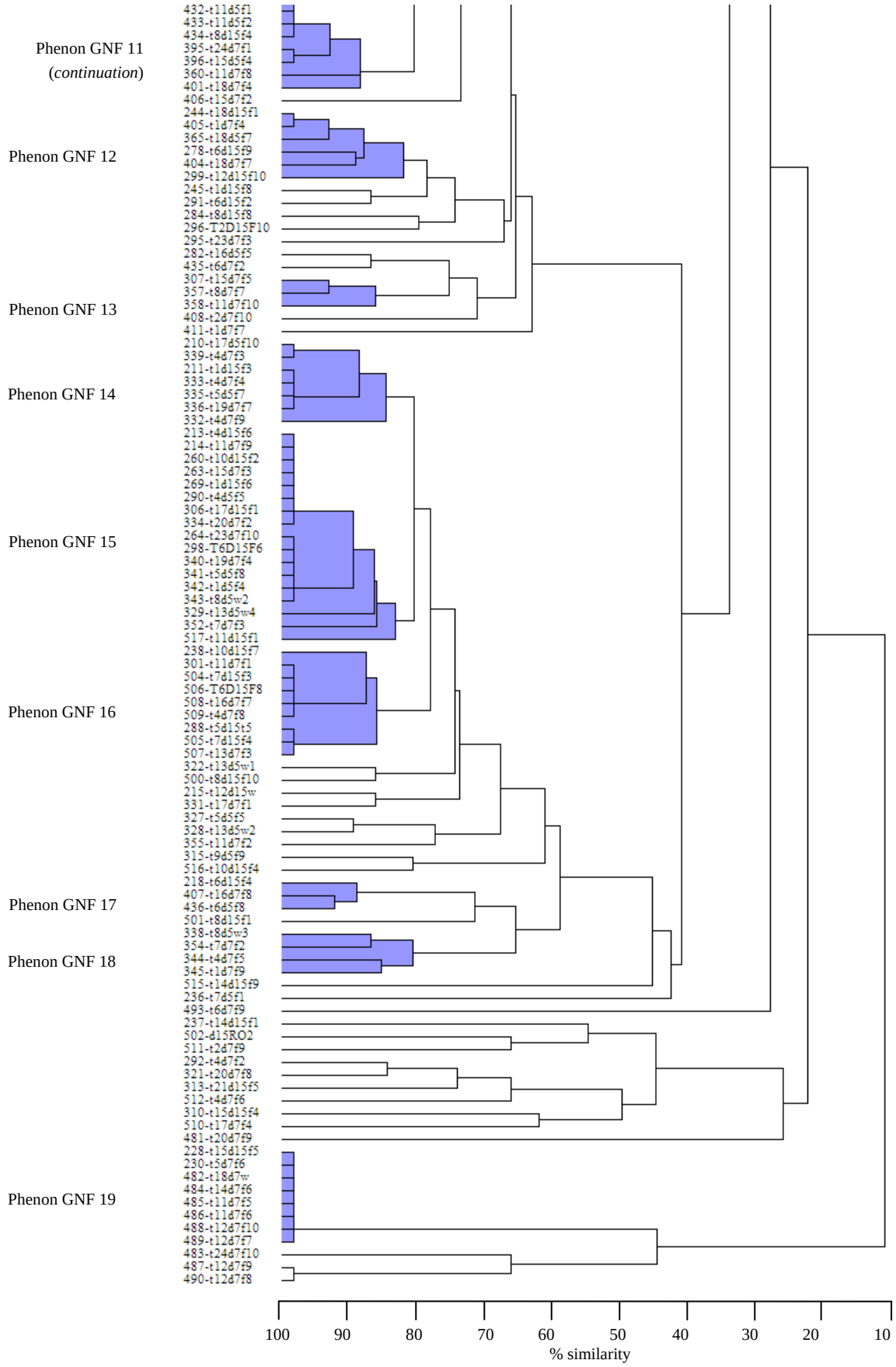


Figure 8.8 Phenogram of glucose non-fermenters from striped trumpeter larvae; proximities calculated by Jaccard's coefficient and clustered by the UPGMA method. Strains from all tank treatments and times. Clusters defined at the 82% similarity level.

Probability identification matrix for glucose non-fermenters

A probability matrix for the identification of glucose non-fermenters was constructed from the data for the 19 phenons; the matrix is given in 8.9 - Appendix. The discreteness of the individual phenons was assessed from the Willcox probability value *P* for the HMO of each phenon group in the matrix. Most of the phenons had *P* values of at least 0.99 (Table 8.9), except for GNF 14 and GNF 16, where the *P* value was less than 0.99. This apparent fuzziness in phenotype, seen by the reduced *P* score, also means that there is some overlap between the two phenons. All phenons are differentiated by at least two tests except for GNF 14 and 16 which are separated by only one test, utilisation of citrate as a sole carbon source.

Table 8.9 Willcox probability values *P* for hypothetical median organisms for glucose non-fermenter strains from the probability identification matrix GNF.

Phenon	Willcox *P*	Overlap
GNF 1	1.00000	
GNF 2	0.99996	
GNF 3	1.00000	
GNF 4	1.00000	
GNF 5	1.00000	
GNF 6	1.00000	
GNF 7	1.00000	
GNF 8	0.99813	
GNF 9	0.99937	
GNF 10	0.99998	
GNF 11	0.99997	
GNF 12	1.00000	
GNF 13	1.00000	
GNF 14	0.98397	GNF 16
GNF 15	0.99923	
GNF 16	0.98933	GNF 14
GNF 17	1.00000	
GNF 18	1.00000	
GNF 19	1.00000	

In retrospective testing of the matrix to assess accuracy and precision, there appeared to be a high level of both accuracy (strain allocated to parent cluster) and precision seen as Willcox probability scores equalling or exceeding the acceptance threshold of $P = 0.99$ (Table 8.10). All 248 strains were correctly allocated to their parent cluster and 89% of strains had *P* values of ≥ 0.99.

Table 8.10 Willcox probability scores *P* for glucose non-fermenting strains (n=248) belonging to phenons GNF1-19.

ID Score Range	Frequency	%	Cumulative %
1.000-0.999	202	81%	81%
0.990-0.998	18	7%	89%
0.98-0.989	10	4%	93%
0.950-0.979	8	3%	96%
0.900-0.949	7	3%	99%
0.800-0.899	3	1%	100%
Total	**248**	**100%**	

Distribution of strains by tank treatment and by time

Labelled strains either, as species of *Vibrio* or as unclassified phenons, were allocated to water type treatment and by time as a means of assessing the emergence of particular types or the frequency of particular types within a given population. Summaries of distributions are given pictorially in Figure 8.9 and Figure 8.10. Temporal changes in bacterial community structure are evident across the two major groups defined, Vibrios and glucose non-fermenters, and three different tank culture conditions. Clearly discernible is the relatively simple community structure that has developed by Day 5, comprising 19 distinct types, while by Day 15 this has increased to 30 types, a marked increase in diversity. For the Vibrios almost half the flora by Day 15 is comprised of just two phenotypes: *A. fischeri* biovar II and *Vibrio* phenon ST9. Evident also is the emergence of two potentially pathogenic species, *V. anguillarum* and *V. ichthyoenteri*. Development in community structure is less apparent with the glucose non-fermenters and there is little change with time in the number of different types found. A consistent finding was that Phenon GNF10 formed the largest and most persistently represented of the types isolated, ranging from 29-45% of the flora.

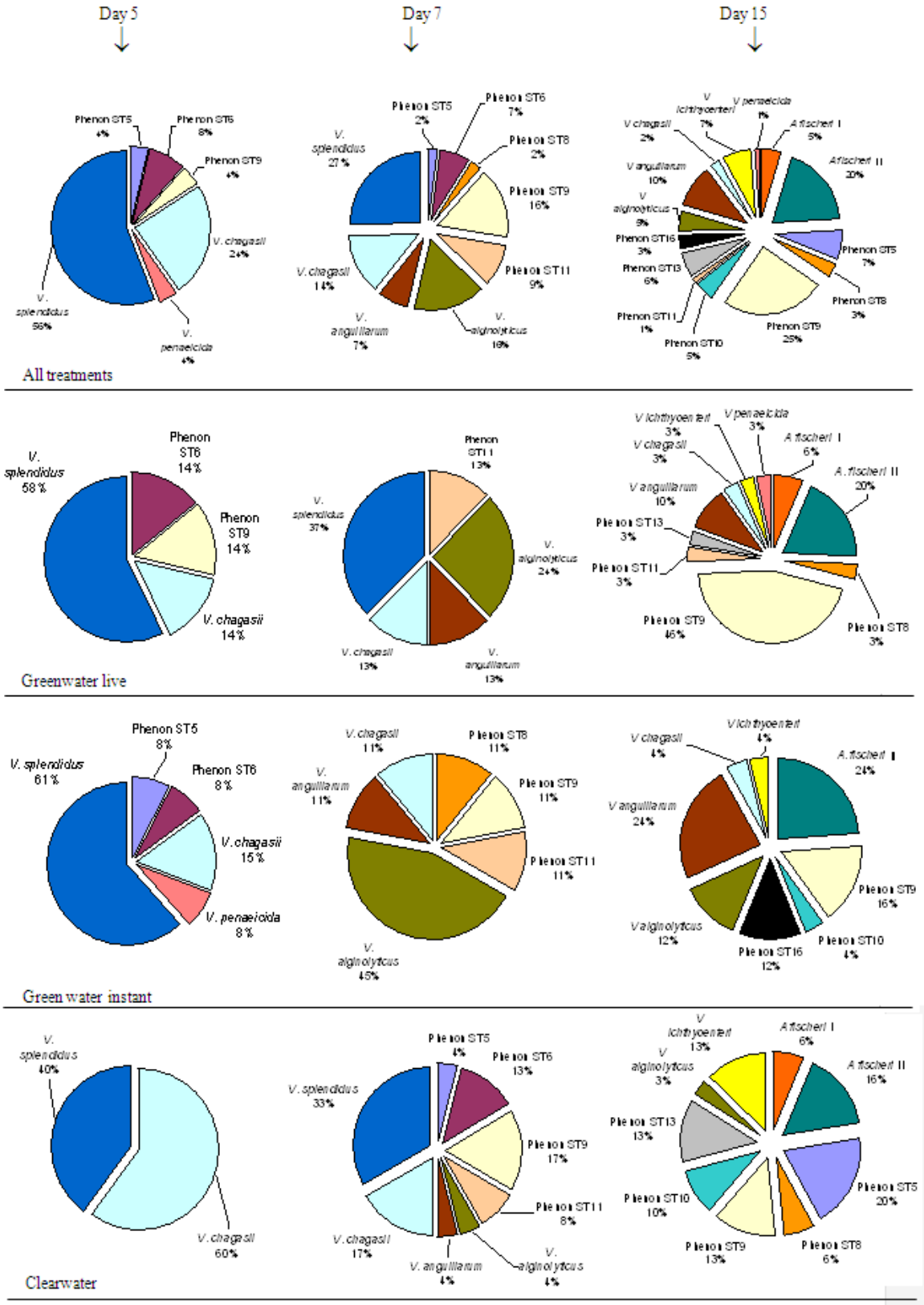
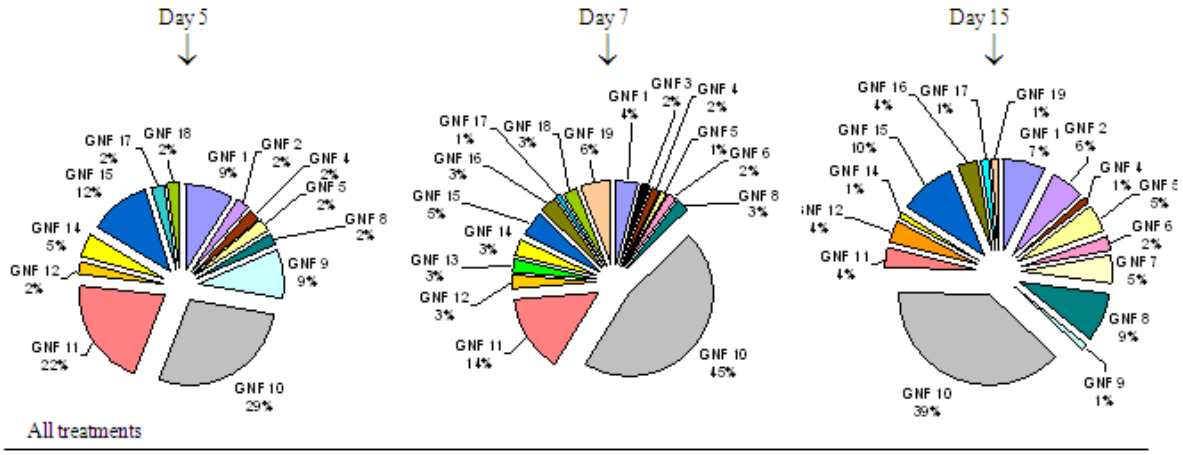
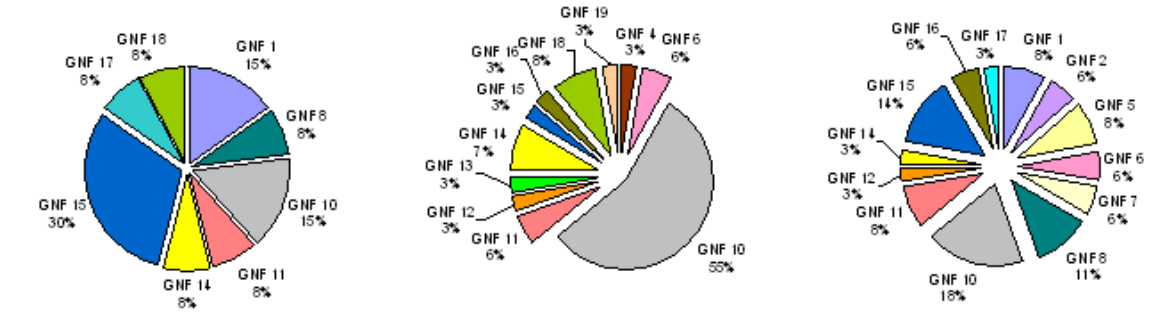
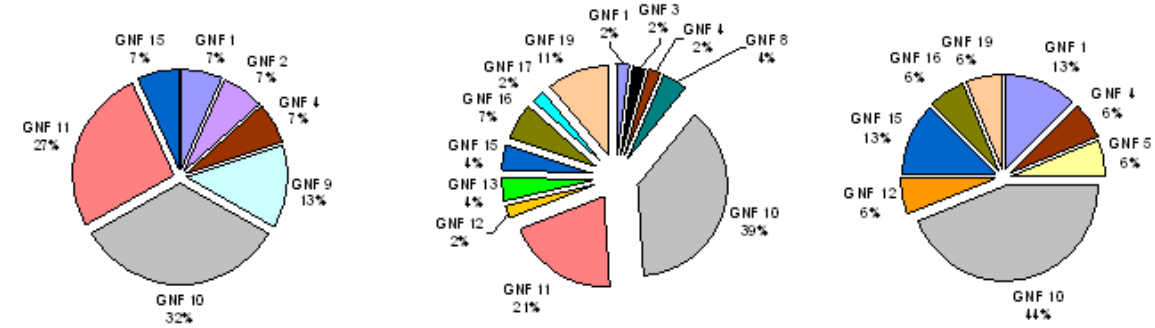


Figure 8.9 Distribution of *Vibrio* phenotypes by treatment and by days post larval hatch


Day 5
Day 7
Day 15
All treatments


Greenwater live


Green water instant

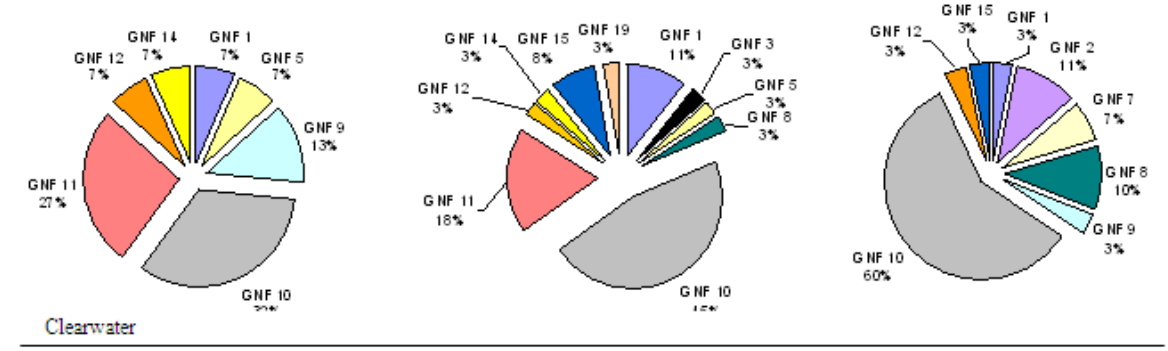

Clearwater

Figure 8.10 Distribution of glucose non-fermenting phenotypes by treatment and by days post larval hatch

The three tank environments were associated with differences in bacterial floras isolated from the striped trumpeter larvae. More complex floras were developed in the greenwater treatments compared to clearwater, seen for both the *Vibrio* and glucose non-fermenter floras (Figure 8.11). With increasing time post-hatch, the diversity in bacterial flora became similar for the greenwater instant and clearwater tanks, but for the greenwater live by Day 15 there was a marked increase in the number of bacterial types present compared to the other two tank conditions.

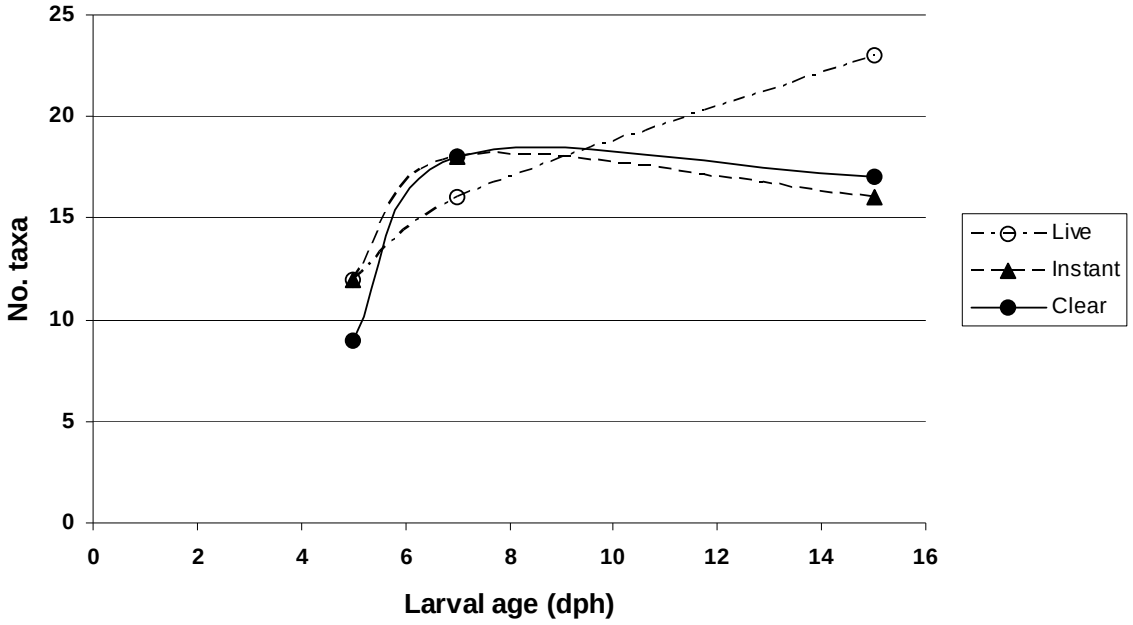


Figure 8.11 Changes in the number of bacterial types isolated from striped trumpeter larvae days post-hatch across different tank environments

8.5 Discussion

The performance of striped trumpeter larvae was optimal in live algae greenwater culture and poorest in the instant algae greenwater system in relation to growth and survival. Whilst final survival was high in clearwater, growth was lower than in live algae greenwater. Many studies report benefits to larval fish performance in greenwater related to direct and indirect larval nutrition, enhanced prey visibility, larval distribution, stable water quality and microbial flora (Reitan, et al., 1997; Cahu, et al., 1998; Cobcroft, et al., 2001; review by Muller-Feuga, et al., 2003; Shaw, 2006; Shaw, et al., 2006). This study supports the advantage of live algae greenwater over clearwater, but demonstrated the risk of using commercial algal paste concentrates (instant) algae for greenwater. Initial larval growth and mortality suggested both greenwater types were superior to clearwater. However, in instant algae greenwater, mortality increased (12 dph) immediately following an increase in bacterial load of larvae (11 dph) and was accompanied by a decline in larval growth rate toward the end of the experiment. This is in agreement with a previous study of striped trumpeter, where a higher total bacterial load of larvae was associated with higher larval mortality (Battaglene, et al., 2006). Muller-Feuga, et al. (2003) reported a higher release of

organic nitrogen from frozen compared with fresh algae and cited this as a potential pollutant issue. In this study, the elevated cultivable bacterial load in the reservoirs and rearing water in the instant algae treatment supports the theory that frozen algal concentrates may provide a growth media for bacteria. In contrast, while there were more bacteria in live algae than in clearwater tanks, numbers were relatively stable in the reservoir acclimation period and in the larval tanks over time, supporting the argument for stabilisation of bacterial flora in live algae (Muller-Feuga, et al., 2003).

The high incidence and severity of grey gut in larvae from the live algae tanks was indicative of high feed intake and an accumulation of lipid in the enterocytes (Bransden, et al., 2005). The long-term effects of this condition are unknown, although it is often high in striped trumpeter larvae that are feeding well on lipid enriched rotifers and decreases with age, potentially as the efficiency of lipid transport improves with larval development. The lower incidence in clearwater and instant algae suggests poorer feed intake and/or lipid absorption. A high incidence of urinary calculi, such as that seen in larvae from clearwater, is usually associated with sub-optimal nutrition (Battaglene, unpublished data), though the importance to ongoing larval success is unknown. The nutritional quality of rotifers in greenwater is superior to that of those in clearwater (Reitan, et al., 1997; Yamamoto, et al., 2009) which may explain the lower incidence of urinary calculi in both the greenwater treatments.

The rapid proliferation of cultivable bacteria in the reservoirs and persistently high counts in the larval culture tanks observed in this study could potentially be ameliorated by alternative application methods with the commercial concentrated algal product. Suspension of the algae in temperature acclimated water immediately prior to addition to larval tanks would avoid the bacteria build-up in the reservoir, although the bloom may still occur in the larval tank. The use of ozone disinfected seawater for larval culture has proven beneficial with striped trumpeter and may also reduce bacterial proliferation in instant algae greenwater (Battaglene and Cobcroft, 2007). Supplementary use of probiotic bacteria in the larval culture may also help control the proliferating flora (Ringø and Birkbeck, 1999; Skjermo and Vadstein, 1999). Several authors recommend concentrated commercial algae pastes (fresh or frozen) for reliable live feed (especially rotifer) production (reviewed in Lee, 2003; Muller-Feuga, et al., 2003). However, methods for disinfection or microbial control in rotifers prior to feeding to fish larvae have reduced bacterial colonisation of larvae and improved larval performance (Davis and Arnold, 1997; Munro, et al., 1999; Skjermo and Vadstein, 1999; Cutts, et al., 2007; Suzer, et al., 2008; Giménez-Papiol, et al., 2009). Given that all culture treatments in this experiment were fed with the same rotifers, differences in bacterial flora were associated with water type rather than live feeds.

Assessment of microbial floras in specific environments has been undertaken using many approaches (Spiegelman et al., 2005). To a great extent the approach used should reflect the type of information required to best describe the bacterial flora, its composition and interactions. If the purpose of an assessment is to determine the range of species present then molecular procedures alone may be all that is required. If however there is a need to make some evaluation of the physical properties of a flora then culture needs to be used for at least part of the investigation. For culture based approaches, community-level physiological profiling (CLPP) is well suited to comparing different floras and in particular for assessing community dynamics (Spiegelman et al., 2005) and to a more limited extent biochemical function if suitable and relevant markers have been used (Folman et al., 2001). All means of describing and investigating community structures, whether by molecular, biochemical or culture have inherent limitations. Culture procedures have been criticised because only a portion of the flora can be brought into culture and therefore are not a true reflection of the

original community in the environment under study (Ritz, 2007). Nevertheless, culture procedures are a useful approach to assessing bacterial communities (Pontes et al., 2007) and are able to reveal aspects of a community not apparent with culture independent methods that rely on genomic signatures (Ellis et al., 2003). It has been argued that culture studies give context to microbial ecology (Nichols, 2007) and provides a source of bacteria that can be used in subsequent studies, such as pathogenicity assessments or to determine other biological properties such as probiotic activity.

Recovery of marine bacteria from aquatic animals has commonly relied on the use of a non-selective medium, usually ZoBell's marine agar and invariably a selective medium for Vibrios. This strategy has been used widely for studying marine aquatic floras such as marine aquaria (Blanch et al. 2001), colonisation of the gut of turbot larvae (Munro et al., 1994) and community dynamics of tropical rock lobster larvae (Bourne et al., 2004). The use of BTB-Teepol agar is more limited but has been used in similar studies to recover *Vibrio* floras from marine animals (Sugita et al., 1989, Verdonck et al., 1997, Sugita et al., 2008). The suitability of BTB-Teepol as a medium was assessed for this study by comparing the efficiency of recovering Vibrios on TCBS and BTBT using environmental samples as well as a range of Vibrionaceae and some commonly occurring freshwater species associated with aquatic animals. The growth index of the test species was uniformly higher on BTBT compared to TCBS a reflection of the different selective agents used in the two media, the detergent Teepol and bile salts respectively. It is apparent that BTBT did not suppress entirely the growth of the freshwater species tested, particularly the aeromonads and *E. coli*. The reduced selectivity of BTBT accounts for the high recovery rates for the brackish and estuarine samples which would contain a proportion of freshwater bacteria. The reduced selectivity of BTBT has been observed in other studies which found that non-target species will grow on the medium (Honda et al., 1982).

Differences in medium performance were less apparent when seawater was used from a marine site without an allochthonous freshwater flora. Counts of presumptive Vibrios were similar for both media but were still higher on BTBT indicating that it is less inhibitory than TCBS, consistent with observations for the panel of Vibrionaceae that were tested. Although the better recovery rates on BTBT is at the expense of reduced selectivity, this was not considered to be a major consideration since striped trumpeter are held in seawater uncompromised by freshwater inputs. Accordingly, a high degree of confidence could be placed in the presumptive recovery of Vibrios on BTBT. If sampling had been from a more estuarine environment, TCBS would have been used in preference because of its better selectivity and hence confidence which could be placed in the presumptive isolation of Vibrios.

Where there is a need to recover components of a flora, culture dependent studies to assess community structure can be justified. For this study of striped trumpeter larvae, community-level physiological profiling was used as the means of determining temporal variations and the effect of tank treatments on larval development. Partitioning of the bacterial profiles was undertaken by numerical taxonomy using cluster analysis. This approach to describing microbial communities has been applied to sediments (Berardesco et al., 1998), colonisation of halibut and cod eggs (Hansen & Olafsen, 1989), rotifer floras used for larval rearing (Verdonck et al., 1997) and salt plain mud flats (Litzner et al., 2006).

From the numerical taxonomy of the library of isolates obtained from the striped trumpeter larvae, well defined groups of bacteria comprising species of *Vibrio* and several types of glucose non-fermenters were obtained. Of particular note is that the Vibrios formed a

relatively small proportion of the flora compared to the glucose non-fermenters. The Vibrios also formed a relatively discrete group of phenotypes comprising 8 well defined species and a further 8 unspeciated types that formed distinct and unique phenotypes. Defined species were identified using the MicroSys V36 panel and VibEx7 probability matrix (Carson et al., 2006) and used in the numerical taxonomy as a means of establishing cluster boundaries which encompassed strains with outlier characteristics. Definitions of some species in the VibEx7 database are known to have limited phenotypic depth which can mean that some wild types may sit on the margins of the phenotypic definition. As more strains are characterised improved, consolidated definitions will be obtained for these taxa.

Of the strains which could be identified, only a few are known to have pathogenic abilities (Table 8.11). Of the species listed *V. anguillarum* and *V. ichthyoenteri* are known to be a cause of disease in fish and have been isolated from farmed salmonids in Tasmania (J. Carson, pers. comm.). From health surveillance studies, *V. anguillarum* has been isolated previously from failed striped trumpeter larvae (Carson et al., 2006) while *V. ichthyoenteri* is a known cause of bacterial enteritis in Japanese flounder (*Paralichthys olivaceous*) (Ishimaru et al., 1996). Isolation of *V. alginolyticus* as part of the bacterial flora is noteworthy as strains of this species are known to have probiotic properties (Verschuere et al., 2000).

Table 8.11 Species of Vibrionaceae and their known pathogenicity for fish

Species	Status
Aliivibrio fischeri biovar I	Pathogenicity unknown
Aliivibrio fischeri biovar II	Pathogenicity unknown
Vibrio alginolyticus	Strains known to have probiotic properties
Vibrio anguillarum	Known fish pathogen
Vibrio chagasii	Pathogenicity unknown
Vibrio ichthyoenteri biovar II	Known pathogen of larval fish
Vibrio penaeicida	Pathogen of crustacea
Vibrio splendidus biovar I	Opportunist pathogen of fish

The identity of the un-named *Vibrio* phenons ST5-16 remains unknown and requires further taxonomic analysis using molecular based phylogenetic techniques.

The glucose non-fermenters represent a large group of taxa isolated from the striped trumpeter larvae. The identity of the phenons remains to be established, but the 19 groups form discrete phenotypic clusters that may represent distinct taxonomic groups. As for the *Vibrio* phenons, further work is required to establish the identity of these glucose non-fermenter types.

There is some similarity of the *Vibrio* flora with previous work on striped trumpeter larvae which found *V. anguillarum*, *V. chagasii* and *V. splendidus* biovar I as common species; in addition 8 phenon groups were isolated from a previous study (Battaglene et al., 2006) but were not found in these trials.

In broader terms, comparisons can be made with floras found associated with other species of larval fish, but making close comparisons could be misleading given the differences between hosts. It is accepted however that larval fish acquire a gut flora even before the yolk sac is consumed and that a primary transient flora is transformed into a persistent flora. A characteristic of the gut microflora is its composition of gut group Vibrios and marine pseudomonads (Hansen and Olafsen, 1999). In this alone, the broad division of flora from

striped trumpeter is consistent with other cold water marine species, being composed of a consortium of Vibrios and glucose non-fermenters, the latter equivalent to marine pseudomonads. As points of reference, 17 distinct types of *Vibrio* were isolated from turbot (*Scophthalmus maximus*) larvae (Munro et al., 1994) but only *V. anguillarum, V. alginolyticus* and *V. splendidus* I of this flora were common to that of striped trumpeter larvae. Similarly, in summer flounder (*Paralichthys dentatus*) 19 *Vibrio* taxa were isolated of which only *A. fischeri, V. anguillarum* and *V. alginolyticus* were found in both flounder and striped trumpeter. Floras of larvae from marine fish may not all include Vibrios as found with herring larvae where the bacterial flora after 14 days post-hatch consisted entirely of glucose non-fermenters (Hansen et al., 1992).

The environment in which larvae develop will effect the development of a microflora and its composition. It has been suggested that the primary gut flora of larval fish arises from the egg epiflora at the time of hatching (Hansen et al., 1992) and is subsequently modified by ingestion of bacteria through drinking (Hansen and Olafsen, 1999). Rapid colonisation and hence potential changes in microflora occur when live prey are ingested and in turbot larvae, there was a high correlation between bacterial types of rotifers and the larvae but less so between the water and larvae (Munro et al., 1993). The effect of environment on bacterial floras may occur through temperature. In juvenile puffer fish, it was found that as water temperature increases, bacterial density in the gut also increases as does the diversity of the bacterial flora though it has been reported that the food source is more important than temperature as a factor determining gut flora (Munro et al., 1993). In this trial of striped trumpeter, there was a correlation between bacterial flora and environment seen as a more complex flora associated with larvae held in greenwater live compared to greenwater instant or the clearwater.

Bacterial composition of the floras from the striped trumpeter clearly differed based on the environment from which the larvae were held and indicates that environment to day 15 post-hatch leads to the development of a diverse bacterial flora. In a study of turbot larvae, it was found that there was little difference between the floras of larvae held under intensive or extensive conditions but that the rate of bacterial colonisation was slower under extensive conditions which led to better survival (Munro et al., 1993).

8.6 Conclusion

The performance of striped trumpeter larvae was best in live algae greenwater. While potentially pathogenic bacteria were identified it is suggested that the overall bacterial load in the culture water and larvae in instant algae was responsible for the high larval mortality. Concentrated algal paste products should be used with caution as greenwater for marine fish larval rearing as they may lead to potentially harmful bacterial proliferation and larval mortality, and the efficacy of probionts to ameliorate this effect remains to be investigated.

8.7 Acknowledgments

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other Participants of the CRC. Experimentation was conducted with the approval of the University of Tasmania Animal Ethics Committee, approval number A0008191.

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8.9 Appendix: Probability matrix

The identification of glucose non-fermenters. Data as % strains positive

Test		GNF 1	GNF 2	GNF 3	GNF 4	GNF 5	GNF 6	GNF 7	GNF 8	GNF 9	GNF 10
Acid:	Sucrose	1	1	1	1	1	1	1	1	1	1
Growth:	7% NaCl	99	99	99	99	99	99	99	99	99	99
	10% NaCl	99	99	99	99	99	99	99	99	99	99
Amylase		25	1	1	1	60	1	20	60	99	1
Gelatinase		99	1	99	1	99	75	60	99	80	1
IXP: alkaline phosphatase		99	1	99	99	99	50	99	99	99	7
PNPG: α-D-galactosidase		99	1	66	99	99	99	1	1	1	87
LGN: γ-glutamyl transferase		1	1	1	1	66	1	1	1	1	1
NPS: sulphatase		1	1	1	1	1	99	1	1	1	1
Aesculin hydrolysis		1	1	1	1	16	1	1	23	60	2
Utilisation:	α-ketoglutarate	1	1	1	1	66	1	1	1	1	99
	Acetate	81	99	99	99	99	99	99	99	99	99
	alanine	1	1	1	1	1	1	1	1	1	2
	citrate	18	20	1	1	1	1	99	1	1	89
	citrulline	93	1	1	1	1	1	1	1	99	1
	galactose	99	99	99	99	99	99	1	1	1	99
	gluconate	1	1	1	99	99	99	1	1	1	99
	glucosamine	1	1	1	99	1	1	99	99	80	1
	glucose	99	99	99	75	99	99	99	99	99	89
	glucuronate	1	1	1	33	1	99	1	1	1	99
	glycerol	1	99	99	99	99	1	1	1	1	99
	histidine	1	1	1	1	99	1	99	1	1	3
	hydroxybutyrate	1	99	99	99	99	33	60	1	1	1
	hydroxyproline	1	1	1	1	1	1	1	1	1	1
	lactate	1	1	33	25	99	99	1	1	1	99
	lactose	99	75	99	99	83	99	1	1	1	1
	propionate	99	99	99	99	99	99	99	99	99	1
	putrescine	1	1	1	25	1	1	1	1	1	89
	succinate	1	1	1	1	1	1	99	99	75	99
	sucrose	99	99	99	99	99	99	99	99	99	1
Resistance:	0/129 10µg	99	50	1	1	20	25	99	99	99	1
	0/129 150µg	6	50	1	1	1	1	99	92	40	1
	Ampicillin 10µg	1	50	1	1	20	1	1	1	1	1
	Novobiocin 5µg	93	50	1	1	1	1	99	99	99	1
	Carbenicillin 100µg	1	50	1	1	20	1	1	1	1	1

Test		GNF 11	GNF 12	GNF 13	GNF 14	GNF 15	GNF 16	GNF 17	GNF 18	GNF 19
Acid:	Sucrose	1	1	1	1	5	1	1	1	1
Growth:	7% NaCl	99	99	99	1	5	1	1	99	1
	10% NaCl	99	99	99	1	1	1	1	99	1
Amylase		1	1	1	1	1	1	1	1	1
Gelatinase		3	16	1	1	1	1	1	1	1
IXP: alkaline phosphatase		86	83	1	1	5	1	33	1	1
PNPG: α-D-galactosidase		99	99	99	1	1	1	1	1	1
LGN: γ-glutamyl transferase		1	16	1	1	1	1	1	1	1
NPS: sulphatase		1	1	66	1	1	1	66	1	1
Aesculin hydrolysis		3	1	1	1	1	1	1	1	1
Utilisation:	α-ketoglutarate	99	99	99	85	99	99	99	99	1
	Acetate	99	99	99	99	99	99	99	99	1
	alanine	99	1	99	99	94	99	99	75	1

Test		GNF 11	GNF 12	GNF 13	GNF 14	GNF 15	GNF 16	GNF 17	GNF 18	GNF 19
	citrate	99	99	66	99	94	1	99	50	1
	citrulline	1	1	99	1	1	1	1	1	1
	galactose	99	99	99	1	1	1	1	1	1
	gluconate	99	99	33	1	5	1	1	99	1
	glucosamine	1	16	99	1	11	1	1	1	1
	glucose	99	99	99	1	99	33	99	99	1
	glucuronate	99	80	1	28	5	1	99	75	1
	glycerol	99	99	99	1	35	1	99	99	1
	histidine	99	1	99	1	5	11	1	1	1
	hydroxybutyrate	1	50	99	99	99	99	99	99	1
	hydroxyproline	1	1	1	1	1	1	99	25	1
	lactate	99	99	99	99	99	99	99	99	1
	lactose	1	99	1	1	1	1	1	1	1
	propionate	1	99	1	99	99	99	99	99	1
	putrescine	99	99	99	1	1	1	99	75	1
	succinate	99	99	99	99	99	99	99	99	1
	sucrose	1	99	1	1	5	1	1	1	1
Resistance:	0/129 10µg	1	1	1	50	99	50	50	1	50
	0/129 150µg	1	1	1	50	1	50	50	1	50
	Ampicillin 10µg	1	25	1	50	1	50	50	1	50
	Novobiocin 5µg	1	1	1	50	1	50	50	1	50
	Carbenicillin 100µg	1	1	1	50	1	50	50	1	50

9 MICROBIAL COMMUNITIES OF POST HATCH STRIPED TRUMPETER (*LATRIS LINEATA*) LARVAE, HELD UNDER DIFFERENT REARING CONDITIONS, DETERMINED USING CULTIVATION-INDEPENDENT APPROACHES

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9.1 Abstract

A knowledge of the microbial bacterial community of the larvae, seawater and live feeds was believed to be necessary in order to develop an understanding of what represents a healthy microbial ecology relevant for larval fish rearing success. 16S rRNA bacterial clone library and terminal restriction fragment length polymorphism (TRFLP) analysis was utilised to examine the microbial community associated with striped trumpeter (*Latris lineata*) larvae cultured under different "green water" conditions. It was discovered that the larvae-associated microbial diversity was restricted but varied considerably between culture conditions. Most bacteria detected belonged to class *Alphaproteobacteria* (predominantly the *Roseobacter* clade), *Gammaproteobacteria* (genus *Psychrobacter* and *Pseudoalteromonas*) and *Actinobacteria* (primarily genus *Microbacterium*). No association was found between larval survival and microbial community structure. Similar results were obtained using TRFLP analysis, though it was found that the larval microbial community was distinct from the bacterial community present in the surrounding water.

9.2 Introduction

Intensive cultivation of most marine larval fish is potentially subject to bacteria-associated problems that result in poor growth and mass mortalities (Skjermo and Vadstein, 1999; Vadstein et al., 2004). An understanding of the microbial bacterial community of the larvae, seawater and live feeds is necessary in order to develop an understanding of what represents a healthy microbial ecology relevant for larval fish rearing (Nicolas et al., 1989; Hansen and Olafsen, 1999; Verschuere et al., 1999). As with all live animals, larval fish have their own bacterial communities that they acquire from their surroundings. It is possible that, as in mammals, fish contain a specific suite of intestinal microbiota that establish during juvenile developmental stages (Hansen and Olafsen, 1999; Verschuere et al., 2000).

New techniques are required to better understand these bacterial communities and their interactions. Traditionally, culture-dependent studies of hatchery fish have been conducted on identifying the major families of bacteria associated with marine fish at different stages of life. Hansen and Olafsen (1989) found that larvae of juvenile cod (*Gadus morhua*) were

made up of predominantly *Vibrio, Lactobacillus* and *Bacillus,* while this changed to *Vibrio, Photobacterium, Pseudomonas,* and *alteromonads* during the adult stages. Studies on the Atlantic halibut (*Hippoglossus hippoglossus*) have also shown a shift during the yolk sac stage through to the larvae stages (Verschuere et al., 1999). During the yolk sac stage, it was observed that the dominant genera present were *Cytophaga, Flexibacter, Flavobacterium and Pseudomonas,* while during the feeding larval stages, *Vibrio and Aeromonas* were detected (Bergh et al., 1992). While using a culture-dependent approach does give an insight into the bacterial communities, often a high proportion of bacteria are overlooked, either due to inadequate surveying or because they cannot be cultured on standard agar media. For this reason, more recent studies have applied molecular-based approaches (Jensen et al., 2004; Romero and Navarrete, 2006; McIntosh et al., 2008; Zhong et al., 2008). These methods have allowed the identification of bacteria without isolation and allow the phylogenetic affiliation of the community present to be accurately determined. Overall, this information has greatly increased our knowledge of the microbiology of fish (Romero and Navarrete, 2006).

Methods based on the amplification of the 16S rRNA gene, have emerged as powerful tools (Holben et al., 2002). Examples of these are DGGE, TRFLP, clone libraries and real time PCR, which have been discussed in Chapter 9. These techniques are subject to PCR biases and resolution limits, meaning that even when precautions, such as multiple PCR reactions are made, PCR can skew community composition data. This may also result in species with a smaller amount of DNA present to remain undetected. Using these methods, it has been found that the microbial diversity of larval fish is relatively similar on a broad level with most taxa belonging to the phyla *Proteobacteria, Actinobacteria,* and *Bacteriodetes*. On a species level, the differences are much greater. It has also been reported that these differences can occur within different parts of the fish, e.g. foregut or hind gut (Verner-Jeffreys et al., 2003) and that skin mucus and the surrounding water also have different microbial populations (Smith et al., 2007). The age of the fish, as well as whether it is either wild or captive, can also influence microbial communities (Romero and Navarrete, 2006). Therefore, by utilising molecular biology techniques, it is possible to obtain insights into the microbiota and thus better understand how the microbial ecology of the fish and its environmental influences health and growth.

While the microbial community of the striped trumpeter larvae has been previously investigated following culture (Battaglene et al., 2006), to gain a greater understanding of the microbial community of striped trumpeter larvae a molecular approach was hereby applied. This study uses 16S rRNA and TRFLP molecular techniques described in Chapter 9. Samples were obtained from 15 day-old striped trumpeter larvae that were grown under three different green water conditions. The main objectives of this study were to:

1. Determine the bacterial communities associated with 15 day-old striped trumpeter larvae reared under different conditions, by using 16S rRNA gene clone library analysis.
2. Identify and track the dominant bacterial species on fish tissue samples using TRFLP analysis.
3. Determine how the microbial communities are influenced by different greenwater rearing approaches and to determine whether microbial communities affect growth and survival of larvae.

9.3 Methods

9.3.1 *Background*

The study formed part of a larger experiment investigating the effects of three water treatments on the survival and growth of striped trumpeter larvae (Cobcroft et al., 2010, see Chapter 8).

Larval rearing

Eggs were collected from a female striped trumpeter broodstock by strip-spawning, and fertilised with the milt of four males. Fertilised eggs were incubated and hatched as previously described in Bransden et al., (2004). Larvae (4500 per tank) were stocked into twenty four, 300 l black hemispherical fiberglass tanks at 1 dph. Larvae were held under static, clear water conditions from 1 to 5 dph. A photoperiod of 18 hours light: 6 hours dark was used throughout the experiment and was produced by a computerised halogen light source (~11 µmol s^{-1} m^{-2} at the water surface) using a gradual fade in and fade out process. During 6 dph, internal screens (390 µm mesh size) were placed into the centre of each tank to allow outflow and removal of rotifers, algae and incoming seawater overnight (see below).

There were three treatments each with eight replicated tanks. The first treatment, designated "Fresh Algae" (FA), consisted of rearing larvae in a 'greenwater' environment that included live *Nannochloropsis oculata* at a turbidity level of 3 Nephelometric Turbidity Units (NTU) (HACH 2100 portable turbidity meter). The second treatment, designated "Instant Algae" (IA) was an algal paste of concentrated *N. oculata* (Reed Mariculture Inc., California, USA) that was also used to produce a green water environment by re-suspension of the concentrate in seawater to a turbidity level of 3 NTU. The final treatment, designated "Clear Water" (CW) consisted of rearing larvae without addition of algae (<0.01 NTU). The algal additions were added to each tank at a rate of 8.8 l min^{-1} (total 100 l) from the reservoirs accompanying each individual tank. The algal suspensions or seawater had been acclimated to tank temperature in the reservoirs overnight and provided with aeration. Tanks remained static during the light phase with gentle aeration (200 ml min^{-1}). A central screen (mesh size 390 µm) was placed in the tank and incoming seawater (112.5 l hr^{-1}) that had been passed through primary filtration was supplied. The screen prevents the loss of larvae but allows the passage of algal cells and live food (see below). All tanks were supplied with live food rotifers (*Brachionus plicatilis*) enriched on Algamac 2000 (Aquafauna Biomarine, USA) as described by Bransden et al. (2004). Enriched rotifers were supplied at 10ml^{-1} every morning. Surface skimmers were used from 8-15 dph to remove surface oil and promote swim bladder inflation (Trotter et al., 2003). Water quality was measured daily with a range of temperatures of 15.5–16.5°C, salinity of 34.1-34.7ppt, pH of 8.1 and dissolved oxygen of greater than 90% saturation. Mortalities were spot siphoned and counted daily, up to the conclusion of the experiment (15 dph) when all live larvae were removed and counted to determine final survival.

9.3.2 *Larval sampling*

Larvae were sampled from each tank 15 dph. Fifty larvae were removed from each tank and following anesthesia; 30 were used to determine morphormetric indices and dry matter content and 20 were used for the assessment of the microbial community. At the same time

20 ml of water was removed via a sterile pipettor from each tank and placed into sterile containers. All sampling took place prior to feeding.

The larvae and water samples selected from the six treatment tanks for the study were chosen from tanks that performed at different levels for each of the three treatments, designated "good performance" and "poor performance" i.e., the best and worst performing tanks for each treatment. Further information on performance of tanks not chosen for analysis can be found in Cobcroft et al. (2010). In addition, water samples relating to each of these six larvae samples were also obtained for TRFLP analysis. No replication was possible because of the costs and time required to process samples.

9.3.3 *DNA extraction*

Extraction of bacterial DNA from the homogenised striped trumpeter larvae was performed utilising the DNeasy Tissue Kit (Qiagen Pty. Ltd., Australia). An initial pretreatment step employing lysozyme was incorporated to lyse Gram-positive bacteria. Additional wash steps were employed in addition to the manual protocol which resulted better yields of DNA as it removed salts and other inhibitors that were present in the samples. Extraction of bacterial DNA was also performed on water samples, utilising the MoBio water extraction kit (MoBio Laboratories, Inc) following the manufacturer's instructions. The amount of DNA present in larval and water extracts was measured by spectrophotometry using a SmartSpec 3000 (BioRad).

9.3.4 *Clone library polymerase chain reaction (PCR)*

Clone libraries were generated using DNA extracted from the six sample types. PCR amplification of the 16S rRNA gene used the primers 10f (GAG TTT GAT CCT GGC TCA G) and 907r (CCG TCA ATT CCT TTG AGT TT). Each reaction was a 50µl reaction mix with 5µl of 10X buffer, 1µl of Advantage Taq (1.1 µg/µl of TITANIUM taq DNA polymerase and TaqStart Antibody, Clontech), 4µl of a deoxynucleotide mix solution, 4µl the forward primer, 4µl of the reverse primer, and approximately 10ng DNA template. A final volume of 50 µl was obtained using milliQ water. The following thermal cycling program was used: initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation for 1 minute, annealing at 50°C for 1 minute, extension for 3 minutes: final extension at 72°C for 10 minutes. The reactions were purified using the Qiagen PCR cleanup kit.

9.3.5 *Clone library construction*

PCR-amplified 16S rRNA gene samples to be cloned were ligated into the pGem-T vector (Promega) according to the manufacturer's instructions and transformed into Epicurian coli XL ultracompetent cells (Stratagene). Transformants were then screened using blue-white selection on Luria agar containing XGal/IPTG and 100 mg l^{-1} ampicillin. Approximately 50 white colonies from each library were then transferred to fresh plates and re-incubated overnight. Plasmids were extracted using the Ultraclean miniplasmid extraction kit (MoBio). Positive clones were sequenced in both directions using primers M13 forward and M13 reverse using the Ready Reaction Dideoxy Cycle Sequencing kit (Beckman-Coulter). Sequencing was performed using a Beckman-Coulter CEQ2000XL automated capillary sequencing system

9.3.6 *Sequence analysis*

Sequences were aligned against reference sequences obtained from GenBank (http:/www.ncbi.nlm.nih.gov/Blast) (Altschul et al., 1997) using BioEdit (version 7.0.9) (Hall, 1999). Automated alignment using Clustal X was performed and aligned data exported to the CLC free workbench (v4) where similarity trees were produced, using the Neighbour-joining algorithm. The 16S rRNA gene sequences from *Thermotoga maritima* and *Coprothermobacter platensis* were used as outgroup references on the *Alphaproteobacteria*, *Gammaproteobacteria* and *Actinobacteria* sequence based-trees, while the plastid 16S rRNA gene sequence from *Fucus versiculosus* was used for the plastid sequence-based tree. Clones with a sequence similarity of 98% were considered to be the same phylotype (Keswani and Whitman, 2001) for the purposes of assessing diversity. Clone library comparisons method utilised the LIBSHUFF v. 1.22 computer program (http://libshuff.mib.uga.edu/) (Singleton et al., 2001) to generate homologous and heterologous coverage curves from clone libraries, which were then compared statistically. The DNADIST program of PHYLIP using the Jukes-Cantor model for nucleotide substitution was used to construct the distance matrix submitted to LIBSHUFF.

9.3.7 *TRFLP sample preparation*

TRFLP was used to determine the changes in bacterial communities between the six larvae samples held under different conditions and to assess whether there were differences in the bacterial community that could have resulted in higher or lower survival rates of the larvae. Fluorescently labeled primers were labeled with Beckman Coulter WellREDtm fluorescent dyes. Primer10f (GAG TTT GAT CCT GGC TCA G) was labeled with the D4 (Blue) dye while primer 907r (CCG TCA ATT CCT TTG AGT TT) was labeled with the D3 (Green) dye. Each PCR reaction comprised of 12.5 µl HotstartTaq MasterMix (Qiagen, Australia), 1.0 µl of each primer and approximately 10ng DNA template, with a final volume of 25 µl obtained with milliQ water. The following thermal cycling program was used: initial denaturation at 94°C for 15 minutes, 34 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute with a final extension at 72°C for 10 minutes. Four identical PCRs per sample as outlined above were run separately and the resulting products were pooled during the PCR purification process. The reactions were purified using the Qiagen PCR cleanup kit.

Purified PCR products were digested with 20U of enzymes *HaeIII, HhaI* and *HinfI* (New England BioLabs) 10µl reaction were prepared using 2 µl of DNA template, 20U of enzyme 1µl of bovine serum albumin solution, 1µl of NEL Buffer and milliQ water was used to obtain a volume of 10 µl. Each digest was incubated at 37°C for 3 h. At the end of the incubation the digestion was stopped using heating in a thermocycler with the *Hha*I digestion stopped by incubating at 65°C for 20 min while *Hinf*I and *Hae*III digestion were stopped by incubation at 80°C for 20 min. Each digest was done separately in duplicate. Digests were then desalted and purified using an ethanol precipitation method. To each well, 1µl of 3M sodium acetate and 0.5 µl of glycogen was added and mixed; 30 µl of ice-cold absolute ethanol was added and the sample vortexed. Plates were covered with an aluminum sealing mat and incubated at -20°C for 20 minutes. Plates were then centrifuged at 4°C for 30 minutes at 4300 rpm (Sorvall Super T21 Thermo Electron Corp. Waltham, USA). Removal of ethanol was achieved by inverting the plate onto size cut absorbent paper, followed by centrifugation for 30 s at 300 rpm (Quantum Scientific, Murarrie, Australia). This was followed by a wash with 200 µl 70% (vol./vol.) ethanol, which was removed as previously described. Plates were left to air dry in a laminar flow cabinet, until there was no more remaining ethanol residue. Each digest was performed in duplicate.

The dried pellets were re-suspended in 30 µl Sample Loading Solution (Beckman Coulter) and 0.3 µl of the 600 bp (base pair) DNA size standard (Beckman Coulter) was added to each well. Samples were then analysed on a Beckman Coulter CEQ800 Genetic Analysis System, using the Frag-4 program, injection 2.0 kV/30sec, run at capillary temperature 50 °C /4.8kV for 60 minutes.

9.3.8 *TRFLP data analysis*

The TRFLP analysis procedures employed by Dann et al. (2009) were used to ensure TRFLP profiles were reproducible and also to minimise baseline noise. In summary this was done by producing *in silico* digests using workbench 4.0. Clone sequences were submitted and digested within the program generating the corresponding base position size for each TRF. Duplicate samples were analysed using T-ALIGN (Smith et al., 2005), with a confidence interval of 0.5 bp in order to generate a consensus TRFLP profile in which TRFs present in both replicates were represented. Multi-dimensional scaling (MDS) plots were obtained using Primer v. 6 (Primer-E Ltd., Plymouth, UK) to determine the similarity of the microbial community between treatment replicates and between separate treatments. Stress values obtained with each MDS plot provide an indication of the "goodness of fit" with the data set with the lower the value indicating a more representative analysis of the dataset. The similarity of treatments was determined using ANOSIM analysis using Primer v. 6. This analysis produces R statistic values that provide an indication of the level of similarity between sample sets within a spatial scale, where 1 indicates completely different sets of samples while a value of 0 indicates sample sets that completely overlap. Permutation analysis was also performed to obtain a significance value. Percent similarity within sample sets and the average percent contributions of individual TRFs to the total peak area TRFLP profile were determined using SIMPER analysis within the Primer 6 software package.

In relation to the MDS plots the actual location of each data point in space is arbitrary, and the axes can be rotated freely. It is the relationship of the data points to each other that is of importance, with two near points representing more similarity to each other than to another point located at a distance. The stress of the plot, generated as part of the MDS analysis in Primer 6, is a measure of how much distortion was introduced to allow the representation of the data in the specified dimensions, i.e. a "goodness of fit". A stress of 0 gives a perfect representation while a measure of stress <0.2 indicates the plot is a good representation of the data set and can be used for interpretation. A measure of >0.3 indicates that the level of distortion that was required to display the data on the map is too high for any reliable inferences to be made from the configuration.

9.4 Results

9.4.1 *Clone library data and comparisons between treatments*

Larval survival, 15 days post-hatch, was most consistent in the clear water treatment tanks (38 ± 5% survival) (Table 9.1) and also yielded larvae of the greatest size. The use of fresh algae resulted in similar outcomes, but was more variable (36 ± 9% survival). The use of "instant algae" preparations resulted in poor outcomes (7 ± 4% survival) (Table 9.1). Overall, the microbial communities directly associated with the larvae were low in diversity (Table 9.2) with the microbial community dominated by members of the class *Alphaproteobacteria* (Figure 9.1 to Figure 9.4). Members of the *Actinobacteria* (Figure 9.6) and *Gammaproteobacteria* (Figure 9.7) were also detected in certain samples. 16S rRNA-

like gene sequences derived from the plastids of *Nannochloropsis* species (Figure 9.5) were detected in all samples (Figure 9.1 to Figure 9.3).

Clear Water tanks

Both clear water-based tank samples were dominated by the seawater species *Sulfitobacter pontiacus, Ruegeria mobilis* and *Microbacterium oxydans*/*M. marinotypicum* (Table 9.2). Collectively, these species made up 95% and 48% of the bacterial clones, respectively in the two samples analysed. The species *Sulfitobacter dubius* and *Sphingomonas paucimobolis* were also detected in the clear water treatment sample with the poorest survival outcome (CWP). In the case of the sample with the best survival outcome (CWG) 38% of bacterial clones were most closely related to the species *Psychrobacter nivimaris* (Figure 9.2). In the same sample additional clones were found to belong to a clade of uncultivated *alphaproteobacteria* found in various marine ecosystems based on unpublished cloned sequence data on GenBank; these are most closely related to the genera *Thalassobius* and *Thalassobacter*.

Instant Algae

Only *Sulfitobacter pontiacus* was found to occur in both instant algae treatment samples. Clones related to *Psychrobacter nivimaris* and *Microbacterium oxydans* were detected in the sample with the poorest survival (designated IAP). On the other hand the *alphaproteobacterial* species *Phaeobacter gallaeciensis* and *Nautella italica* were only detected in a second sample (designated IAG) that had better but still comparatively poor survival (Table 9.12).

Table 9.1 Striped trumpeter larval survival and size for three different water treatments: fresh algae, instant algae and clear water. Survival of fish in single tanks chosen for analysis from the best and worst tanks in the treatment ("good" and "poor" n=1), along with overall treatment means for survival and Total Length (TL) (n=8, see Cobcroft et al., 2010)

	Fresh Algae	Instant algae	Clear water
		% survival:	
Good Performance	50	13.4	45.6
Poor Performance	22.5	1.1	30.1
Tank means (SD)	36 ± 9	7 ± 4	38 ± 5
		size (mm):	
Larval size (SD)	7±0.075	6.6±0.11	6.6±0.014

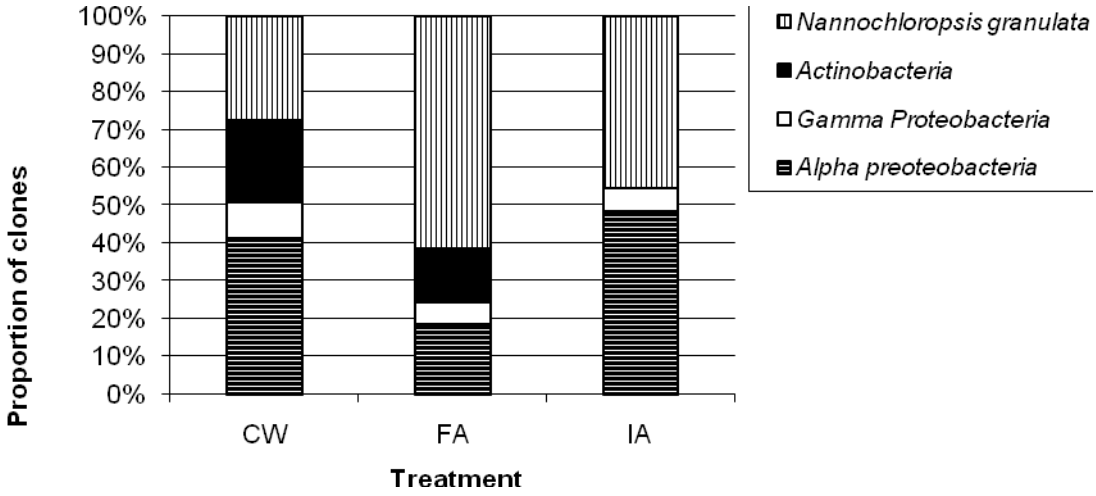

Figure 9.1 Percent proportion of major taxonomic group occurring between the samples divided between the three different green water treatments. Abbreviations: clear water, CW; fresh algae, FA; and instant algae, IA.

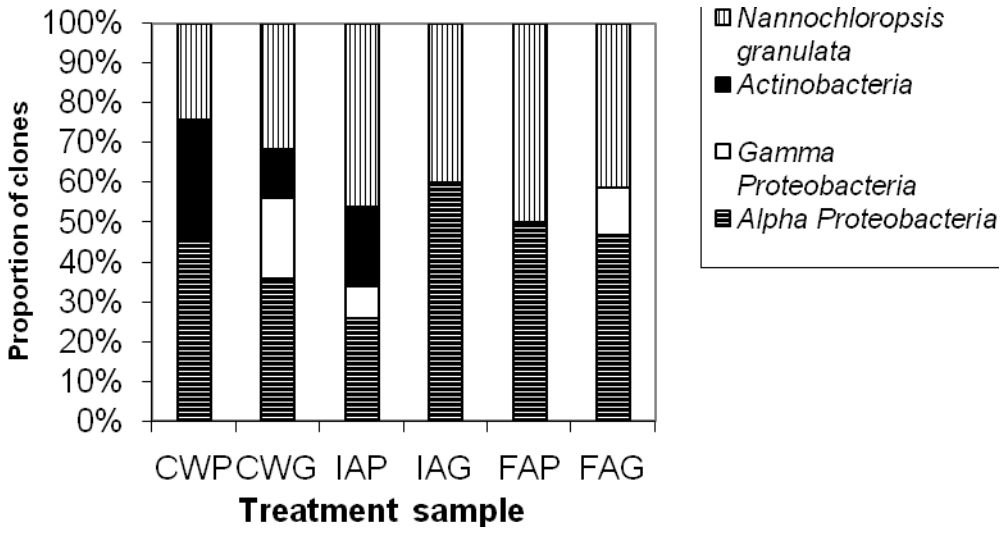

Figure 9.2 Percent proportion of major taxonomic group occurring between the different treatment samples analysed.

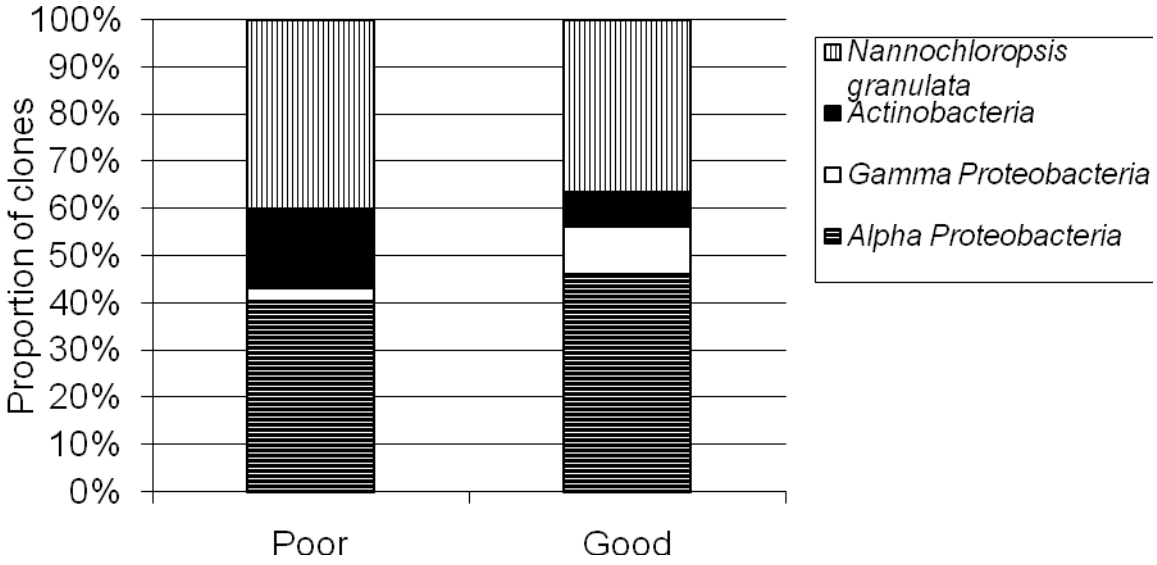

Figure 9.3 Percent proportion of major taxonomic groups occurring in samples pooled on the basis of poor and good larval survival performance as indicated in Table 9.1.

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Fresh Algae

Overall diversity was found to be greatest in the fresh algae supplied tanks and the communities differed almost completely between the samples analysed. The only phylotypes in common were related to *Ruegeria mobilis* and to deep-branching *alphaproteobacteria* distantly related to the family *Rickettsiales*, very similar to clones derived from the gut microbiota of zebrafish (Rawls et al., 2004). The sample with the best survival outcomes (FAG, 50% survival, Table 2.1), also possessed phylotypes related to uncultured marine *alphaproteobacteria*, *Psychrobacter* and *Pseudoalteromonas*. The comparatively poorer performing tank (FAP), though still several times better than both IA samples, had phylotypes related to *Phaeobacter gallaeciensis* as well as *Methylobacterium aquaticum* and *Sphingomonas paucimobilis*.

Comparison of rearing performance based on clone library data comparisons

In order to find whether microbial communities influenced larvae rearing, samples were defined in terms of performance 'pools' (Table 9.1), designated simply as either "good" and "poor" performers. From the clone library data (Table 9.2) most species detected were found in tanks with different performance outcomes and thus could not be expected to be influencing larvae survival. The species *Methylobacterium aquaticum, Sphingomonas paucimobilis* and *Sulfitobacter dubius* were only found in poorly performing tanks based on fresh algae and clear water treatments.

Table 9.2 Distribution of cloned bacterial 16S rRNA sequences detected in striped trumpeter larvae grown under different green water conditions.

Species:	Similarity (%)	Clear water		Instant algae		Fresh algae	
		Sample name and relative larvae survival (performance):					
		CWP	CWG	IAP	IAG	FAP	FAG
		Proportion of clones sequenced (%):*					
Class *Alphaproteobacteria:*							
Sulfitobacter pontiacus	>99	36	27	50	36		35
Sulfitobacter dubius	>99	2					
Uncultivated species (closest species *Thalassobius mediterraneus*)	96		10				
Phaeobacter gallaeciensis	>99				43	16	
Nautella italica					21		
Ruegeria mobilis	>99	6	13			16	23
Uncultivated species (closest species *Bartonella bacilliformis*)	90						3
Methylobacterium aquaticum						16	
Sphingomonas paucimobilis	>99	2				8	
Uncultivated species (most similar to *Rickettsia* an relatives)	<80					41	19
Class G*ammaproteobacteria*:							
Psychrobacter nivimaris/P. glacincola	98-99	38		15			9
Pseudoalteromonas elyakovii	>99						9
Phylum *Actinobacteria*:							
Marinobacterium oxydans; *M. maritypicum*	>99	16	50	35			

However, as these species were not detected in the corresponding instant algal treatments it cannot be suggested they are linked to poor survival outcomes. Furthermore, the species were encountered only sporadically and at lower proportions than other species detected. Similarly, no one species can be indicated as being responsible for improving outcomes based purely on the clone library data.

The null hypotheses that the three tank treatment libraries derived from 15 day post hatch larvae would not be significantly different were tested by using a statistical approach useful for comparing clone library data - the LIBSHUFF method (Singleton et al., 2001)**.** It was found that all sample clone libraries were significantly different from one another ($p<0.01$) (Table 9.3) due to the low diversity but distinct speciation was encountered. Under-sampling may thus contribute to this finding as LIBSHUFF analysis sensitivity is considerably affected by the sampling effort (Singleton et al., 2001). When the performance data is pooled, coverage values derived from LIBSHUFF analysis are relatively small, suggesting that performance variations are not linked to microbial community composition within the resolution limits of the available data.

Table 9.3 Libshuff comparisons of the heterologous and homologous coverages (delta-C) of bacterial clone library data on the basis of larval sample green water treatments and larval survival outcomes.

Comparison Number	Homologous Data	Heterologous Data	*P*[b]	delta-C
1	FAP[a]	FAG	0.003	4.198
	FAG	FAP	0.003	5.232
2	IAP	IAG	0.003	4.364
	IAG	IAP	0.009	0.616
3	CWP	CWG	0.012	0.173
	CWG	CWP	0.003	1.766
4	IAP	CWP	0.003	1.96
	CWP	IAP	0.006	0.634
5	IAP	CWG	0.003	0.899
	CWG	IAP	0.009	0.615
6	IAP	FAG	0.003	1.232
	FAG	IAP	0.006	1.107
7	IAP	FAP	0.006	1.087
	FAP	IAP	0.003	2.205
8	FAG	IAG	0.003	2.276
	IAG	FAG	0.003	3.01
9	FAG	CWG	0.003	1.394
	CWG	FAG	0.003	1.893
10	FAG	CWP	0.003	2.95
	CWP	FAG	0.003	2.303
11	FAP	CWP	0.003	4.198
	CWP	FAP	0.003	5.232
12	FAP	IAG	0.003	3.93
	IAG	FAP	0.003	3.11
13	CWP	IAG	0.003	2.692
	IAG	CWP	0.003	0.844
14	FAP	CWG	0.003	1.812
	CWG	FAP	0.003	5.246
15	IAG	CWG	0.003	3.093
	CWG	IAG	0.003	4.474
16	Poor	Good	0.003	0.287
	Good	Poor	0.003	0.457

[a]Abbreviations for treatments are provided in the Methods section.
[b]*P*-values were Bonferroni-corrected to account for the possibility of false positive significance values.

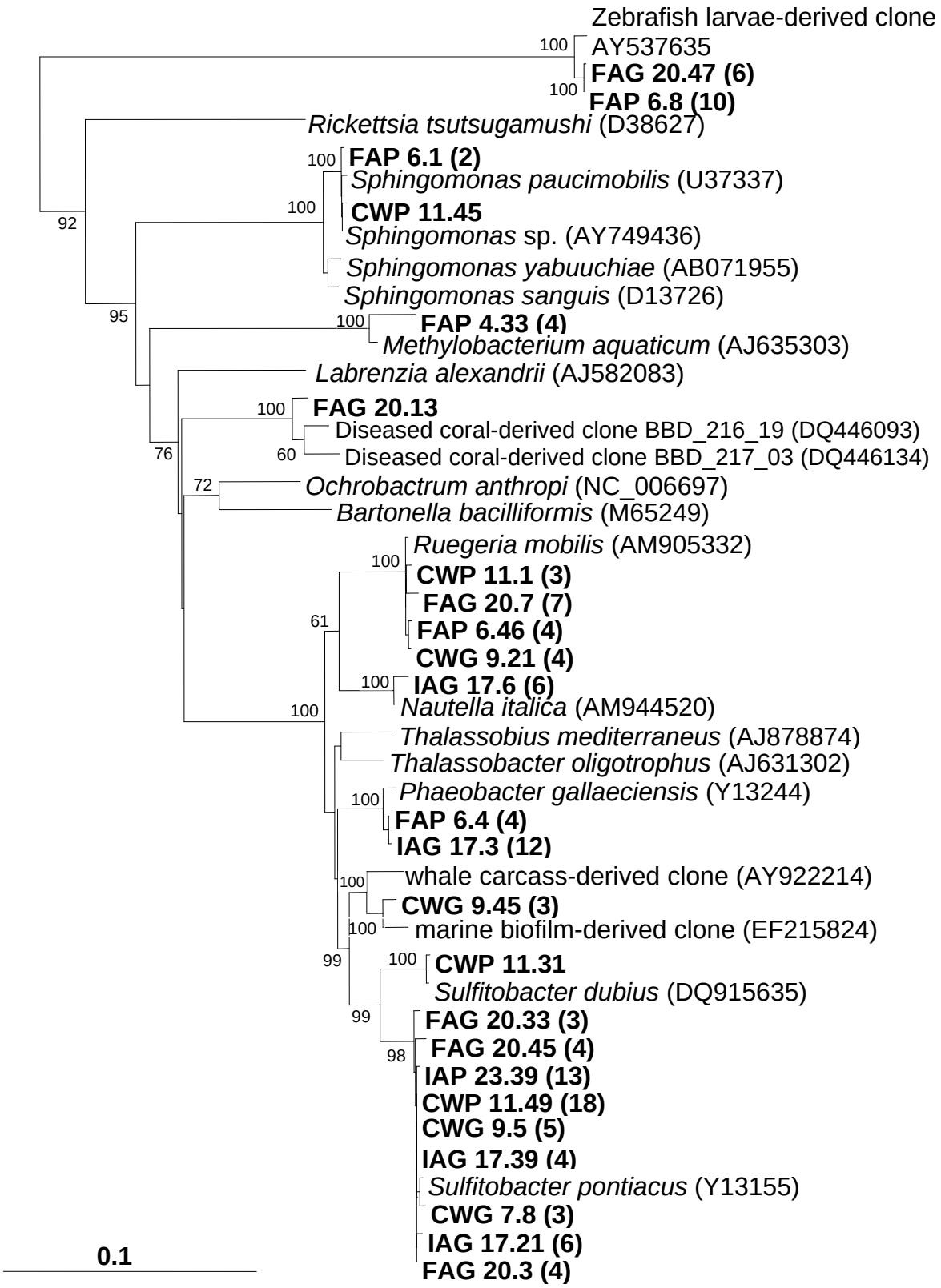

Figure 9.4 16S rRNA gene-based phylogenetic tree showing position of phylotypes in comparison to other members of the class *Alpha-proteobacteria*. Values in parentheses indicate the number of clones found for each phylotype. Numbers at each branch point are bootstrap values. See methods section for abbreviations.

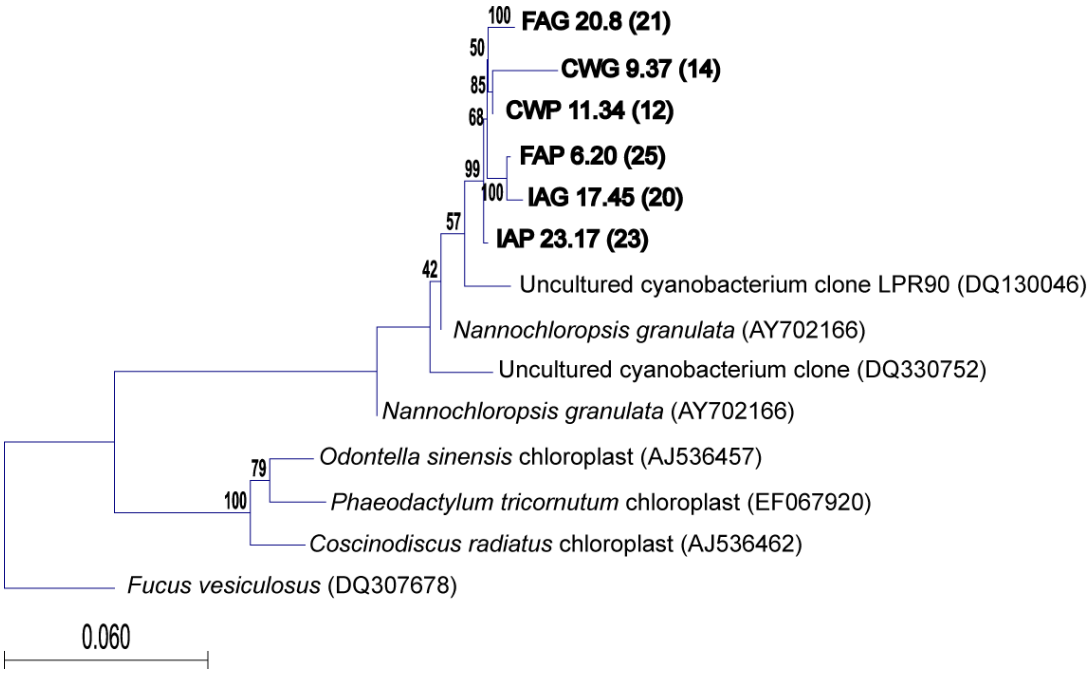

Figure 9.5 Phylogenetic tree showing position of clones in comparison to algal plastid-derived 16S rRNA gene sequences. Clones labelled as "cyanobacteria" are erroneously labelled in the GenBank database. Values in parentheses indicate the number of clones found for each phylotype. Numbers at each branch point are bootstrap values. See methods section for abbreviations.

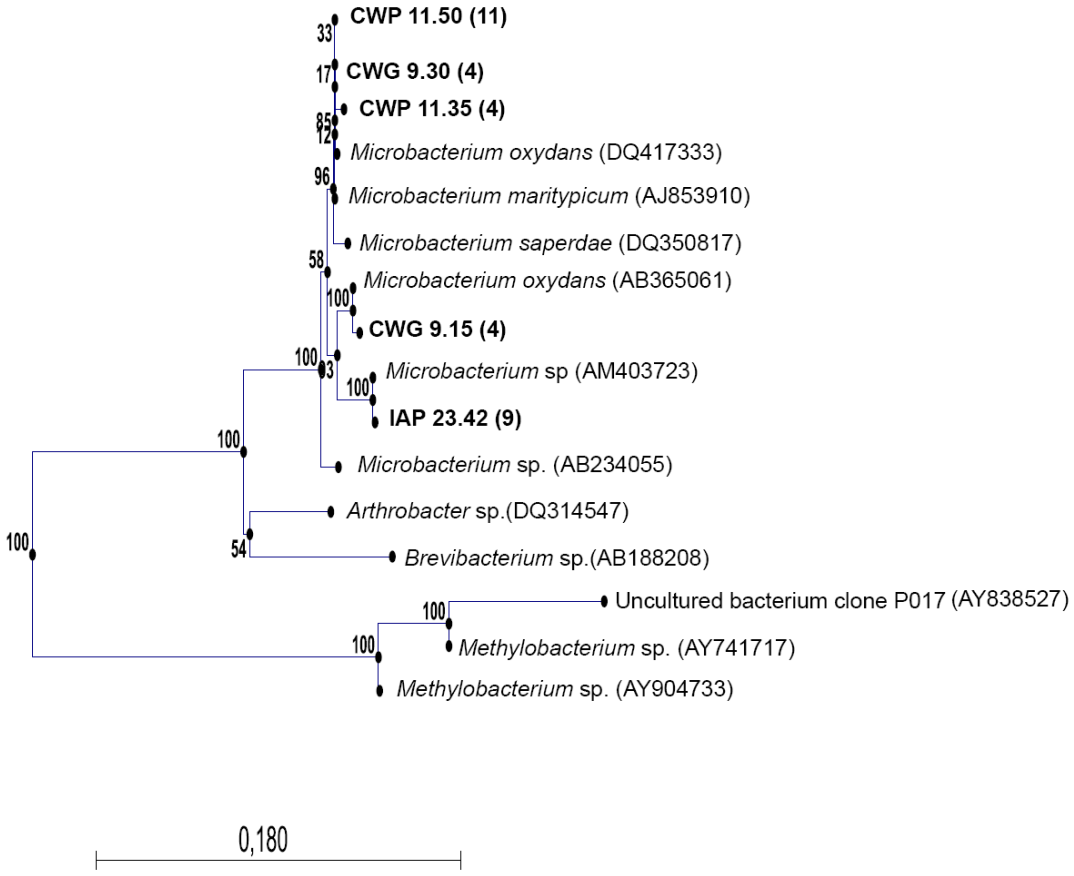

Figure 9.6 16S rRNA gene-based phylogenetic tree showing position of clones in comparison to closely related members of the phylum *Actinobacteria*, in particular genus *Microbacterium*. Values in parentheses indicate the number of clones found for each phylotype. Numbers at each branch point are bootstrap values. See methods section for abbreviations.

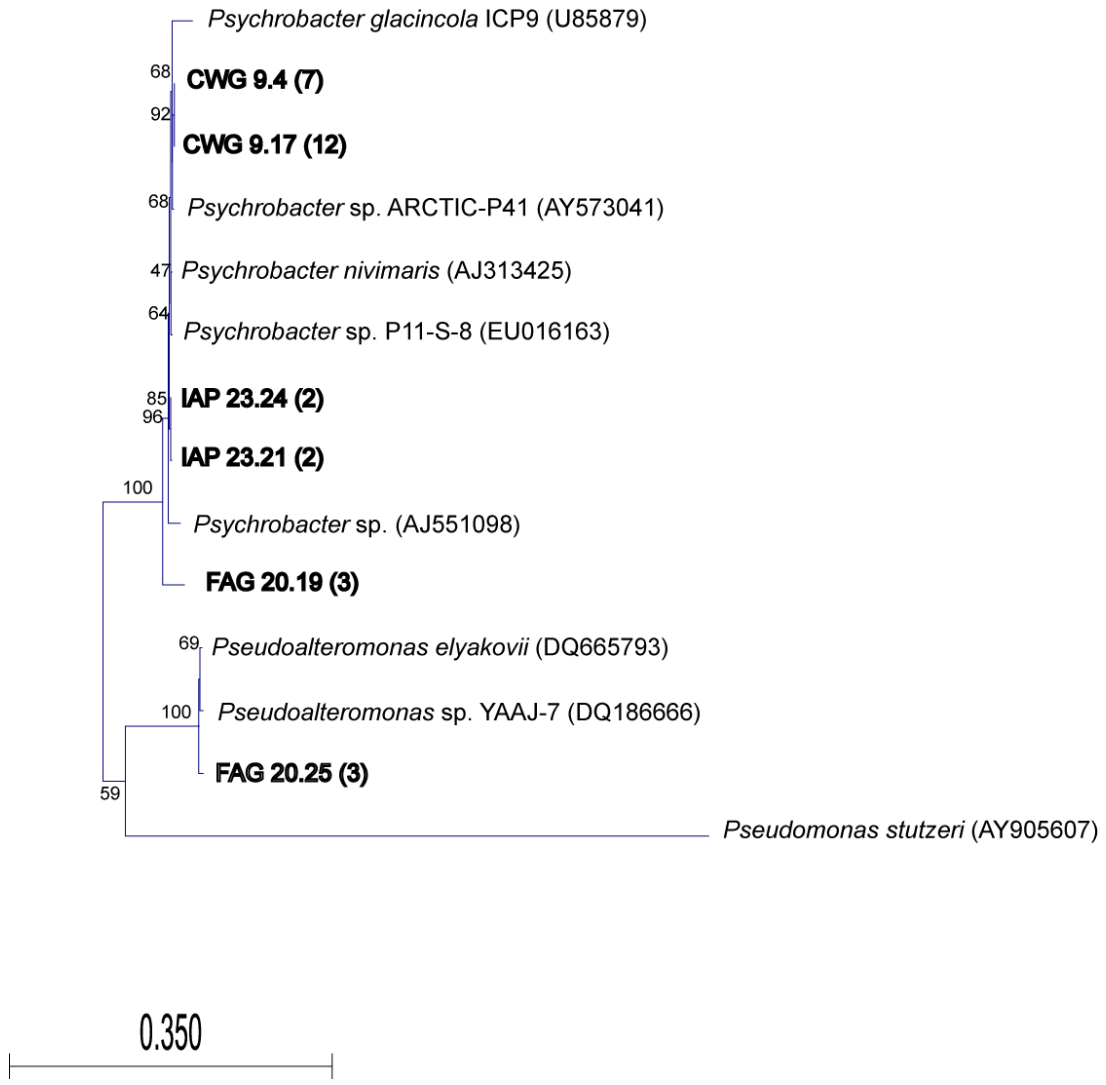

Figure 9.7 16S rRNA gene-based phylogenetic tree showing position of clones in comparison to other members of the class *Gammaproteobacteria*. Values in parentheses indicate the number of clones found for each phylotypes. Numbers at each branch point are bootstrap values See methods section for abbreviations.

9.4.2 *TRFLP results*

The relationship between the composition of total bacterial communities of the six larval samples, including treatment effects and performance outcomes, with the surrounding tank water was investigated using TRFLP analysis. In order to further understand microbial communities associated with 15 day post-hatch larvae TRFs were compared to clone library sequence data that was digested *in silico* (Table 9.4).

Changes in the composition of the larval bacterial communities within good versus poor performing tanks.

On the basis of ANOSIM no differences in the structure of the total bacterial community of the tanks defined on the basis of performance (Table 9.1) were detected (Table 9.1, Figure 9.8). To further evaluate the data, SIMPER analysis was performed to specifically delineate TRFs that may differ on the basis of performance (Table 9.5). It was found that for poor and good performance pooled samples there was on average 61.7% dissimilarity. Two TRFs were found that were significant contributors to the microbial communities in both tanks while four TRFs were identified that contributed the greatest to differences on the basis of performance (Table 9.5). The source species of several of these TRFs could not be confidently identified on the basis of the clone library data digests (Table 9.4). In the poor performance sample pool TRF 568 *HinfI* (r) was likely derived from a *Nannochloropsis* plastid; TRF 257 *HaeIII* (f), observed as a significant contributor to the better performing tanks, is also likely derived from the same source. The tanks contained a number of slightly different plastid phylotypes (Figure 9.7), suggesting that different source *Nannochloropsis* strains may be present across the different greenwater tank treatments with TRFLP analysis results suggesting that they are not present equally between tanks.

Similarity based SIMPER analysis of the poor performance tanks revealed only a low average similarity between replicates of the CWP, IAP and FAP samples (31.4% similarity). TRF 66 *HhaI* (r) was the largest contributor in the dataset, possibly deriving from members of the *Roseobacter* clade. The next highest was TRF 570 *HinfI* (r), likely deriving from chloroplasts of ingested *Nannochloropsis* (Table 9.5). SIMPER analysis indicated slightly higher congruence between the CWG, IAG and FAG samples (42.5% similarity) with TRFs 66 *HhaI* (r), 66 *HinfI* (f) and 257 *HaeIII* (f) contributing approximately equally in these samples (10-14%; Table 9.5). As previously indicated, some of these TRFs could not be identified based on clone library data. This suggests cloned 16S rRNA genes were not retrieved from the microorganisms that are the source of these TRFs during the clone library analysis.

Table 9.4 Restriction enzyme TRFs of microorganisms detected in larval samples determined by *in silico* digestion[a].

Clone identification:	*HinfI* (f)	*HinfI* (r)	*HhaI* (f)	*HhaI* (r)	*HaeIII* (f)	*HaeIII* (r)
Sulfitobacter pontiacus	296	538	60	66	~	568
Sulfitobacter dubius	296	538	60	66	~	568
Uncultivated species (closest species *Thalassobius mediterraneus*)	282	538	60	66	~	483
Phaeobacter gallaeciensis	296	538	60	66	~	483
Nautella italica	296	538	60	66	~	483
Ruegeria mobilis	296	538	60	66	~	483
Uncultivated species (closest species *Bartonella bacilliformis*)	296	563	~	~	192	483
Methylobacterium aquaticum	81, 298	367	515	68	62	292
Sphingomonas paucimobilis	102	272	81	68	70	~
Uncultivated species (most similar to family *Rickettsiales*)	~	~	~	~	197	71
Microbacterium oxydans/M. marinotypicum	123, 129	79	138, 144	228	223, 229	~
Psycrobacter nivimaris/P. glacincola	118	473	~	~	254	~
Pseudoalteromonas elyakovii	324	588	366	545	~	~
Nannochloropsis plastids	199	567-569, 575	~	~	258, 260	~(489)

[a]TRFs <60 bp are not included.

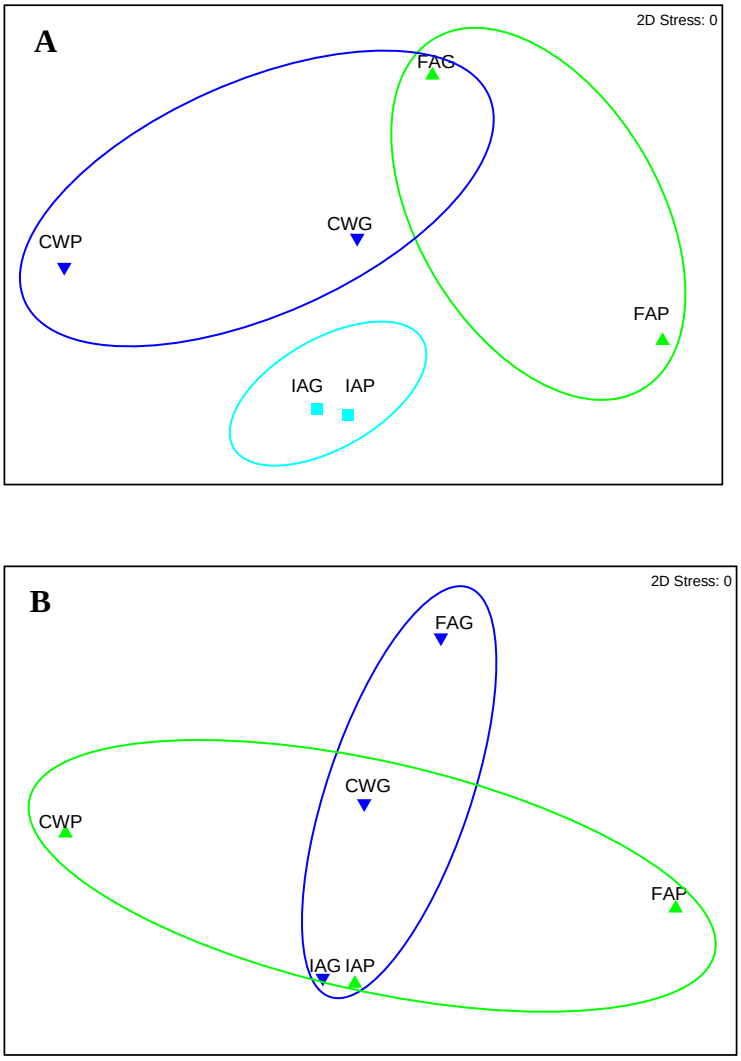

Figure 9.8 MDS plot of pairwise comparisons between 16S rRNA gene-based TRFLP profiles from larvae reared using - (A) instant algae (IA), clear water (CW) and (FA) fresh algae green water treatments. (B) Data compared on the basis of survival performance (P, poor; G, good performance).

Table 9.5 SIMPER analysis results for TRFLP profile data obtained from larvae-associated bacteria derived from tanks samples pooled on the basis of survival performance and treatment.

TRF[a]	Predicted species:[b]	Green water treatment			Larval survival Performance	
		Fresh Algae	Clear Water	Instant Algae	Poor	Good
		% contribution to % similarity:				
66 *HhaI* (r)	*Sulfitobacter/ Roseobacter* clade	23.95	33.58	35.78	28.12	34.08
66 *HaeIII* (f)	-	-	15.67	15.45	8.37	12.37
66 *HinfI* (f)	-	28.67	-	32.92	12.26	3.42
67 *HhaI* (r)	*Methylobacterium/Sphingomonas*	-	11.10	-	9.38	4.59
69 *HaeIII* (f)	*Sphingomonas*	25.49	-	-	7.59	-
76 *HaeIII* (r)	-	-	-	-	-	2.62
90 *HhaI* (f)	-	7.03	-	-	4.35	-
197 *HinfI* (f)	*Nannochloropsis* plastids	10.74	-	7.49	10.80	8.79
202 *HaeIII* (r)	-	-	-	-	-	4.06
257 *HaeIII* (f)	*Nannochloropsis* plastids	16.48	12.87	-	-	17.24
296 *HinfI* (f)	*Sulfitobacter/ Roseobacter* clade	-	-	-	-	3.73
326 *HinfI* (f)	*Pseudoalteromonas*	-	-	2.79	-	2.11
484 *HaeIII* (r)	*Sulfitobacter/ Roseobacter* clade	-	5.42	-	-	-
568 *HaeIII* (r)	*Sulfitobacter pontiacus*	-	-	6.09	6.91	2.65
570 *HinfI* (r)	*Nannochloropsis* plastids	17.29	10.68	12.27	16.33	10.49
Similarity%:		32.2	44.0	42.3	31.4	42.5

[a]TRFs indicated are the main contributing TRFs matching between replicate samples.
[b]TRF peak values can vary by ±1-2 bp due to inaccuracies in the size estimation during electrophoresis. The species or genera indicated are the closest matches based on the *in silico* digests indicated in Table 9.4. However, a significant caveat should be noted that the identification is at best a prediction.

Changes in the composition of the larval bacterial communities between fresh algae, Instant algae and clear water treatments.

TRFLP analyses indicated that CW and FA treatments had higher similarity to each other (R = 0.25) compared to that of the IA supplied tanks (Figure 9.9, Table 9.6, R = 0.5 & 0). Similarity for the FA samples was only 32.2% while the other treatment similarities were 42-44% (Table 9.6). However variation in the microbial communities between tanks of the same

treatments was high, indicating that specific relationships between microbial community differences are difficult to determine. This is suggested by the fact that only one TRF was common between all treatments – *66 HhaI* (r). The comparatively higher similarity between the CW and FA treatments despite high inter-tank variation is suggested by three TRFs contributing 47-55% of the peak area (Table 9.6).

TRFLP analysis of tank water samples comparing treatment and performance

Water samples from the corresponding tanks were also analysed using TRFLP. Data mining of the possible TRF originators again relied on the clone library data derived from larvae samples as it was assumed that microorganisms colonising the larvae mainly derived from surrounding tank water. As previously described water samples were collected from two tanks for each treatment including one in which survival was the best and a second tank in which survival was the least.

TRFLP profile data indicated water samples for the IA treatment were very similar (63.8% similarity) but the other treatments had less similar communities particularly the FA treatments (23.3% similarity) (Table 9.6). No differences in community structure are present for samples pooled on the basis of larval survival (R=-0.07, p=0.8). ANOSIM data indicates the FA and CW water samples based TRFLP profiles were similar overall (R=0) but that IA and CW water samples are completely different (R=1). Some overlap was observed for the FA and IA samples (R=0.25). MDS analysis (Figure 9.9) suggests a polarisation occurs between TRFLP profiles when based purely on the basis of performance *independent* of treatment. IAP, IAG and FAP (survival 1-22.5%, Table 9.1) water sample profiles were completely separate to the profiles from water from the better performing tanks (FAG, CWP, CWG, survival >30.1%). MDS analysis also demonstrates the high divergence between FA tanks samples and homogeneity for the IA samples. This suggests that poor larval survival may not be linked to colonisation by deleterious bacteria but is rather more dependent on the nature of the microbial community present in the water.

SIMPER based analysis reveals that fundamentally the greenwater treatments lead to considerably different communities that may have as much variation between individual tank samples as it does between treatments, especially in the case of the FA tanks. A high proportion of TRFs could not be identified from the clone library data and also suggests greater diversity occurs in the water as compared to what is observed in the larval samples.

Comparison of water and larval communities

Using TRFLP analysis bacterial communities were compared between the larval samples and the surrounding water. MDS, ANOSIM and SIMPER analysis results suggests the communities are different. Overall, dissimilarity was high at 84.3% correlating with an R value of 0.91 (p<0.02) (Figure 9.10). Several TRFs were more abundant in larval samples including 60-*HhaI* (f), 66-*HhaI* (r), 66-*HinfI* (f), 257-*HaeIII* (f) and 570-*HinfI* (r). Most of these TRFs likely derive from *Roseobacter* clade members and *Nannochloropsis* plastids. Several water sample TRFs [e.g. 81-*HinfI* (f), 228-*HhaI* (r)], were found to be specific to water samples. By comparison pooling water and larvae-derived TRFLP data revealed no significant difference on the basis of treatment-dependent performance (R=-0.11, Figure 9.10). In the case of individual treatments the CW and IA treatment tanks appear to form rough subsets of the overall FA community diversity.

Table 9.6 SIMPER analysis results for TRFLP profile data obtained from larvae-rearing tank water compared on the basis of survival performance.

TRF	Predicted species	Green water treatment			Treatment Performance:	
		FreshAlgae	ClearWater	InstantAlgae	Poor	**Good**
		% contribution to % similarity:				
60 *HhaI* (f)	*Sulfitobacter/ Roseobacter* clade	-	8.39	4.81	5.84	2.96
61 *HaeIII* (f)	*Methylobacterium*	-	-	-	-	1.49
61 *HinfI* (r)	*Sulfitobacter/ Roseobacter* clade	-	10.75	-	-	-
64 *HaeIII* (f)	-	2.27	5.12	-	3.00	2.65
66 *HaeIII* (f)	-	-	7.38	-	-	-
66 *HhaI* (r)	*Sulfitobacter/ Roseobacter* clade	-	19.14	13.53	7.40	5.83
69 *HaeIII* (f)	*Sphingomonas*	5.57	-	7.82	4.00	7.47
70 *HhaI* (r)	*Methylobacterium/Sphingomonas*	-	7.88	5.28	2.67	6.10
71 *HinfI* (r)	-	-	-	-	-	4.39
74 *HhaI* (r)	-	-	3.20	-	-	2.12
76 *HhaI* (f)	-	-	4.38	-	-	2.03
76 *HaeIII* (r)	-	-	3.71	-	-	1.62
81 *HaeIII* (f)	-	6.11	-	-	-	2.54
81 *HinfI* (r)	*Methylobacterium*	-	19.21	-	13.69	4.50
90 *HaeIII* (f)	-	-	-	-	-	1.78
90 *HinfI* (f)	-	-	11.21	-	-	-
100 *HaeIII* (f)	-	-	-	-	-	1.71
100 *HhaI* (f)	-	-	-	-	-	2.04
100 *HinfI* (f)	*Sphingomonas*	13.59	-	-	-	8.56
113 *HaeIII* (r)	-	3.47	-	-	4.51	2.14
115 *HaeIII* (r)	-	-	-	-	3.27	-
183 *HaeIII* (f)	-	-	-	3.33	-	-
190 *HaeIII* (f)	-	-	-	3.94	-	2.74
197 *HinfI* (f)	*Nannochloropsis* plastids	-	-	-	5.00	-
202 *HaeIII* (r)	-	4.73	8.39	-	7.11	2.72
228 *HhaI* (r)	*Microbacterium*	-	12.68	-	3.89	10.24
241 *HinfI* (r)	-	-	-	4.62	2.08	-
267 *HinfI* (f)	-	4.95	-	-	-	4.21
270 *HinfI* (r)	-	5.54	-	-	-	-
296 *HinfI* (f)	*Sulfitobacter/ Roseobacter* clade	-	-	3.88	2.04	-
326 *HinfI* (f)	*Pseudoalteromonas*	4.90	3.29	-	4.81	-
349 *HhaI* (r)	-	-		24.12	16.40	11.93
366 *HinfI* (r)	*Methylobacterium*	-	-	7.40	1.58	-
461 *HinfI* (f)	-	-	-	-	-	1.24
484 *HaeIII* (r)	*Roseobacter* clade	-	-	23.10	-	1.36
524 *HhaI* (r)	-	-	-	10.56	-	-
563 *HinfI* (r)	Uncultivated alphaproteobacterium	2.76	-	6.11	2.42	3.48
572 *HaeIII* (r)	*Sulfitobacter*	-	-	10.02	-	-

587 *HinfI* (r)	*Pseudoalteromonas*	4.57	-	4.23	1.99	4.83
Similarity%:		23.3	33.8	63.8	23.1	31.8

[a]Tanks are compared on the basis of survival performance.

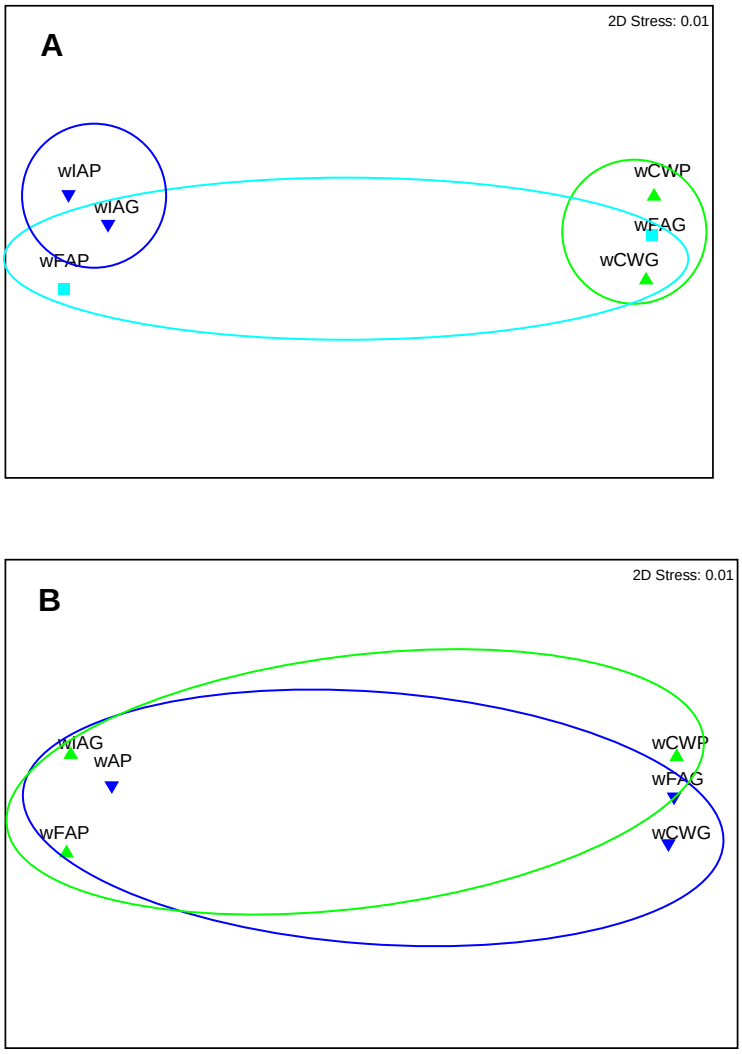

Figure 9.9 MDS plot of pair wise comparisons between 16S rRNA gene-based TRFLP profiles from water samples in which larvae are reared – (A) using instant algae (wIA), clear water (wCW) or (wFA) fresh algae treatments. (B) Data pooled in regards to survival performance (P, poor; G, good performance).

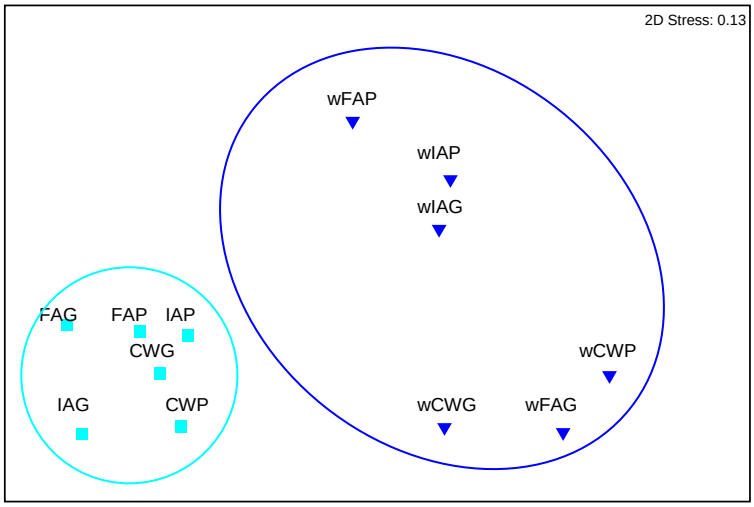

Figure 9.10 MDS plot of green water treatment samples showing a comparison of TRFLP profiles obtained from tank water and larvae samples: ■ larvae; ▼ water.

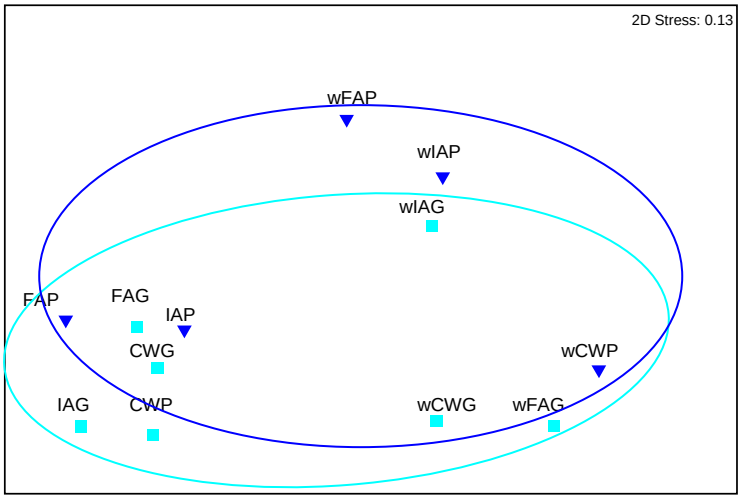

Figure 9.11 MDS plot of green water treatment samples showing a comparison of TRFLP profiles obtained from samples defined on the basis of larval survival performance. Performance: ▼ Poor; ■ Good

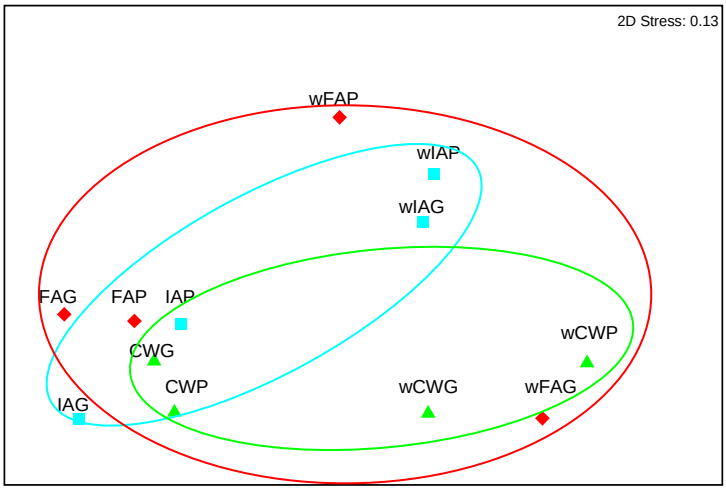

Figure 9.12 MDS plot of green water treatment samples showing a comparison of TRFLP profiles obtained from water samples and larvae samples. Treatments: ▲ Clear Water; ■ Instant algae; ◆ Fresh Algae.

9.5 Discussion

The overall results of this study showed that the microbial community of the 15 days post hatch larvae was relatively low in complexity, in comparison to other microbial environments, such as soils and marine sediment, with majority of phylotypes belonging to class *Alphaproteobacteria*. The bacterial diversity in this study is in general agreement with other studies of larvae microbiota (Jensen et al., 2004; Romero and Navarrete, 2006; Schulze et al., 2006) that show only low diversity occurs and the predominance of proteobacteria. No *Vibrio* spp. were detected; however, *Vibrio* spp. are likely to have been present since in culture based studies they have been isolated, following enrichment using a selective media (see Chapter 8). However, in the *Vibrio* populations are likely too low to be detected in the randomised clone library survey performed here. It has been found that it can be difficult to generate good quality genomic DNA preparations from a number of *Vibrio* spp. (Wong and Kuo, 2006), however this could be due to interfering components derived during extraction of relatively large amounts of biomass.

This low complexity in diversity observed may be a result of the shortcomings of PCR based studies, as reported by Wintzingerode et al. (1997). Other groups may have been missed through selection of the primers used to construct the libraries, such that only the predominant species in a microbial community were detected through the use of clone library construction and the use of TRFLP (Vlasov et al., 1998; Smith et al., 2007). It has also been suggested that bacteria already present in hatchery environments may influence the composition of subsequent larvae-associated microbiota (Cahill, 1990; Ringo and Olsen, 1999; McIntosh et al., 2008).

Differences in communities suggested by the clone library may have been caused by the different treatments that changed the microbial community structures. However, as the time frame for colonisation is relatively short, the colonisation process could be stochastic in its early phases. This is suggested by the presence of several similar phylotypes that occurred sporadically from sample to sample. This is similar to what is observed during juvenile

development of animals and humans, in which heterogeneous communities tend to occur in neonates but with maturation the communities converge towards homogeneity (Palmer et al. 2007). These different colonisation outcomes may have produced an effect that was deleterious to larvae survival; however, based on TRFLP data, no differences in performance in the larvae-associated communities could be observed, either dependent on or independent of the green water rearing approach. In the case of the tank water, survival outcomes could potentially be related to tank water microbial communities (Figure 9.9). This suggests that the deleterious effects on larval survival could be due to microorganisms occurring in the water but not necessarily active in larval fish colonisation.

The predominant species in the larvae-associated microbial community belong to the *Roseobacter* clade (Table 9.2). This is also apparent in the TRFLP data, which shows that the major taxonomic group present was comprised of *Roseobacter* clade members (Table 9.2). The only detected gammaproteobacteria belonged to the genera *Psychrobacter* and *Pseudoalteromonas*, both of which are commonly found in marine samples and are not noted for pathogenicity to fish (Hjelm, et al., 2004; Bowman, 2006). It is possible that another potentially antagonistic species may become more prevalent in the absence of typical pathogens like *Vibrio*.

Members of the genus *Microbacterium* have been isolated from marine samples but have never been shown previously to cause problems related to fish health, although they have been found on rare occasions to be associated with animal infections (Funke et al., 1997). It is remotely possible that particular conditions provided in the samples examined in this study allowed for *Microbacterium* strains to become sufficiently abundant to have deleterious effects on the larvae.

As no *Vibrio* spp. were detected, they cannot immediately be painted as an indicator of poor health as has been done in other studies. When comparing data from other studies, it is sometimes difficult to compare culture-based studies to molecular-based studies. Culture-based studies select and enrich for certain bacteria, for example with TCBS agar, which selects for *Vibrio* spp. and has additives (bile salts) that prevent most other species from growing. This may explain why no *Vibrio* spp. were detected in this study and why they were detected in previous culture-based studies (Battaglene et al., 2006). Battaglene et al. (2006) used selective TCBS to study microbiota associated with striped trumpeter larval cultures, but did not use other media to culture other heterotrophic bacteria. Although it is thought that *Vibrio* spp. are an indicator of poor tank hygiene (Villamil et al., 2003), some *Vibrio* strains have probiotic properties, including, for example, *V. alginolyticus* (Ringo and Vadstein, 1998; Ottesen and Olafsen, 2000; Huys et al., 2001; Makridis et al., 2001).

No concrete reason can be given for why one tank of a treatment performed better than another, but it may be proposed that the conditions that they provided do influence the bacterial community. There is a suggestion that the differences that occur are possibly induced by the initial availability of nutrients and what subsequently occurs during the feeding process (Romero and Navarrete, 2006).

The change in the community when different conditions occur is seen also in the water samples. A more diverse community is present in the seawater while larvae harbour a more restrictive range of microbiota (Jensen et al., 2004). This can perhaps be explained by the bacteria being part of either autochthonous (adherent) or allochthonous (transient) communities. Investigation of the intestinal bacterial community of Atlantic salmon found that when fish were fed two varying diets, the population changed in regards to

autochthonous and allochthonous communities (Ringo and Olsen, 1999; Schulze et al., 2006). As larvae get older, their microbiota also changes, as seen by Jensen et al. (2004), who reported that feeding Atlantic halibut larvae had more complex DGGE profiles than non-feeding larvae. Hansen and Olafsen (1989) proposed that bacteria in the surrounding water mass are involved in the initial colonisation of larval fish and dominate subsequent communities in adult fish. The TRFLP results from the current investigation also support this idea, as the dominant species found in the larvae were also detected in the water.

This investigation found that the water sample TRFs, compared to those of the larvae, were different (R=1, Figure 9.10) but compositionally analogous; that is, certain members of communities co-occurred in both locations but in different proportions. A similar observation was also reported by Smith et al. (2007) who observed that the flora of the outer mucus layer of whiting (*Merlangius merlangus*) is more diverse than that of its mouth and gut. Smith et al. (2007) also saw found that the bacteria associated with whiting had limited similarity to the bacterial community of the surrounding water when analysed using culture-independent approaches, including 16S rRNA gene-based clone libraries and TRFLP. These findings support what has been observed in this investigation, because although larvae and surrounding tank water did share some of the same TRFs, they were in different proportions and some were not present at all in the larvae. This may be explained by the diet of the fish and that certain bacteria are better suited to colonise the larval gut than others.

9.6 Conclusions

This investigation provided an insight into the bacterial diversity of striped trumpeter using 16S rRNA bacterial clone libraries and TRFLP. It showed that under different green water culture conditions the bacterial diversity appears relatively low. Definite conclusions cannot be drawn about the influences of the bacterial community of the larvae from the information obtained, although the surrounding water seems to have a potential influence on larval survival performance outcomes.

From this initial knowledge of the bacterial diversity of the striped trumpeter and the changes that different rearing conditions bring upon it, further investigations can now be conducted by screening isolated bacteria from these larvae cultures for their probiotic capabilities. Assessment of ways of altering the bacterial communities of live feeds, including *Artemia* and rotifers, and devising methods to track probionts within changing bacterial communities will also be explored.

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10 ASSESSMENT AND TRACKING OF BACTERIAL PROBIONTS WITHIN A STRIPED TRUMPETER LARVAE REARING SYSTEM

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10.1 Abstract

Potential probiotic candidates were identified by using antimicrobial *in vitro* plate testing against known pathogenic *Vibrio* species, with six out of 25 isolates tested selected for further testing. In *Artemia* challenge trials, it was determined that *Pseudoalteromonas agarivorans* ST18 and *Aliivibrio fischeri* ST7 had the least effect on *Artemia* survival. To further assess the probiotic capability of strains ST18 and ST7, rotifer and *Artemia* cultures were challenged with pathogenic strain *V. proteolyticus* V760 mixed with strains ST18 or ST7. Strain ST18 was found to have a probiotic effect in that cultures containing both V760 and ST18 were not significantly different from the control system but produced significantly better survival compared to the pathogen-only treatments. To further investigate ST18 and ST7 in a mixed cultured system terminal restriction fragment length polymorphism (TRFLP) analysis was applied to monitor the change in bacterial community. Through tracking probiont strain specific terminal restriction fragments (TRF) the probionts could be distinguished within the microbial community associated with rotifers and appeared to be readily taken up by rotifers. However, in *Artemia* experiments, uptake of the probionts appeared to be less successful.

10.2 Introduction

A review of the literature indicates bacterial probionts may have protective and/or beneficial effects in aquaculture processes. It was also discussed that since rotifers and *Artemia* are common live feeds, they could act as vectors for *Vibrio* spp., many of which are pathogenic (Lopez-Torres and Lizarraga-Partida, 2001). With this in mind, research has been undertaken to explore ways of manipulating the bacterial community associated with rotifers and *Artemia.* The manipulation is first designed to reduce harmful *Vibrio* spp. populations. Once successful the live feeds are fed to the larvae with the knowledge that they contain reduced amounts of harmful bacteria. Also once reduced it is also possible to introduce beneficial bacteria to the live feeds, by adding them during the enrichment stages. By doing this after the live feeds have been reduced in bacterial populations it increases the probability of the beneficial bacteria being incorporated into the live feed (Douillet, 2000).

The aim of the research was to identify potential probiotic bacteria using previously obtained isolates. This was done by assessing their ability to produce antimicrobial compounds that

inhibited growth of known pathogens. These potential probionts were then inoculated into bacteria-reduced rotifer and *Artemia* cultures and survival was assessed. This was achieved by:
1) Assessment of the probiotic properties of 22 bacterial isolates obtained from striped trumpeter against five known fish-pathogenic *Vibrio* strains by employing *in vitro* antimicrobial plate tests.
2) Assessment of four strains of bacteria isolated from striped trumpeter larvae that were shown to have some level of antimicrobial activity against at least one of the five known fish-pathogenic *Vibrio* strains by:
i) Assessing the four candidates for probiotic capacity in rotifer cultures when challenged with a pathogen.
iii) Evaluating probiotic capacity in cultures when challenged with a pathogen in *Artemia* cultures.
iii) Terminal restriction fragments (TRFs) were determined for each potential probiont so that it could be specifically identified when in a mixed community. This allowed an assessment of probiont uptake by the rotifer or *Artemia.*

10.3 Materials and Methods

10.3.1 *Bacterial Isolates*

A total of 22 bacterial isolates obtained by enrichment from striped trumpeter cultures (Table 10.1) (Chapter 9) were tested against five pathogenic *Vibrio* strains (Table 10.2) that were obtained from Dr Jeremy Carson of the Fish Health Unit of the Department of Primary Industries, Water and the Environment, Launceston, Tasmania. The bacterial isolates obtained from striped trumpeter larvae were sampled from three days post hatch to 15 days post hatch, at two day intervals. The larvae were washed in sterilised sea water (three washes to remove external bacteria) and homogenised, and plated on marine agar media and thiosulfate citrate bile salts sucrose agar (TCBS; Difco) after serial dilutions. After incubation at 25°C for 48 h isolated colonies were randomly selected and purified on ZoBell's marine agar (Oxoid) plates. All strains were phenotypically identified with the MicroSys ® V48 kit for the identification of *Vibrio* spp. (Carson et al., 2001) and the data matched with the probabilistic identification software for Windows (PibWin) (Bryant, 2004). The five pathogenic strains tested were isolated from culture tanks in which rotifers, *Artemia* or larval fish or rock lobsters had been cultivated at the Marine Resarch Laboratories, Taroona, Tasmania, and undergone mass mortalities (J. Carson personal communication). The culture methods are as described for striped trumpeter samples.

10.3.2 *In vitro antimicrobial activity assay*

Antimicrobial activity was assessed against five known fish-pathogenic bacteria (Table 10.2) using the cross-streak assay described by Lemos et al. (1985). The assay was performed by heavily inoculating each isolate onto one third of a marine agar plate and incubating at 25˚C for 7 days. This was done by swabbing and covering one third of the agar with enriched isolates grown for 48 h at 25˚C grown in marine broth. Pathogens were then inoculated as a single streak at right angles to the isolate growth and the plates re-incubated for a further 48 h. Extensive zones of inhibition of a known pathogen were scored as "++", narrow zones of inhibition were scored as "+" and no inhibition was scored as "-" .

10.3.3 *Bacterial production*

Bacterial strains were grown in marine broth, which was made in the same manner as marine agar, but with the omission of the agar; 250 ml Schott bottles were filled with 100 ml of marine broth and autoclaved. The broths were loop inoculated with bacteria that had been previously sub- cultured on marine agar for 24 h and then incubated at 25˚C for 48 h.

Table 10.1 Species identification of potential probiotic bacteria used in the *in vitro* antimicrobial activity test, all isolates were obtained from striped trumpeter larvae

Strain no.	MicroSys ID
ST1	*Vibrio alginolyticus*
ST2	*Vibrio alginolyticus*
ST3	*Vibrio anguillarum*
ST4	*Vibrio anguillarum*
ST5	*Vibrio chagasii*
ST7	*Vibrio fischeri I*
ST8	*Vibrio fischeri II*
ST9	*Vibrio ichthyoenteri II*
ST11	*Vibrio penaeicida*
ST12	*Vibrio splendidus I*
ST13	*Vibrio splendidus I*
ST14	Phenon 36
ST15	Phenon 36
ST16	Type 1
ST17	Type 2
ST18	Type 3a
ST19	Type 3b
ST20	Type 5
V4	Phenon 29
V8	Phenon 29
V17	Phenon 59
V52	Phenon 59

Table 10.2 Pathogenic *Vibrio* species used in the *in vitro* antimicrobial activity tests. All were isolated from water or animals that had been identified to have had a bacterial issue that resulted in mortalities

Strain no.	MicroSys ID	Host species or source
V34	*Vibrio alginolyticus*	Seawater
V568	*Vibrio anguillarum*	Rotifers
V760	*Vibrio proteolyticus*	*Artemia*
V886	*Vibrio alginolyticus*	Rock lobster phyllosoma
V890	*Vibrio harveyi*	Rock lobster phyllosoma

The densities of the cultures were calculated by taking a subsample of the broth and counting populations using a haemocytometer. For the haemocytometer counts the following formula was used:

Total cells counted x (25×10^4) x no. of triple ruled squares counted = Number of cells/ml x the dilution factor (if applied).

10.3.4 *Rotifer production*

Rotifers (*Brachionus plicatilis*) were harvested daily from semi- continuous stock cultures raised on a diet of the microalgae *Nannochloropsis* sp. (Battaglene et al., 2006). Harvested rotifers were rinsed in ozonated seawater and then transferred to 500 ml Schott experimental glass vessels for 12 h enrichment, at 400 rotifers ml^{-1} and 23˚C. Rotifers were enriched with AlgaMac (Aquafauna Biomarine, Hawthorne, California, USA) at 0.2 g per million rotifers. AlgaMac was blended with seawater and otherwise added according to the manufacturer's instructions. Aeration and oxygen were provided to maintain dissolved oxygen above 4 mg l^{-1}. Probionts were added to the corresponding rotifer enrichment vessel at the same time as the AlgaMac was added.

10.3.5 *Artemia production*

Decapsulated *Artemia* cysts (E.G. Artemia Systems, INVE, Belgium) were hatched in a 100 l conical tank at 28˚C in sea water with vigorous aeration and 24 h light. After 24 h hatched nauplii were rinsed for 5 min with seawater after passing through a 180µm screen filter to remove the hatched and unhatched cysts and then through a 60 µm screen filter to catch the *Artemia* nauplii, which were then placed into a 1 l beaker to a final approximate density of 50 *Artemia* ml^{-1}. The *Artemia* bacterial load was reduced using Sanocare Hatch control (INVE) at a rate of 0.1 ml l^{-1}. Prior to stocking Artemia were also washed in ozonated sea water for 5 min.

10.3.6 *DNA and extraction and purification*

DNA was extracted from bacterial cells using the same methods set out in Chapter 9.

10.3.7 *PCR amplification of 16S rRNA genes*

PCR amplification of the 16S rRNA gene was performed using the Hotstart Taq kit (Qiagen) and universal bacterial primers 10f (5'-GAG TTT GAT CCT GGC TCA G-3') and 907 (5'-CCG TCA ATT CCT TTG AGT TT-3'). Each reaction was a 25 µl reaction mix with 12.5 µl of HotStart mastermix, 1 µl of the forward primer, 1 µl of the reverse primer, and approximately 10 ng of DNA template. A final volume of 25 µl was adjusted with sterile milliQ water. The following thermal cycling program was used: initial denaturing at 94°C for 15 min, 34 cycles of denaturing for 1 min, annealing at 55°C for 1 min, extension for 3 min: final extension at 72°C for 10 min. The reaction was purified using the Qiagen PCR cleanup kit.

10.3.8 *DNA Sequencing and TRFLP analysis*

Both DNA sequencing and TRFLP analysis was performed as described in Chapter 9.

10.3.9 *Probiotic activity assessment of V52, ST18, V8, ST14, ST7*

Experimental design

The experimental vessels were 500ml Schott bottles with aeration inlets and outlets. Each treatment had three replicates. The air inlet had a sterile, 0.2 µm pore size hydrophilic filter in place to stop any airborne contaminants entering and the outlet had sterile 0.2 µm hydrophobic filters to stop bacteria escaping from the cultures. Fig.10.1 provides a schematic of the set up. Oxygen was provided using an oxygen concentrator (Millennium M5, Respironics, Pennsylvania, USA). At the point at which the glass tube entered the lid of the bottle a piece of rubber tube was fitted to make an airtight fit. The cultures were placed into a climate-controlled room set at a 12:12 light:dark cycle and a temperature of 25˚C ± 1˚C.

To 400 ml of ozonated sea water a total population of 2×10^5 rotifers (500 ml^{-1}) or 2×10^4 *Artemia* nauplii (50 ml^{-1}) was added. Rotifers and *Artemia* were enriched in the same process as that used in the TAFI hatchery to mirror the process in a working hatchery, by adding the required amount of AlgaMac as outlined above. Bacterial isolates were inoculated to achieve a final concentration of 5×10^5 cells ml^{-1}, to assess if they have any detrimental effects on the rotifer population. This was determined by plate counts performed on the broth cultures. The broths were homogenised and the calculated amount of broth required to achieve the final concentration was taken aseptically and transferred into sterile vials. All experimental units were treated the same with all receiving the same amount of broth by adding uncultured sterilised marine broth to those which needed more and to the controls. As a pathogenic control *Vibrio proteolyticus* was used as it is a known marine pathogen (Vadstein et al., 2004). The cultures were enriched for 14 h after which survival was assessed and samples taken for further analysis. Survival was assessed by taking triplicate 1 ml subsamples and counting them under a dissection microscope. If the rotifers or *Artemia* moved freely and were active, they were considered to have survived. If they were moribund, they were classified as effectively deceased. Samples were taken for TRFLP analysis by separating the rotifers and *Artemia* from the culture water using a 62 µm screen, and washing three times in sterilised water to remove external bacteria resulting in a 500 µl sample. All samples were kept on ice until frozen within 2 h.

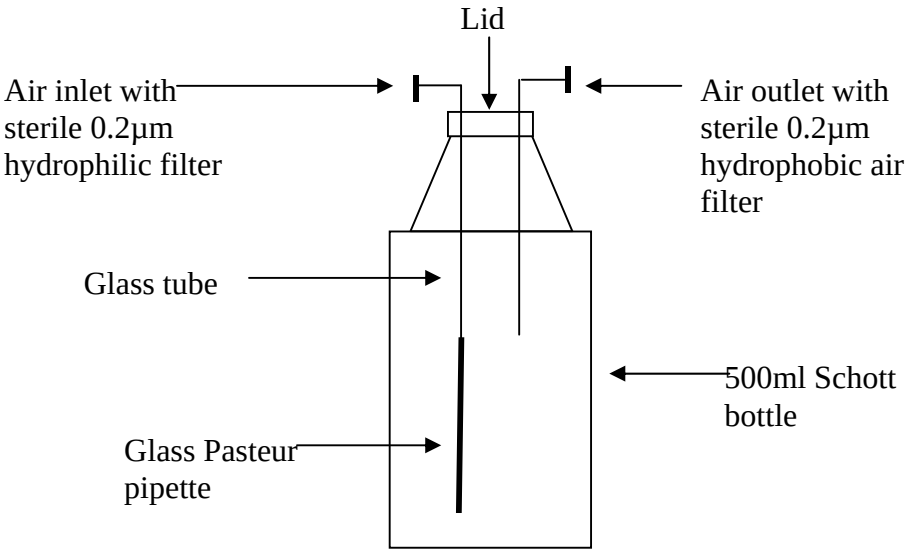

Figure 10.1 Experimental Schott bottle vessel set-up used for survival assessment experiments.

10.3.10 *Assessment of potential probiotic bacteria V52, ST18, V8, ST14, ST7.*

Assessment of potential probiotics V52, ST18, V8, ST14, ST7 and effects on rotifer and *Artemia* cultures during the enrichment process was performed through a series of experiments described below. In all experiments survival of the rotifers was assessed after 14 h.

Screening of V8, V52 and ST18 on rotifer survival during lipid enrichment (Experiment 1).

The aim of the experiment was to determine if potential probionts had any effect on rotifer survival when added to achieve a final concentration of 5 x 10^5 cells ml-1. Six different treatments were assessed: 1) a control that had no bacteria added; pathogen-only containing controls had a known pathogen added, including 2) *V. proteolyticus* V-760, 3) *V. alginolyticus* V-34; 4 to 5) treatments that included isolates V8, V52, and ST18.

Screening of ST7, ST14 and ST18 on rotifer survival during lipid enrichment (Experiment 2).

Experiment 2 repeated the first experiment but tested five different treatments. They included: 1) a control that had no bacteria added; 2) a pathogen-containing control with *V. proteolyticus* V-760 added; and 3-5) the probiont-containing treatments including strains ST14, ST18, and ST7 added to achieve a final concentration of 5×10^5 cells/ml.

Survival of rotifers when challenged with a known pathogen V-760 and combinations of potential probionts (Experiments 3 and 4)

The aim of the experiments was to determine if potential probionts could reduce the pathogenicity of a known pathogen when added to rotifer cultures. There were six different treatments: 1) a control that had no bacteria added; 2) a pathogen control that *V. proteolyticus* V-760 added**,** and 3-6) the test probionts (ST7, ST18) were added singularly or as mixtures with the pathogen (ST7 +V-760, and ST18 + V-760). Strain V-760 was added to achieve a final concentration of 5×10^4 cells ml^{-1} (Experiment 3) or 5×10^5 cells ml^{-1} (Experiment 4). The test probionts were added at 5×10^4 cells ml^{-1}.

Survival of *Artemia* when challenged with a known pathogen V-760 and combinations of potential probionts (Experiments 5 and 6).

The aim of the experiment was to determine that potential probionts had on *Artemia* cultures when the known pathogen V-760 and the test probionts are added at the same time in order to assess whether the probionts can reduce the effect of the pathogen. The experiment consisted of 1) a control that had no bacteria added; 2) a pathogen-containing control in which *V. proteolyticus* V-760 was added**,** 3-4) treatments in which the test probionts (strains ST7, ST18) were added singularly; 5-6) and added with strain V760 as done in rotifer Experiments 3 and 4. Experiments 5 and 6 were except involved different initial bacterial loading (to achieve a final concentration of 5×10^5 cells ml^{-1} for Experiment 5 and 5×10^4 cells ml^{-1} for Experiment 6).

10.3.11 *TRFLP analyses*

TRFLP was analysed as outlined in Chapter 9. In addition TRFLP analysis was performed on selected strains ST7, ST14, ST18, V52, V34, V760, V8, (Tables 10.1 and 10.2) so that their TRFs could be determined experimentally (not just *in silico*), thus enabling them to be potentially identified within a mixed population sample. PCR pseudo-TRFs can be formed with the left over primer bases and thus give false peak readings. This is why it is advisable to perform virtual digestion of corresponding sequence data (i.e. clone library-derived sequences). It is also important to design the primers being used correctly, to optimise primer levels in the TRFLP PCR thermocyling process, and to take account of PCR run-to-run variations (Schutte et al., 2008) thus minimising pseudo-TRF artifacts. By using SIMPER analysis (Primer v.6) an estimation of the average contribution to the total profile peak area of the probiont-derived TRFs within the sample was obtained.

10.3.12 *Statistical analyses*

Statistical analysis was performed by one-way analysis of variance (ANOVA). For all tests a significance level of $P<0.05$ was adopted. Tukey's post hoc test was used to compare means. Homogeneity of variance was evaluated using residual plots. The statistical package SPSS V.10 was used.

10.4 Results

10.4.1 *In vitro Antimicrobial plate results*

Using *in vitro* antimicrobial plate tests it was found that five of the 22 species had some antimicrobial activity against the test pathogens (Table 10.3). Strains ST7, ST18, V8 and V52 exhibited the most inhibition. All of the five strains were found to inhibit *V. alginolyticus* (V-34) with ST18 also inhibiting *V. proteolyticus* (V760). Strain ST18 was seen to have the most inhibition across the test pathogens having an effect on all but *V. harveyi* (Table 10.3). A typical *in vitro* antimicrobial plate is shown in Fig. 10.2.

Table 10.3 Results of the *in vitro* antimicrobial plate test. ++ indicates strong inhibition + indicates slight inhibition and – indicates no inhibition seen on test pathogen growth

	Test Pathogens:				
Bacterial Isolate	*V. algino-lyticus* (V34)	*V. anguillarum* (V568)	*V. proteo-lyticus* (V760)	*V. algino-Lyticus* (V886)	*V. harveyi* (V890)
ST1	-	-	-	-	-
ST2	-	-	-	-	-
ST3	-	-	-	-	-
ST4	-	-	-	-	-
ST5	-	-	-	-	-
ST7	++	-	+	-	-
ST8	-	-	-	-	-
ST9	-	-	-	-	-
ST11	-	-	-	-	-
ST12	-	-	-	-	-
ST13	-	-	-	-	-
ST14	+	-	-	-	-
ST15	-	-	-	-	-
ST16	-	-	-	-	-
ST17	-	-	-	-	-
ST18	++	+	++	+	-
ST19	-	-	-	-	-
ST20	-	-	-	-	-
V4	-	-	-	-	-
V8	++	-	+	-	-
V17	-	-	-	-	-
V52	++	-	-	-	-

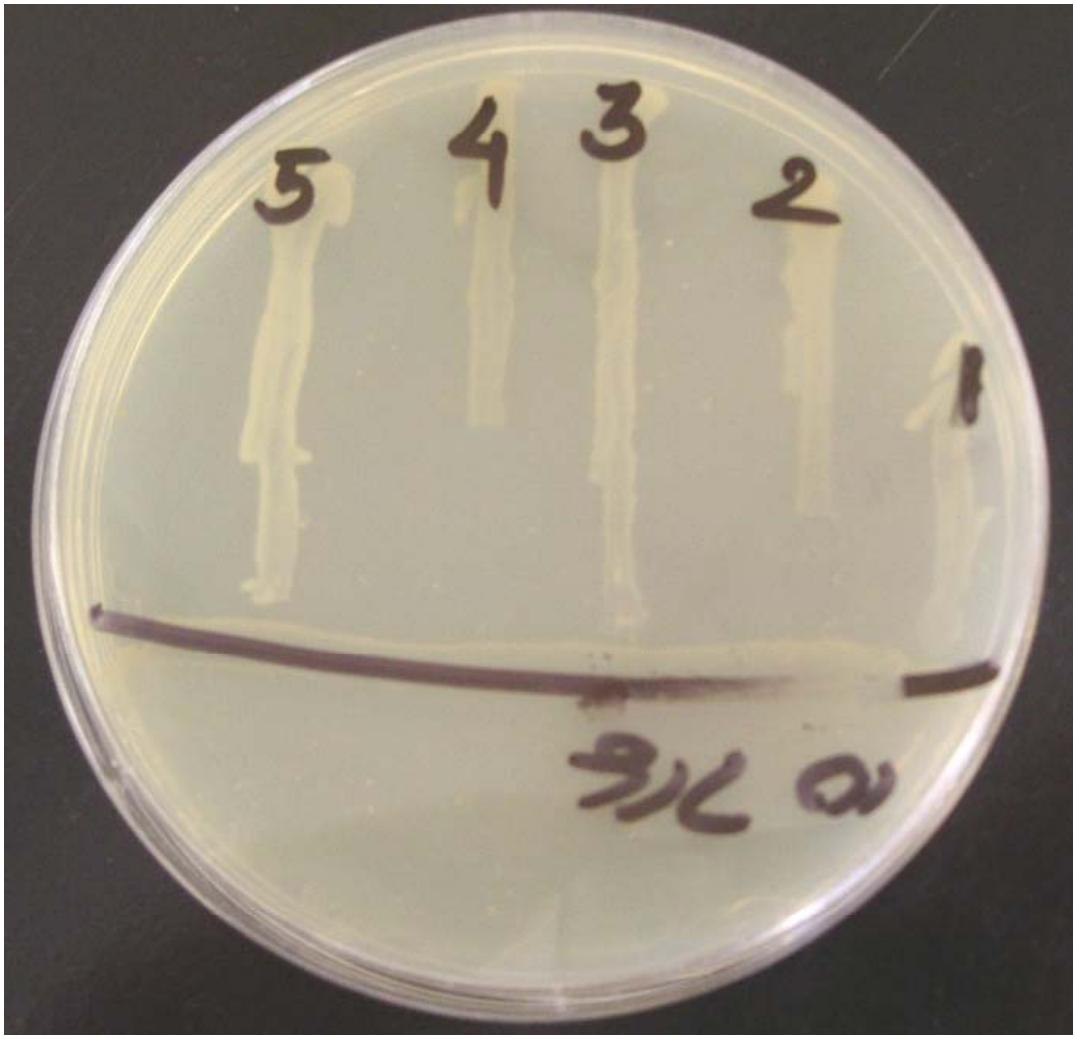

Figure 10.2 Photograph of an *in vitro* antimicrobial plate showing inhibition of *Vibrio* test pathogens by putative probiont isolate ST18. A maximum inhibition score of "++" was recorded against streaks of strains numbered 4 and 2. Strain streaks 1 to 5, are *V. anguillarum* (V568), *V. alginolyticus* (V34), *V. harveyi* (V890), *V. proteolyticus* (V760) and *V. alginolyticus* (V886), respectively.

Seventeen isolates had no detectable activity against any of the *Vibrio* test pathogens (Table 10.3). Of the pathogens examined *V. alginolyticus* (V34) showed a tendency to be more readily inhibited.

10.4.2 *Identification of putative probiont isolates*

Putative probiont isolates not identifiable with the MicroSys system (strains ST14, ST15, ST16, ST17, ST18, ST19, ST20, V4, V8, V17, and V52; Table 10.2) were identified using 16S rRNA gene sequencing. The following strains were grouped as glucose non-fermenters: ST16, ST17, ST18, ST19 and ST20. Strains V4, V8, V17, and V52 were identified as *Vibrio* spp. by MicroSys but these could not be given a species name as the data obtained could not be matched with any known members of the family *Vibrionaceae*. Table 10.4 shows the 16S rRNA gene sequencing results determined by GenBank BLAST matches (http:/www.ncbi.nlm.nih.gov/Blast (Altschul et al., 1997). Strain ST7 is likely a strain of *Aliivibrio fischeri* (Urbanczyk et al. 2007). ST14 was most likely a strain of *Vibrio penaeicida* (99% similarity to the type strain LMG 19663^T). Strain ST18 was likely a strain of *Pseudoalteromonas agarivorans* (>99% similarity to type strain KMM 255^T). Strain V8 was found to group in the *V. splendidus/V. tasmaniensis/V. lentus* complex of strains (99% similarity to *Vibrio* sp. V004 isolated from *Latris lineata*; Gudkovs et al, unpublished).

Table 10.4 Comparison of MicroSys system identifications to 16S rRNA gene sequence data results. The five isolates with potential as probionts are highlighted in bold.

Strain no.	MicroSys ID	IDS	Host	Closest 16S rRNA gene sequence match (GenBank accession number):	% similarity
ST1	*V alginolyticus*	0.99906	Striped trumpeter	*Vibrio alginolyticus* strain UQM 2770 (AY264938)	99
ST2	*V alginolyticus*	0.99888	Striped trumpeter	*Vibrio alginolyticus* strain SR1 (DQ269211)	98
ST3	*V anguillarum*	0.99999	Striped trumpeter	*Vibrio anguillarum* (EF467287)	99
ST4	*V anguillarum*	0.99984	Striped trumpeter	*Vibrio anguillarum* strain MHK11 (EF091706)	99
ST5	*V chagasii*	0.99703	Striped trumpeter	*Vibrio pomeroyi* (AJ491290)	99
ST7	***V fischeri I***	**0.99967**	**Striped trumpeter**	***Allivibrio fischeri* strain VFISC2 (AY780014)**	**99**
ST8	*V fischeri II*	1.00000	Striped trumpeter	*Aliivibrio fischeri* strain 082205 (DQ174503)	99
ST9	*V ichthyoenteri II*	1.00000	Striped trumpeter	*Vibrio ichthyoenteri* (AJ437192)	99
ST11	*V penaeicida*	1.00000	Striped trumpeter	*Enterovibrio norvegicus* (AJ437193)	97
ST12	*V splendidus I*	0.99715	Striped trumpeter	*Vibrio tasmaniensis* strain 562 (AY620964)	99
ST13	*V splendidus I*	0.99964	Striped trumpeter	*Vibrio splendidus* (AJ874367)	99
ST14	**Phenon 36**	**0.99927**	**Striped trumpeter**	***Vibrio penaeicida* (AJ437191)**	**99**
ST15	Phenon 36	0.99992	Striped trumpeter	*Enterovibrio norvegicus* (AJ437193)	99
ST16	Type 1	0	Striped trumpeter	*Marinomonas aquimarina* (AJ843079)	99
ST17	Type 2	0	Striped trumpeter	*Marinomonas aquimarina* (AJ843079)	99
ST18	**Type 3a**	**0**	**Striped trumpeter**	***Pseudoalteromonas* sp. P11-B-12 (EU016154)**	**99**
ST19	Type 3b	0	Striped trumpeter	*Pseudoalteromonas agarivorans* (AB049728)	99
ST20	Type 5	0	Striped trumpeter	*Enterovibrio norvegicus* (AJ437193)	99
V4	Phenon 29	0.99995	Striped trumpeter	*Vibrio splendidus* (AJ874367)	98
V8	**Phenon 29**	**0.99995**	**Striped trumpeter**	***Vibrio* sp. (DQ146970) (*V. splendidus* strain complex)**	**99**
V17	Phenon 59	1.00000	Striped trumpeter	*Aliivibrio fischeri* (DQ090767)	97
V52	**Phenon 59**	**1.00000**	**Striped trumpeter**	***Aliivibrio fischeri* (DQ090767)**	**97**
V34	*V. alginolyticus*	0.99994	Seawater	*Vibrio alginolyticus* (DQ269211)	98
V568	*V. anguillarum*	1.00000	Rotifers	*Vibrio anguillarum* strain MHK11 (EF091706)	99
V760	*V. proteolyticus*	1.00000	Artemia	*Vibrio proteolyticus* (AF513463)	98
V886	*V. alginolyticus*	0.99787	Rock lobster phyllosoma	*Vibrio alginolyticus* strain SR1 (DQ269211)	97
V890	*V. harveyi*	1.00000	Rock lobster phyllosoma	*Vibrio harveyi* (AY967728)	99

10.4.3 *Determination of the probiotic capabilities of possible candidates on two live feeds - Rotifer and Artemia Challenge Experiments.*

Results from Experiment 1 and 2 indicated that the controls with no bacteria added survived significantly better (75±2%) (F=19.713, Df=5,12 P<0.001) than other treatments (Figure 10.3 and Figure 10.4). Addition of strain ST18 lead to significantly better survival (63 ± 2%) than the other isolates (Figure 10.3). Experiment 2, adding ST14 significantly reduced survival (43 ± 4%) (F= 156.288, Df=5,12 P<0.001) .

Experiments 3 and 4 confirmed that adding ST18 had no significant impact on rotifer survival (Figure 10.5 and Figure 10.6). Results from Experiment 3 where lower inoculums of bacteria were added indicated that adding both ST18 and ST7 together provided significantly better survival than ST7 added alone but was not significantly different from the control in which probiotics were not added (F= 32.198, Df =4,10 P<0.001) (Figure 10.5) while adding ST18 or ST7 to cultures challenged with V-760 significantly improved survival compared to the negative controls in both experiments (F=33.854 Df=5,12 P=<0.001), F=42.11 Df=5,12 P=<0.001) (Figure 10.5 and Figure 10.6).

Experiments 5 and 6 confirmed that the addition of ST18 and ST7 to *Artemia* showed no significant difference to the control (F=63.129 Df=5,12 P<0.001). When added with V-760 it improved survival (F=42.612 Df=5,12 P<0.001) compared to treatments that received only strain V-760 (Figure 10.7 and Figure 10.8).

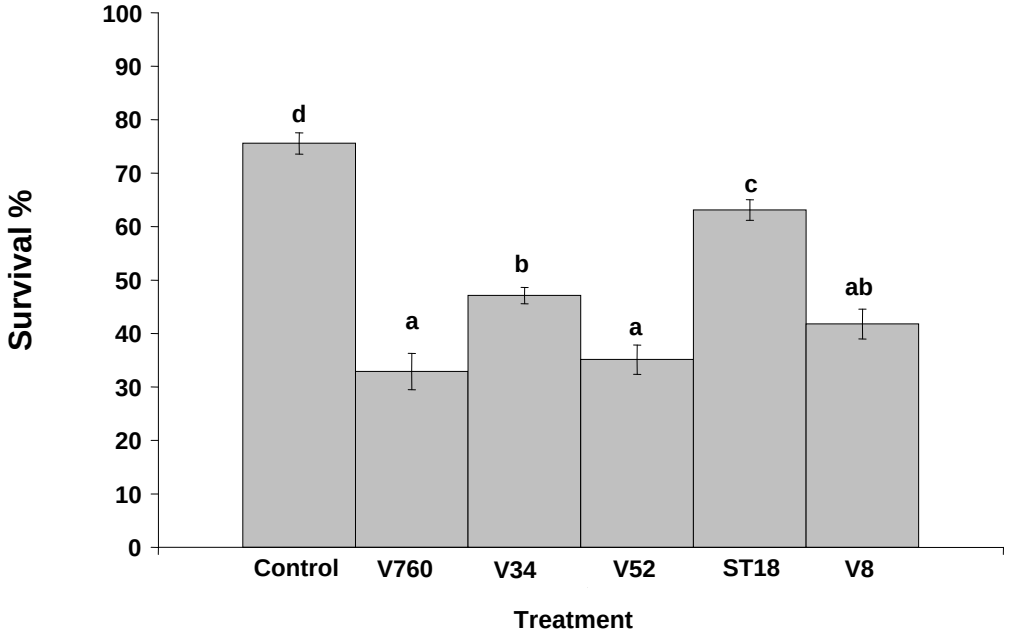

Figure 10.3 Mean survival (± standard deviation) of rotifers when challenged with *V. proteolyticus* V760, *V. alginolyticus* V34, and isolates V52, ST18 and V8, over a 14 h enrichment period. Bacterial cell concentrations used were 5×10^5 cells ml^{-1}. Columns sharing the same letter are not significantly different (P< 0.05).

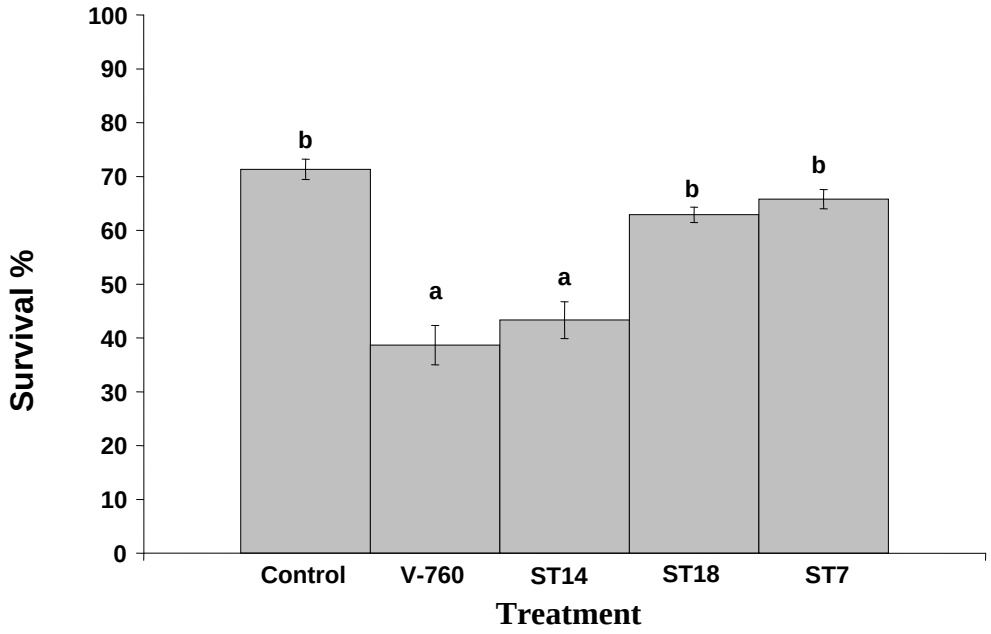

Figure 10.4 Mean survival (± standard deviation) of rotifers when challenged with *V. proteolyticus* V-760, ST14, ST18, and ST7, over a 14 h enrichment period. Bacterial cell concentrations used were 5×10^5 cells ml^{-1}. Columns sharing the same letter are not significantly different ($P< 0.05$) in mortality between each treatment.

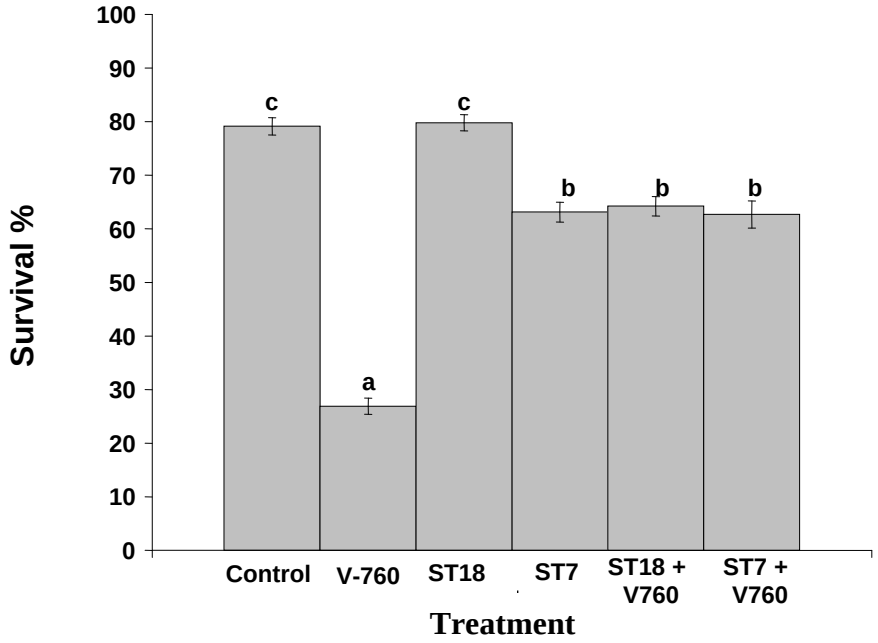

Figure 10.5 Mean survival (± standard deviation) of rotifers when challenged with *V. proteolyticus* V-760, ST18, ST7 over a 14 h enrichment period. Bacterial cell concentrations used were 5×10^4 cells ml^{-1}. ST18 + V-760 and ST7 + V-760 had both the bacteria isolates added at the same time at a concentration of 5×10^4 cells ml^{-1} for each isolate. Columns sharing the same letter are not significantly different ($P< 0.05$) in survival among treatments.

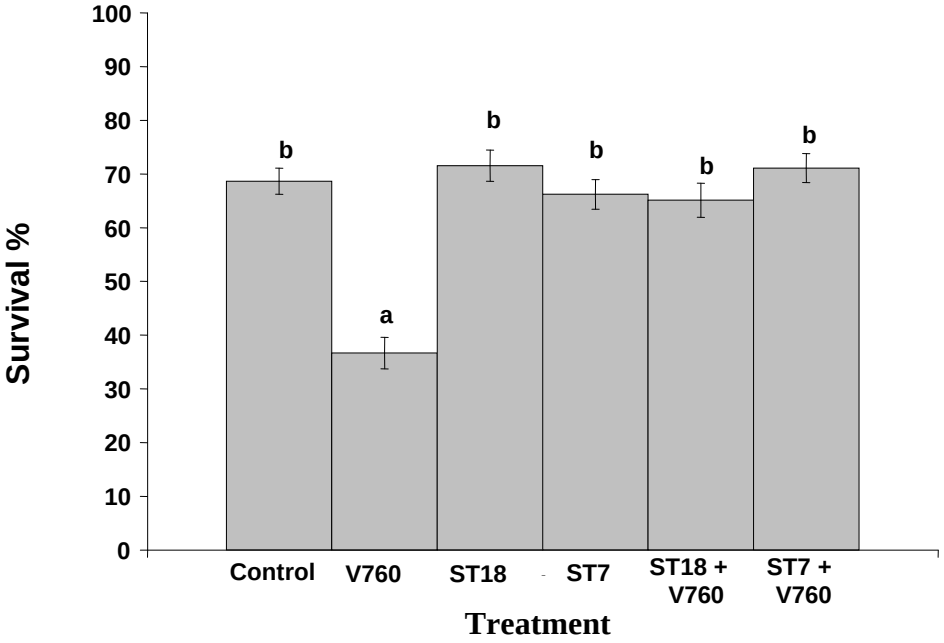

Figure 10.6 Mean survival (± standard deviation) of rotifers when challenged with *V. proteolyticus* V-760, ST18, ST7 over a 14 h enrichment period. Bacterial cell concentrations used were 5×10^5 cells ml^{-1}. ST8 + V760 and ST7 + V760, had both the bacterial isolates added at the same time at a concentration of 5×10^5 cells ml^{-1} for each isolate. Columns sharing the same letter are not significantly different ($P < 0.05$) in mortality between each treatment.

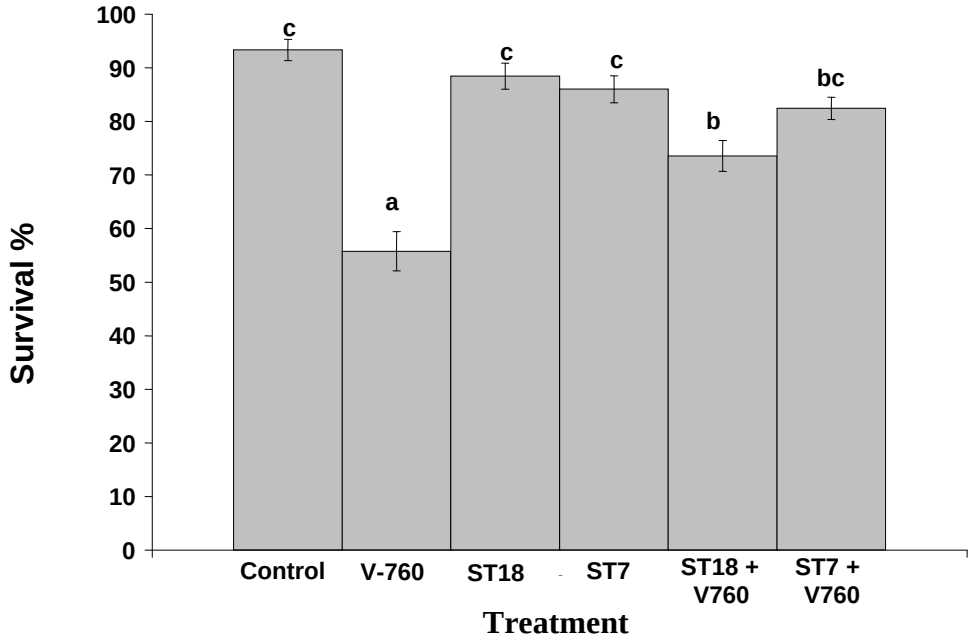

Figure 10.7 Mean survival (± standard deviation) of *Artemia* when challenged with *V. proteolyticus* V-760, ST18, ST7 over a 14 h enrichment period. All initial bacterial cell concentrations used were 5×10^5 cells ml^{-1}. Columns sharing the same letter are not significantly different ($P < 0.05$).

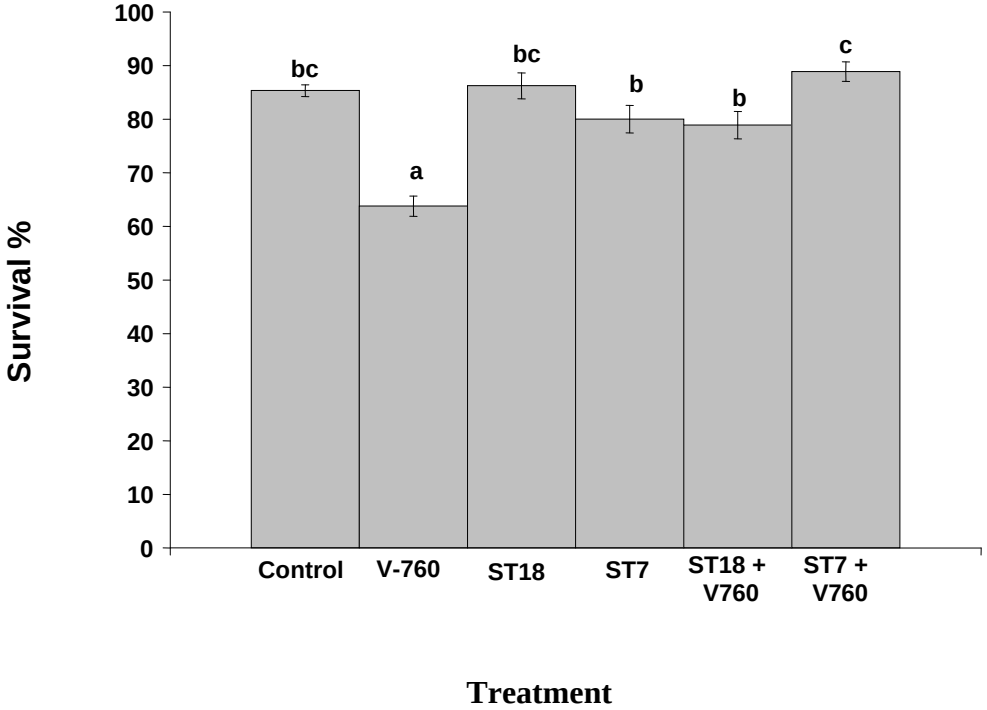

Figure 10.8 Mean survival ± SE of *Artemia* when challenged with *V. proteolyticus* V-760, ST18, ST7 over a 14 h enrichment period. All initial bacterial cell concentrations used were 5×10^4 cells ml^{-1}. Different letters indicate significant differences ($P < 0.05$) in mortality between each treatment for individual days (n=3).

Table 10.5 TRFs representing putative probiont strains utilising restriction endonucleases enzymes *HaeIII*, *HinfI* and *HhaI* determined using TRFLP

	HaeIII	
Strain:	**Forward fragments:**	**Reverse Fragments:**
ST14	-	190
ST18	64	508
ST7	84	-
V52	84	-
V34	78, 84	190
V760	78, 84	190
V8	78, 84	-
	HinfI	
Strain:	**Forward fragments:**	**Reverse Fragments:**
ST14	71, 172, 338	270
ST18	104	270
ST7	268	270,
V52	328	270, 588
V34	72, 338	270, 587
V760	72, 338	270, 587
V8	-	270
	HhaI	
Strain:	**Forward fragments:**	**Reverse Fragments:**
ST14	179, 226,	350, 549
ST18	60	366
ST7	68	177
V52	179, 226,	349
V34	475	349, 579
V760	179, 226,	579, 475
V8	178	349

10.4.4 *TRFLP analysis and TRF tracking results*

The MDS plot derived from the TRFLP data for probiotic assessment Experiment 1 and 2 (Figure 10.9 and Figure 10.10) shows that all samples have some overlap with the control bacterial community in which putative pathogens or probionts were not added. The addition of strains V760, V34, V52, V8, and ST14 resulted in TRFLP profiles not significantly different to the control sample ($p>0.7$, Figure 10.9 and Figure 10.10). ANOSIM values, however indicated the treatment in which strain ST18 is added alone to rotifer cultures (at 5 × 105 cells/ml) was most consistently different to the control as well as the other treatments ($R = 0.185$ to 0.667, significance $p<0.3$, and only 28% similar to the control profile). Using SIMPER analysis it was possible to track ST18 and detect it through the observation of distinctive TRFs (Table 10.5) amongst the other TRFs present. Strains V760, V34 and V52 could not be readily tracked (Table 10.6) as their TRFs were not distinct from each other and thus TRF information could not be used for confident identification with any of these strains. The same identification problem was also observed in the other experiments using these isolates. It was found that with addition of a bacterial isolate to the rotifers the overall similarity between the replicates increased. The control with no addition of bacteria were only 27% similar to each other, while the addition of V760, V52, ST18, V8 and V34, increased the similarity between the replicates (53, 43, 42, 44.2 and 25% respectively).

Table 10.6 indicates which TRF distinct to ST18 that they were detected and the percent contribution they represent within the overall profile. It is possible other related members of *Pseudoalteromonas* or *Aliivibrio* may be present in the samples, however ST18 or ST7 distinct TRFs were not detected in the controls suggesting the populations are below the sensitivity level of the TRFLP method to detect. When strains ST18 and ST7 were added at higher numbers (5×105 cell ml^{-1}, Experiment 2) TRFLP profiles obtained were significantly different ($R >0.78$) and were only 18-21% similar to the control. ST18 and ST7 could also be more readily identified through the observation of distinct TRFs, which showed increased contributions to the total peak area (Table 10.7).

The same trend in similarity between treatments increasing with addition of isolates ST18 was also seen in Experiment 3 with the control profiles being 42% similar. This similarity increased when other isolates were added to about 46-57%. When added in combination with V760 fewer differences were observed in the profiles than when they were added independently (Figure 10.11, Table 10.8). When a lower population of V760 was added a more marked difference was apparent ($R=0.30$, $p<0.1$). The effect of adding an order of magnitude more bacterial cells, as examined in Experiment 4, made no specific difference to profiles. However ST7 and ST18 additions created distinct TRFLP profiles while sole V760 additions did not differ from controls (Figure 10.12, Table 10.9). It was observed that the control and V760 were 53% similar, while ST18 and ST18+V760 were 63% similar; by comparison ST7 and ST+V760 were 48% similar, resulting in clustering of these groups (Figure 10.12). TRF tracking data for ST18 and ST7 were in the same general range, though TRF abundances were approximately halved when they are added with strain V760 (Table 10.8 and Table 10.9) which is possibly indicative of competition between strains for uptake into rotifers.

In Experiments 5 and 6 the addition of V760, ST7 and ST18 to *Artemia* had a different response to what was observed for rotifers. In general the strain differences were not largely different to the controls with SIMPER similarities mostly ≥ 50%. This may be due to a higher level of bacteria in the *Artemia* samples at the beginning of the experiments. TRFLP

patterns of the treatment in which V760 was added at 5 x 10^4 was not different from the control (R=0.07, p=50%, Figure 10.11; 73.6% similar). However, when added at higher populations a greater difference was observed (R=0.26, p<0.1). In the case of sole additions of strains ST18, ST7 or V706, differences to the control were not significant (Table 10.10, Table 10.11) with similarity between 50-60%. The TRFLP profiles were significantly different to the profile of the treatment in which ST18 was added with V706 suggesting together the strains more significantly influence the overall community. Large variances were observed between replicates, especially for the additions of ST7 in combination with V706, suggesting ST7 populations may have fluctuated widely between treatments and replicates making separation of differences difficult. ST18 and ST7 unique TRFs were detected using SIMPER (Table 10.10, Table 10.11), but only occurred in small numbers, which could be due to the higher abundances of other bacterial species present.

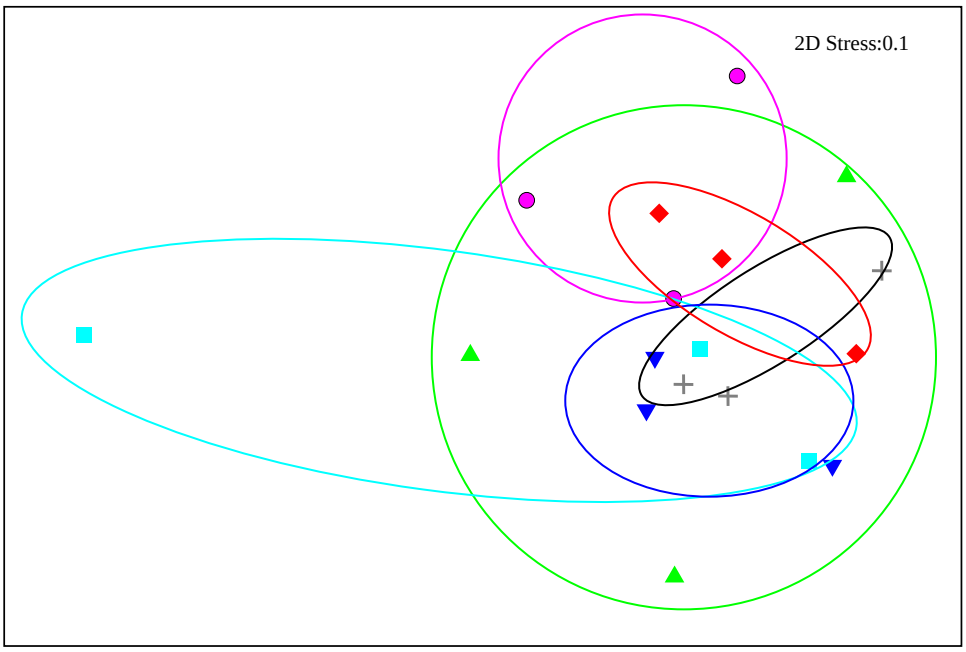

Figure 10.9 MDS plot of TRFLP profiles of bacterial communities associated with rotifers challenged with different bacterial strains added at 5 × 10^5 cells ml^{-1} and incubated over a 14 h period (Experiment 1): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ V. *alginolyticus* V-34; (4) ◆ strain V52; (5) ● strain ST18; and (6) + strain V8.

Table 10.6 Summarised SIMPER output utilised for tracking of probiont strain ST18 through detection of distinguishing TRFs for ST18 within the rotifer challenge treatment experiment (as shown above in Figure 10.9).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7	**ST18:**				
			177 *HhaI*(r)	**60 *HhaI*(f)**	**64 *HaeIII*(f)**	**104 *HinfI*(f)**	**366 HhaI(r)**	**508 HaeIII(r)**
1	Control	26.66	-	-	-	-	-	-
2	V760	53.46	-	-	-	-	-	-
3	V34	24.57	-	-	-	-	-	-
4	V52	42.95	-	-	-	-	-	-
5	ST18	42.04	-	8.53	5.17	4.50	-	5.23
6	V8	44.20	-	-	-	-	-	-

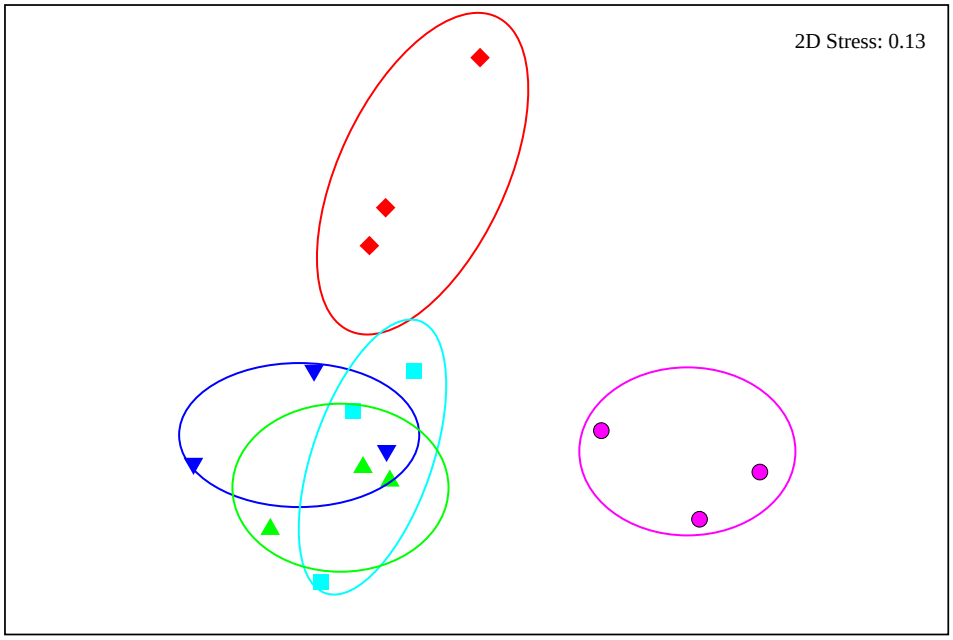

Figure 10.10 MDS plot of TRFLP profiles of bacterial communities associated with rotifers challenged with different bacterial strains added at 5×10^5 cells ml^{-1} and incubated over a 14 h period (Experiment 2): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ strain ST14; (4) ◆ strain ST18; (5) ● strain ST7.

Table 10.7 Summarised SIMPER output utilised for tracking of probiont strain ST18 through detection of distinguishing TRFs for strains ST7 and ST18 within the rotifer challenge treatment experiment (as shown above in Figure 10.10).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7	**ST18:**				
			177 *HhaI*(r)	**60 *HhaI*(f)**	**64 *HaeIII*(f)**	**104 *HinfI*(f)**	**366 HhaI(r)**	**508 HaeIII(r)**
1	Control	41.98	-	-	-	-	-	-
2	V760	45.15	-	-	-	-	-	-
3	ST14	46.77	-	-	-	-	-	-
4	ST18	55.00	-	12.76	-	-	15.87	15.87
5	ST7	57.29	20.07	-	-	-	-	-

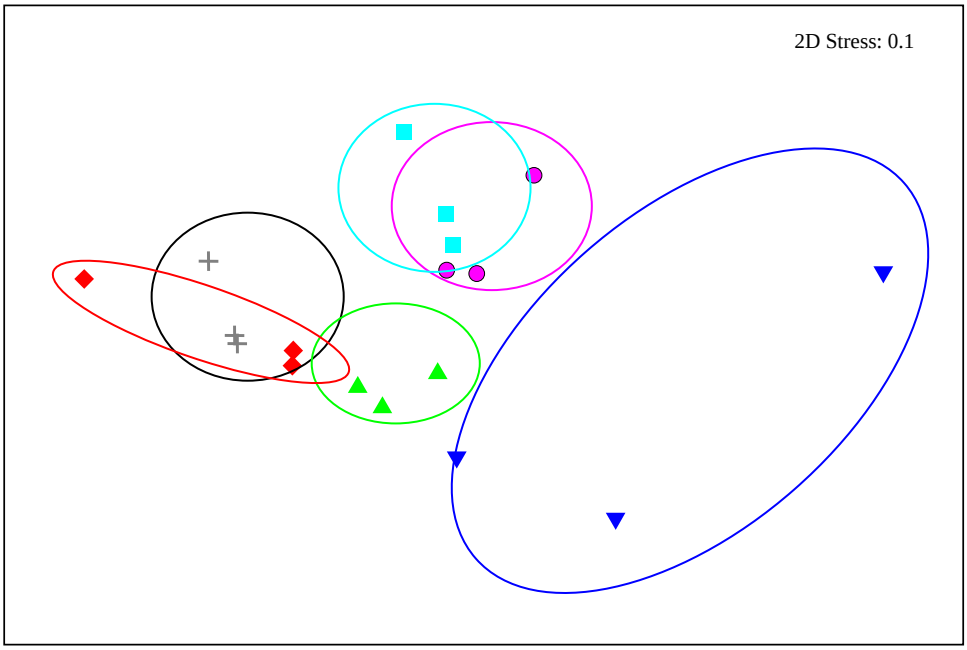

Figure 10.11 MDS plot of TRFLP profiles of bacterial communities associated with rotifers challenged with different bacterial strains added at 5×10^5 cells ml^{-1} and incubated over a 14 h period Experiment 3): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ strain ST18; (4) ◆ strain ST7; (5) ● strains ST18 + V760; (6) + ST7 + V760.

Table 10.8 Summarised SIMPER output data utilised for tracking of probiont strains through detection of distinguishing TRFs for strains ST7 and ST18 within the rotifer challenge treatment experiment (as shown above in Figure 10.11).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7:	**ST18:**				
			177 *HhaI*(r)	**60 *HhaI*(f)**	**64 *HaeIII*(f)**	**104 *HinfI*(f)**	**366 HhaI(r)**	**508 HaeIII(r)**
1	Control	46.05	-	-	-	-	-	-
2	V760	39.52	-	-	-	-	-	-
3	ST18	50.52	-	23.23	14.13	-	10.90	22.02
4	ST7	56.40	16.77	-	-	-	-	-
5	ST18 + V760	42.19	-	11.90	11.48	-	7.67	12.96
6	ST7 + V760	51.56	9.93	-	-	-	-	-

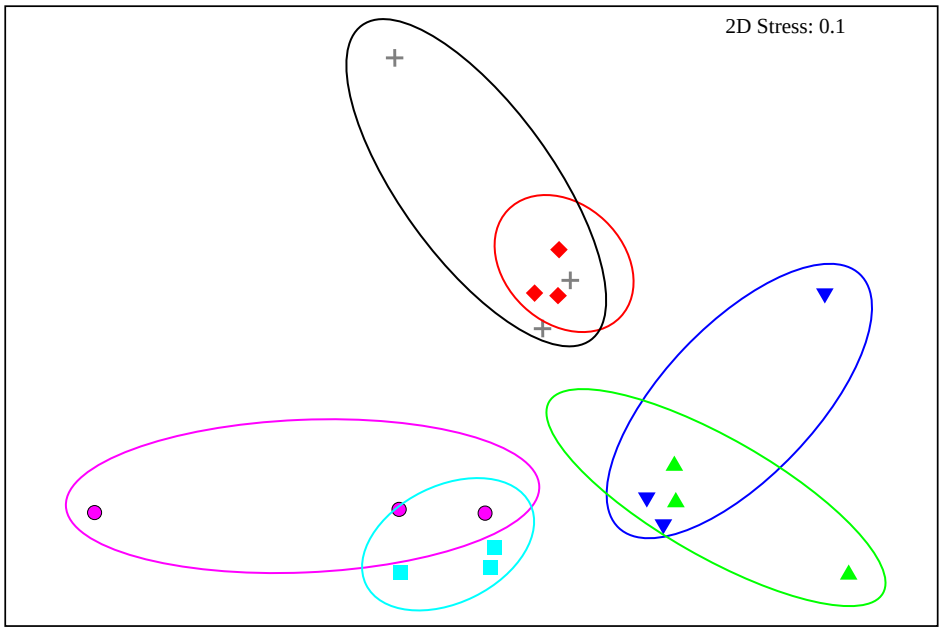

Figure 10.12 MDS plot of TRFLP profiles of bacterial communities associated with rotifers challenged with different bacterial strains added at 5×10^4 cells ml^{-1} and incubated over a 14 h period (Experiment 4): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ strain ST18; (4) ◆ strain ST7; (5) ● strains ST18 + V760; (6) + ST7 + V760.

Table 10.9 Summarised SIMPER output data utilised for tracking of probiont strains through detection of distinguishing TRFs for strains ST7 and ST18 within the rotifer challenge treatment experiment (as shown above in Figure 10.12).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7:	**ST18:**				
			177 *HhaI*(r)	**60 *HhaI*(f)**	**64 *HaeIII*(f)**	**104 *HinfI*(f)**	**366 HhaI(r)**	**508 HaeIII(r)**
1	Control	60.04	-	-	-	-	-	-
2	V760	44.23	-	-	-	-	-	-
3	ST18	56.54	-	13.17	-	19.94	-	5.29
4	ST7	67.04	15.97	-	-	-	-	-
5	ST18 + V760	43.15	-	13.98	-	19.50	-	13.77
6	ST7 + V760	55.57	13.23	-	-	-	-	-

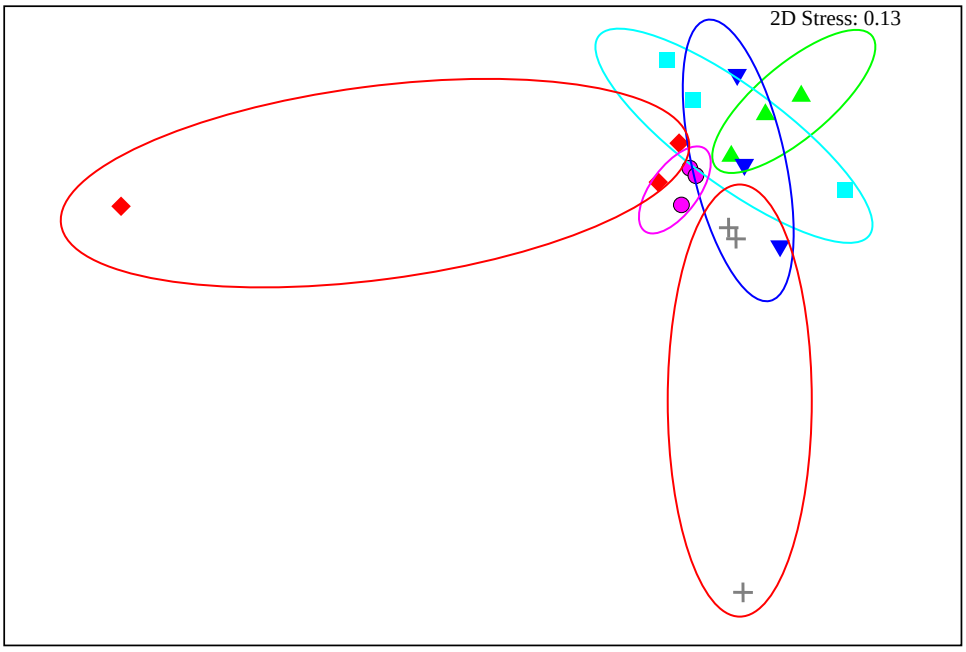

Figure 10.13 MDS plot of TRFLP profiles of bacterial communities associated with *Artemia* challenged with different bacterial strains added at 5×10^5 cells ml^{-1} and incubated over a 14 h period (Experiment 5): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ strain ST18; (4) ◆ strain ST7; (5) ● strains ST18 + V760; (6) + ST7 + V760.

Table 10.10 Summarised SIMPER output data utilised for tracking of probiont strains through detection of distinguishing TRFs for strains ST7 and ST18 within the *Artemia* challenge treatment experiment (as shown above in Figure 10.13).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7:	**ST18:**				
			177 *HhaI*(r)	**60 *HhaI*(f)**	**64 *HaeIII*(f)**	**104 *HinfI*(f)**	**366 HhaI(r)**	**508 HaeIII(r)**
1	Control	79.01	-	-	-	-	-	-
2	V760	69.30	-	-	-	-	-	-
3	ST18	66.26	-	-	-	-	2.53	-
4	ST7	40.02	-	-	-	-	-	-
5	ST18 + V760	82.51	-	-	-	-	4.14	1.06
6	ST7 + V760	68.61	-	-	-	-	-	-

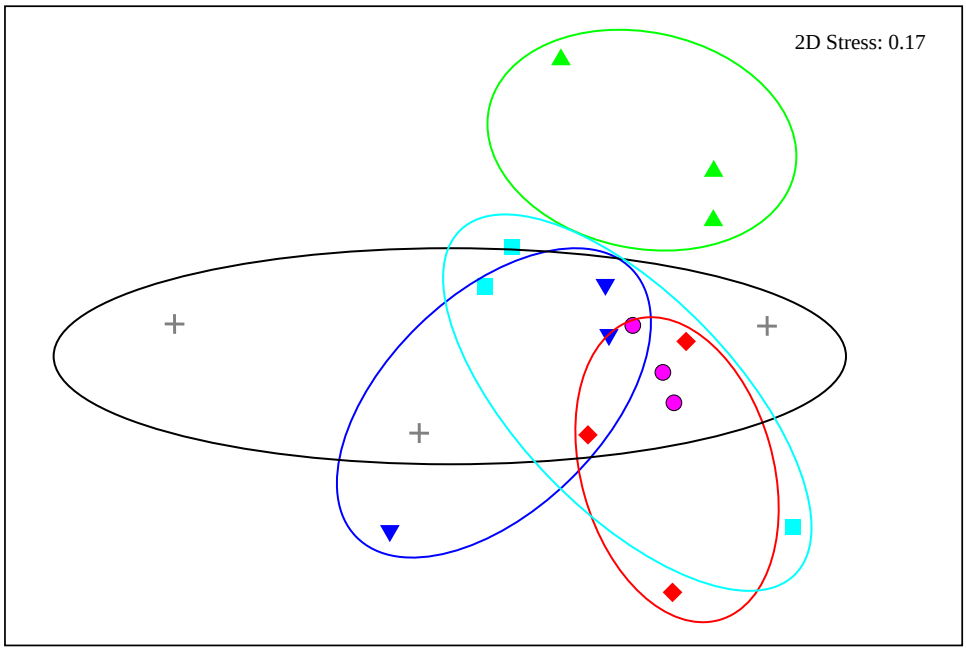

Figure 10.14 MDS plot of TRFLP profiles of bacterial communities associated with *Artemia* challenged with different bacterial strains added at 5×10^4 cells ml^{-1} and incubated over a 14 h period (Experiment 6): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ strain ST18; (4) ◆ strain ST7; (5) ● strains ST18 + V760; (6) + ST7 + V760.

Table 10.11 Summarised SIMPER output data utilised for tracking of probiont strains through detection of distinguishing TRFs for strains ST7 and ST18 within the *Artemia* challenge treatment experiment (as shown above in Figure 10.13).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7:	**ST18:**				
			177 *HhaI*(r)	**60 *HhaI*(f)**	**64 *HaeIII*(f)**	**104 *HinfI*(f)**	**366 HhaI(r)**	**508 HaeIII(r)**
1	Control	64.57	-	-	-	-	-	-
2	V760	59.23	-	-	-	-	-	-
3	ST18	55.81	-	-	-	-	-	-
4	ST7	61.39	-	-	-	-	-	-
5	ST18 + V760	85.34	-	-	-	-	-	2.49
6	ST7 + V760	43.86	-	-	-	-	-	-

10.5 Discussion

Vibrio proteolyticus has been previously recorded as a pathogen of both rotifers and *Artemia* (Puente et al., 1992; Vandenberghe et al., 1999; De Schrijver and Ollevier, 2000; Verschuere et al., 2000). For example, Verschuere et al. (2000) found that a strain of *V. proteolyticus* (CW8T2) at 3×10^6 cells ml^{-1} killed 80 % of *Artemia* within two days. Isolates ST18 and ST7 demonstrated probiotic characteristics when added individually or mixed together. These two isolates were identified as *Aliivibrio fischeri* and *Pseudoalteromonas agarivorans*.

Verschuere et al. (2000) suggested that a probiont may remove toxic metabolic substances that could otherwise adversely affect the growth and survival of the *Artemia.* Furthermore, they suggested that bacteria that are well adapted to the conditions prevailing in intensive *Artemia* culture (as used here) may also be able to prevent the proliferation of opportunistic bacteria. Given the rapidity with which *V. proteolyticus* V-760 was able to increase in numbers and to affect both *Artemia* and rotifers, it appears to be an effective opportunistic colonising bacterium.

TRFLP analysis of the homogenised rotifers and *Artemia* demonstrated that the background bacteria detected in the controls had no obvious effect on the survival of the rotifers or *Artemia*, as the mortality was only high in the pathogen controls where no putative probiont was added. Even though efforts were made to reduce the background bacteria as much as possible, it was observed from the TRFLP data, using SIMPER analysis, that the use of ozone reduced the bacterial microbiota diversity (data not shown). It is likely that *Vibrio* spp. are part of the rotifer- and *Artemia*-associated microbiota and are able to survive this decontamination step (Tolomei et al., 2004). From the TRFLP analysis, clear shifts in community structure away from the controls could be observed when strain ST18 was added. When ST18 was challenged with *V. proteolyticus* V706, it out-competed the pathogen, resulting in a community shift to be more similar in structure to the sole ST18 treatment than the sole *V. proteolyticus* V706 treatment. Based on the TRFLP profiles, strain ST18 also generated the largest changes in the initial starting profiles. This suggests that it both persisted and competed well for the 14 h treatment period and that other *Pseudoalteromonas*

strains were not a major community component in the control rotifer samples. In *Artemia* samples, fewer differences were observed and a lower abundance of specific TRFs, suggesting that existing *Pseudoalteromonas* strains or alteromonads were present. This makes ST18 specifically more difficult to differentiate from the background community TRFs.

The ability of ST18 to protect rotifers and *Artemia* against a challenge from pathogen *V. proteolyticus* V760 could result from its ability to: 1) inhibit colonisation and growth of strain V760; 2) inhibit pathogenicity of V760 through destruction of secreted toxins or by preventing their expression; 3) improve the immune response of the *Artemia*; 4) or, a combination of mechanisms (Kesarcodi-Watson et al., 2008). As the numbers of *V. proteolyticus* V760 did not appear to vary much between treatments with or without the putative probiont, it is unlikely that the probiont inhibited colonisation by *V. proteolyticus* V-760 *per se*. This implies that the action of the probiont was directed towards inhibiting the virulence factor expression in *V. proteolyticus* V-760.

Lately, quorum-sensing has been demonstrated as a mechanism for controlling virulence factor expression in some *Vibrio* spp. (Defoirdt et al., 2004) and (Bassler, 2002). Interference in quorum-sensing (i.e., due to the activity of enzymes that cleave the signal compounds, such as acylated homoserine lactones or furanone-derivatives) may reduce virulence expression e.g. of *V. harveyi* in *Penaeus monodon* (Manefield et al., 2000). Whether or not interruption of quorum-sensing in *V. proteolyticus* by ST18 can explain the improved survival of rotifers and *Artemia* that was observed remains to be determined. The data from the current study supports the notion that selected naturally occurring bacteria can be encouraged to dominate the normal microbiota of rotifers and *Artemia* and can protect them from subsequent pathogen challenge. Combined with decontamination procedures (Tolomei et al., 2004) and sound husbandry, this suggests a useful way of reducing striped trumpeter larval mortality when rotifers and *Artemia* are used as live feed. The rotifers and *Artemia* can be used as a vector to introduce desirable strains into the host microbiota instead of allowing entry of pathogens (Lopez-Torres and Lizarraga-Partida, 2001). The strength of this colonisation approach is that the bacteria need only be added once or infrequently. In contrast, other approaches such as using *Bacillus* spp. require the organism to be added continually, as they often do not colonise the larvae effectively (Vine et al., 2006).

10.6 Conclusion

Of the 22 isolates obtained from striped trumpeter culture systems, five isolates ST7, ST14, ST18, V8 and V52 showed some antimicrobial activity against known marine animal pathogens. *Pseudoalteromonas* sp. ST18 and *Aliivibrio fischeri* ST7, when inoculated at 5 x 10^4 cells ml^{-1} and 5 x 10^5 cells ml^{-1}, respectively, were found to cause the least mortality in rotifers and *Artemia.* In addition, they protected *Artemia* against a challenge by a virulent strain of *V. proteolyticus*, which could cause >60% mortality of rotifers within 14 h and approximately 50% mortality of *Artemia* within 24 h. Survival of rotifers and *Artemia* when exposed to the strains ST7 and ST18 were not significantly different from the control. Furthermore, survival of rotifers and *Artemia* in mixtures of ST18 and *V. proteolyticus* V760 and ST7 *V. proteolyticus* V760 was significantly greater than in *Artemia* exposed only to *V. proteolyticus* V760 alone.-Improved survival of *Artemia* required the presence of isolate ST18 or ST7 or a mixture of both. Through TRFLP analysis, it was possible to detect the probionts in the rotifers and to a lesser extent *Artemia* by observation of distinctive TRFs.

The practical use of probionts ST7 and ST18 is further explored in Chapter 11.

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11 ADDITION AND TRACKING OF PROBIONTS TO YOLK SAC AND FIRST-FEEDING STRIPED TRUMPETER LARVAE

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11.1 Abstract

The protective capacity and most effective delivery mode of putative probionts *Pseudoalteromonas agarivorans* ST18 and *Aliivibrio fischeri* ST7 was investigated in the rearing of yolk sac and first-feeding striped trumpeter (*Latris lineata*) larvae. In these experiments 4500 larvae were randomly stocked into 24, 300 l black hemispherical fiberglass tanks at 1 dph and held under static conditions for 5 days after which 300% daily water changes was applied. Terminal restriction fragment length polymorphism (TRFLP) was used to monitor the changes in bacterial community. The addition of strain ST18 to yolk sac larvae showed no significant reduction in survival (70 ± 6%) versus a control group (83 ± 5%) reared without potential probionts being added. The addition of strain ST7 with and without strain ST18 was found to be more disadvantageous (58 ± 7% and 55 ± 8% survival respectively). By tracking distinct 16S rRNA-derived TRFs, strain ST18 was specifically detected in treatments where it was added by both bioencapulsation and by direct addition. When strain ST18 was added directly to the water it resulted in decreased survival, due to the high bacterial load and possibly potential oxygen demand. The introduction of ST18 to the larvae bioencapsulated in rotifers resulted in the introduction of comparatively smaller numbers of bacteria that did not compromise the growth of the developing larvae.

11.2 Introduction

There has been a realisation that throughout development, fish intestinal microbiota is strongly dependent upon the characteristics of the aquatic environment and this has consequences for fish health and productivity. There is therefore a need to investigate the influences and modifications both induced by changes in the local environment during development, as well as those influenced through the introduction of probiotics. It is necessary to better understand how probiotics, either directly released into the surrounding water or administered within the diet, alter larval fish microbiota, thus allowing us to determine the mode of action that most effectively introduces the probiont to fish larvae (Nikoskelainen et al., 2003; Vine et al., 2006). Studies involving the use of beneficial bacteria in aquatic production systems have focused on increased performance, measured in terms of improvements in survival and growth. This can come about by boosted disease resistance in the animals, as well as increased stress tolerance. Such improvements have

been observed in some cases after just a single species probiotic treatment; for example, the whiteleg shrimp (*Litopenaeus vannamei*), Indian prawn (*Fenneropenaeus indicus*) (Ziaei-Nejad et al., 2006; Wang, 2007), Indian carp (*Labeo rohita*), and red drum (*Sciaenops ocellatus)* (Ghosh et al., 2003).

Microbial probionts have been defined previously in Chapter 10. Probiotics may inhibit the colonisation of the harmful bacteria through competitive exclusion, for example by out-competing for attachment sites and nutrients. They may also use other mechanisms such as secretion of antimicrobial compounds that may inhibit other bacterial growth or through interference of quorum sensing by enzymatic cleavage of autoinducer molecules (Olsen, et al., 2000). Probiotic research has centered on two major strategies: bioremediation (modification of an established flora to one that is supportive of the host) and biocontrol (use of a specific microorganism to minimise the impact of a specific pathogen) (Verschuere, et al., 2000). Methods for selecting potential probionts have been generally based on *in vitro* tests, such as the test used in Chapter 10, or predominance in gut microbiota in fish groups that come from high survival and better performance (Makridis, et al., 2005; Planas, et al., 2006; Vine, et al., 2006). Challenge tests can be applied to determine the ability of probiotic bacteria to prevent disease, as outlined in Chapter 10. They can also be used to determine if the bacterial isolate is harmful in any way to live feeds. The selection of potential probionts was conducted in Chapter 10 and the best candidates were isolates *Aliivibrio fischeri* ST7 and *Pseudoalteromonas* sp. ST18. TRFLP also revealed that it is possible to track these isolates in culture and during enrichment of both rotifers and *Artemia*, using the isolate's distinctive fingerprint TRFs (Chapter 10). The next step in assessing the potential efficacy of the identified probionts was to test them in larval culture through the ability to bioencapsulate the potential probiont within rotifers and *Artemia*.

Within this Chapter two major questions are explored:

1) What is the effect of adding potential probionts to striped trumpeter yolk sac larvae, either singularly or in combination?

To answer this question, strains ST7 and ST18 were introduced to yolk sac larvae held under static clear water conditions, with the aim of modifying the microbial community of both the yolk sac larvae and the surrounding water. Assessment of larval performance when reared with different combinations of both probionts was undertaken.

2) What is the effect of adding combinations of potential probionts using different strategies, either through rotifer bioencapsulation, direct addition to the culture water or both? These additions would occur when the larvae are in a phase of active rotifer feeding, from 6 to 15 days post-hatch (dph). To answer this question, the performance of the feeding larvae was assessed under a series of different treatments, using TRFLP analysis to track the uptake. In this case, the changes in the microbiota of larvae and the surrounding water in association with the treatment were investigated.

11.3 Material and Methods

11.3.1 *Culture of yolk-sac larvae*

Eggs were collected from a female striped trumpeter broodstock by strip-spawning, and fertilised with the milt of four males. Fertilised eggs were incubated and hatched as previously described (Bransden et al., 2004). Larvae (4500) were randomly stocked into 24,

300 l black hemispherical fiberglass tanks at 1 dph. Larvae were held under static conditions in seawater from 1 to 5 dph. A photoperiod of 18 h light: 6 h dark was used throughout the experiment, produced by a computerized halogen light source (~11 µmol s^{-1} m^{-2} at the water surface) with a gradual fade in and fade out. Dead larvae were siphoned twice daily and deducted from the original numbers stocked to estimate survival in each tank.

Experiment 1: Effect of potential probionts on yolk-sac larvae

The aim of this experiment was to determine the effect that the potential probionts ST18 and ST7 have on non-feeding yolk sac larvae. The null hypothesis was that were would be no significant difference in survival of the yolk sac larvae over the six days in which they rely on endogenous food reserves. A further aim was to determine if the uptake and changes in the bacterial communities of the larvae could be explored using the capabilities of TRFLP analysis to track the isolates.

Four treatments were assessed, each with six replicate tanks. The control treatment consisted of rearing larvae in seawater with no added bacteria. The second treatment involved addition of live bacterial cultures of both strains ST7 and ST18 at a final concentration of 5 x 10^{-5} cells ml^{-1}. The third and fourth treatments involved individual addition of ST7 or ST18 at a final concentration of 5 x 10^{-5} cells ml^{-1} each.

Initial samples of 50 yolk sac larvae were siphoned from each tank at stocking to determine the size and condition of the larvae. TRFLP analysis was also undertaken on these samples to gain a baseline assessment of the bacterial community. At 1, 3 and 6 dph further larval and water samples were taken for TFRLP. Larvae (n=50) were removed from each of the experimental tanks using a siphon and anesthetized in 0.06% 2-phenoxyethanol as described in Battaglene et al. (2006). Of these larvae twenty were examined using an Olympus SZ microscope to determine morphormetric indices, including standard length, swim bladder inflation, grey gut and condition. Samples of a further 20 larvae were analysed by TRFLP analyses. At the same time 50 ml water samples for TRFLP analysis were removed with a beaker (assigned to each tank) and placed into 50 ml Falcon tubes and frozen at -20°C.

11.3.2 *Culture of feeding larvae*

Egg collection, incubation and yolk sac rearing is as described in Section 4.2. At 6 dph internal 390 µm mesh screens were placed into the centre of each tank to allow seawater outflow and removal of rotifers, algae and incoming seawater overnight. A 'greenwater' environment was then provided with live *Nannochloropsis oculata* at a turbidity level of 3 Nephelometric Turbidity Units (NTU) (HACH 2100 portable turbidity meter). Each morning the various feed treatments were added to each tank at a rate of 8.8 l min^{-1} (total 30 l) from the 80 l reservoirs accompanying each individual tank. The algal suspensions or seawater had been acclimated to tank temperature in the reservoirs overnight and provided with aeration. Tanks remained static during the light phase with gentle aeration (200 ml min^{-1}) (Shaw, 2006). All tanks were supplied with live rotifers (*B. plicatilis*) enriched on Algamac 2000 (Aquafauna Biomarine, USA) from 6 dph, as described by Battaglene et al. (2006). Enriched rotifers were supplied at 10 ml^{-1} each morning following probiont enrichment as described below in section 4.2.3. Surface skimmers were used from 8-15 dph to remove surface oil and promote swim bladder inflation (Trotter et al., 2003). Water quality was measured daily. Temperatures ranged from 15.5 – 16.5°C, salinity 34.1-34.7 ‰, pH 7.9-8.2 and dissolved oxygen was greater than 90% saturation. Larvae were spot siphoned to

determine mortality levels and counted daily up to the conclusion of the experiment (15 dph) when all live larvae were removed and counted to determine final survival. The final survival tally was determined by a series of volumetric counts on each tank. Prior to counts heavy aeration was applied to the tanks so that the larvae were distributed evenly.

Experiment 2: Effect of potential probionts added directly to the culture water, to the enriched rotifers or a mixture of both

The aim of this experiment was to determine the effect that the potential probionts ST18 have on first-feeding larvae. The null hypothesis was that there would be no significant difference in survival of the larvae over the seven days compared to the control reared larvae

Four treatments were assessed each with 6 replicate tanks:
Treatment 1: control rotifers enriched with Algamac 2000 and fresh algae (*Nannochloropsis oculata*) greenwater culture at 3 NTU.
Treatment 2: ST18 enriched rotifers plus ST18 added to the culture water to achieve a concentration of 5×10^{-5} cells ml^{-1}.
Treatment 3: ST18 enriched rotifers but not added to the culture water. Treatment 4: ST18 added to the culture water to achieve a concentration of 5×10^{-5} cells ml^{-1}.

An initial sample of 20 larvae and a 50 ml water sample were taken from the incubator before stocking to assess the existing bacterial community using TRFLP. At 5 dph before first-feeding occurred, water samples from each tank were taken, followed by samples at days 5, 10 and 13 as well as larval samples (n=50) at days 5, 6, 10 and 13 dph.

Fifty larvae were siphoned from each of the treatments at each sampling. All sampling took place in the morning prior to feeding.

11.3.3 *Rotifer enrichment*

Rotifer enrichment was as described in Chapter 10 but modified by the rotifers being transferred to 200 l vessels for 12 h enrichment, at 400 rotifers ml^{-1} and 23°C. Rotifers were enriched with AlgaMac at 0.3 g per million rotifers according to the manufacturer's instructions. Aeration and oxygen were provided to maintain dissolved oxygen above 4 mg l^{-1} in the enrichment vessels. Probionts were added to the corresponding rotifer enrichment vessel at the same time as the AlgaMac was added, to achieve a concentration of 5×10^{-5} cells ml^{-1}. At the end of the enrichment period rotifers were rinsed and cleaned with ozonated seawater and counted using a Coulter counter.

11.3.4 *Bacterial preparation*

Bacterial strains were grown as in Chapter 10.

The bacterial inoculums were grown in 2000 ml broth and shaken daily, the final volume was standardised for each tank so that they all received the same amount of broth being added, the short fall if any was made up using sterilised marine broth.

11.3.5 *DNA extraction*

Extraction of DNA was performed as in Chapter 9.

11.3.6 *16S rRNA gene PCR.*

PCR conditions were performed as in Chapter 10.

11.3.7 *TRFLP sample preparation*

Preparation for TRFLP was performed as in Chapter 9.

11.3.8 *TRFLP data analysis*

TRFLP analysis was done as in Chapter 9.

11.3.9 *Survival Statistical analyses.*

Survival statistical analyses were performed as in Chapter 10.

11.4 Results

11.4.1 *Determination of the probiotic capabilities of possible candidates on yolk sac larvae*

The inclusion of strain ST7 to the culture water resulted in significantly reduced survival of 55 ± 8% compared to 83± 5% survival in the controls (Figure 11.1). The addition of ST18 resulted in 70 ± 6% survival. A combination of ST18+ST7 resulted in 58 ± 7% survival. Only the addition of ST7 was significantly different to the control (Figure 11.1).

The dissolved oxygen (DO) levels within the tank declined in the treatments in which the bacterial probionts were added (Table 11.1). Treatments 2 and 4 had the lowest DO levels of 73.4 and 68.9% of saturation (Table 11.1) which coincided with lowest survival levels (58 ± 7% and 55 ± 8%). A bacterial bloom was also visible to the eye in the form of a floating mat. The water had slightly increased acidity with pH dropping from 8.1 to 7.9 (Table 11.1).

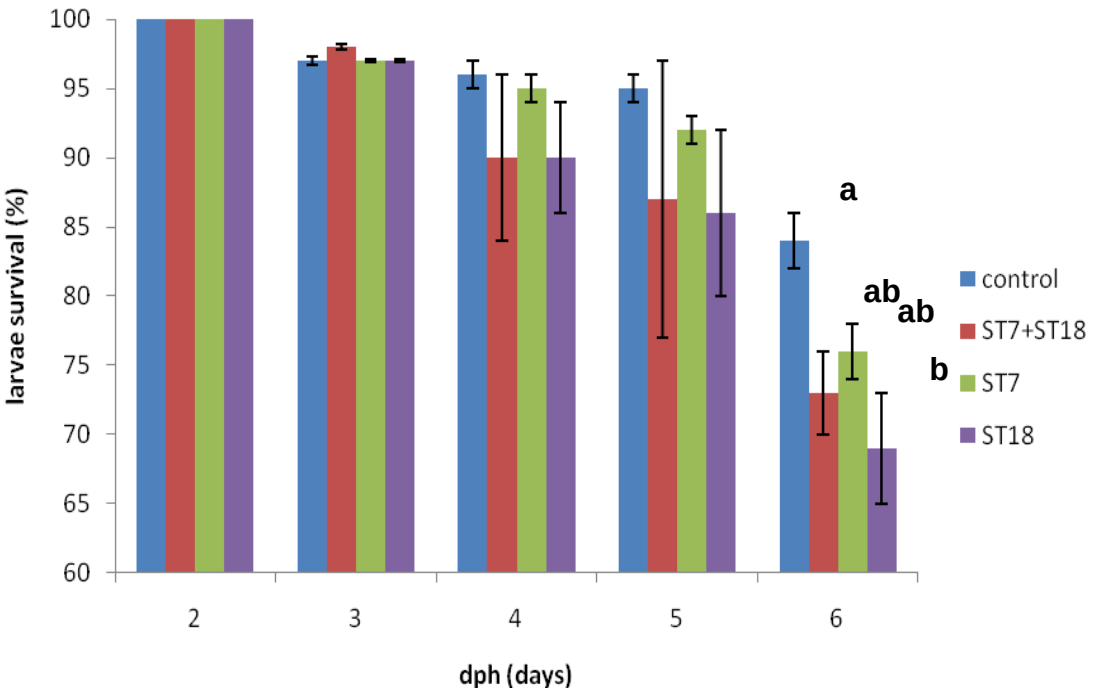

Figure 11.1 Yolk sac larvae survival over the time for Experiment 1. Values for the 6 dph samples not sharing a common letter are significantly different (p<0.05).

Table 11.1 Yolk sac larvae Experiment 1 (Chapter 9) showing larval size and survival and water physicochemical parameters.

Treatment		Control	ST7+ST18	ST18	ST7
Age		1DPH			
Standard length	mm	5.1±0.03			
Survival	%	100.0			
pH		8.1±0.0			
DO	%	90±1			
Temperature	°C	15.4±.0.1			
Age		2DPH			
Standard length	mm	-	-	-	-
Survival	%	100*	100*	100*	100*
pH		8.14±0.1a	8.1±0.0b	8.2±0ab	8.1±0.01b
DO	%	81.65±1.2a	74.6±0.6b	73.1±0.7b	76.7±1.61b
Temperature	°C	15.3±.0.1*	15.38±.0.16*	15.4±0.02*	15.4±0.02*
Age		3DPH			
Standard length	mm	-	-	-	-
Survival	%	97 ± .0.3*	98 ± 0.2*	97 ±0.1*	97 ± 0.1*
pH		8.1±.0.1a	8.0±0.0ab	8.0±0.0b	7.9±0.0b
DO	%	86.1±0.8a	77.0±1.1ab	75.2±0.7b	72.4±4.0b
Temperature	°C	16.1±0.1*	16.1±0.1*	16.1±0.1*	16.0±0.0*
Age		4DPH			
Standard length	mm	-	-	-	-
Survival	%	96 ± 1 *	90 ± 6*	95 ± 1*	90 ± 4*
pH		8.1±0.0a	7.9±0.0b	7.9±0.0b	7.9±0.0b
DO	%	86.2±0.9a	73.9±2.1b	71.9±0.9b	69.2±4.1b
Temperature	°C	16.2±0.0*	16.2±0.0*	16.2±0.0*	16.2±0*
Age		5DPH			
Standard length	mm	-	-	-	-
Survival	%	95 ± 1*	81 ± 10*	92 ± 1*	86 ± 6*
pH		8.1±0.0a	7.9±0.0b	7.9±0.0b	7.9±0.1b
DO	%	85.5±1.31a	73.1±1.9b	74.8±1.15b	67.9±5.1b
Temperature	°C	16.2±0*	16.2±0*	16.2±0*	16.2±0*
Age		6DPH			
Standard length	mm	5.3±0.1*	5.3±0.1*	5.3±0.1*	5.3±0.1*
Survival	%	83 ± 5a	58 ± 7ab	70 ± 6ab	55 ± 8b
pH		8.3±0.1a	7.9±0.1b	7.9±0.1b	7.9±0.3b
DO	%	83.6±2.2a	73.4±3.1ab	75.8±1.5ab	68.8±4.5b
Temperature	°C	16.2±0*	16.2±1*	16.2±2*	16.2±3*

a,b,cValues within rows sharing a common letter indicates statistically significantly differences (P>0.05) determined using one-way ANOVA.

11.4.2 *TRFLP analysis of yolk-sac larvae (Experiment 1)*

The MDS plot derived from the TRFLP data obtained from yolk sac larvae at 1, 3 and 6 dph was inconclusive when all replicates were presented due to the overwhelming similarity between groups (data not shown). The only significant change found was between the treatments and the initial larval sample taken from the incubator before stocking (TRFLP profiles, treatment 1, R=0.45; treatment 2, R=0.22; treatment 3, R=0.30; and treatment 4, R=0.52). There were no significant differences in treatment samples between sampling days. Similar patterns were observed for the tank water TRFLP profiles. For the water samples there were no significant differences in profile patterns with time and only very slight changes were apparent in comparison with the initial sample (R values <0.10).

After addition of the ST7, ST18 and ST7 and ST18 in combination at 1 dph it was possible to track both strains the culture water and the larvae through the presence of their distinctive TRFs using SIMPER analysis. In both water and larvae samples ST18 and ST7-derived TRFs were not detected in the control samples (Table 11.2).

When ST7 and ST18 were added in combination within the larval samples ST18 was only detected at 6 dph, however ST7 was detected at all sample times and made a greater contribution to average similarity, which was albeit rather low. In water samples, however ST18 appeared to be much more abundant while ST7 was not detected. When added singularly, ST7 was detectable in larvae at 4 and 6 dph but again was not detected in the water samples. ST18, when added singularly on the other hand was readily detected in both larval and water samples, though in the case of larvae, the abundance appeared to decline considerably by dph 6.

Based on the TRFLP data ST18 thus appears to become a major component of the microbial community in the tank water, however direct uptake of the probionts appeared to be limited. It was observed that the inoculation of bacteria at the yolk sac larvae stage provided no boosting of larval survival and direct addition generally resulted in slightly reduced survival, however, this apparent reduced survival appeared to be attributable to dissolved oxygen availability.

Table 11.2 Summarised SIMPER output data in which TRFLP analysis was used to track probionts ST7 and ST18 in treatments in which the strains were added directly to tanks holding 1 dph yolk sac larvae. Samples analysed included larval and water samples.

Treatment	dph	Sample	Similarity%	ST18 TRFs			ST7
				104 *HinfI* (f)	366 *HhaI* (r)	508 *HaeIII* (r)	268 *HinfI* (f)
				Contribution to average similarity (%):			
Control	2	larvae	10.61	-	-	-	-
Control	4	larvae	24.49	-	-	-	-
Control	6	larvae	12.60	-	-	-	-
Control	2	Water	37.27	-	-	-	-
Control	4	Water	55.91	-	-	-	-
Control	6	Water	45.72	-	-	-	-
ST7+ST18	2	larvae	7.62	-	-	-	10.65
ST7+ST18	4	larvae	5.22	-	-	-	15.45
ST7+ST18	6	larvae	9.71	-	2.43	-	11.24
ST7+ST18	2	Water	48.86	-	10.36	15.95	-
ST7+ST18	4	Water	44.66	-	11.28	10.78	-
ST7+ST18	6	Water	48.78	-	-	14.07	-
ST7	2	larvae	6.36	-	-	-	-
ST7	4	larvae	23.57	-	-	-	5.64
ST7	6	larvae	3.14	-	-	-	3.95
ST7	2	Water	53.73	-	-	-	-
ST7	4	Water	46.69	-	-	-	-
ST7	6	Water	50.54	-	-	-	-
ST18	2	larvae	12.65	26.03	10.29	-	-
ST18	4	larvae	22.41	14.39	7.29	-	-
ST18	6	larvae	13.88	-	5.23	-	-
ST18	2	Water	34.72	8.12	-	19.24	-
ST18	4	Water	56.36	14.63	-	18.60	-
ST18	6	Water	62.60	20.61	-	24.93	-

11.4.3 *Determination of the probiotic capabilities of possible candidates on first feeding larvae (Experiment 2)*

The larval response to the introduction of ST18 enriched rotifers, ST18 to the water, or both in combination held under greenwater conditions was found to accelerate between 10 to 13 dph (Figure 11.2). At 13 dph, survival in treatments in which ST18 was provided bioencapsulated in rotifers were not significantly different to the controls (F=6.099, df=20,3 P=0.06) with the control having the best survival of 87.5 ± 0.4% while the ST18/rotifer addition was similar at 84.4 ± 1.9%. The combined addition of ST18 in the form of rotifer bioencapsulation and direct addition to the tank water was the worst performing treatment with only 70.9 ± 3.3% larvae survival occurring at 13 dph (Figure 11.2, Table 11.3) while direct water addition alone provided more intermediate survival outcomes.

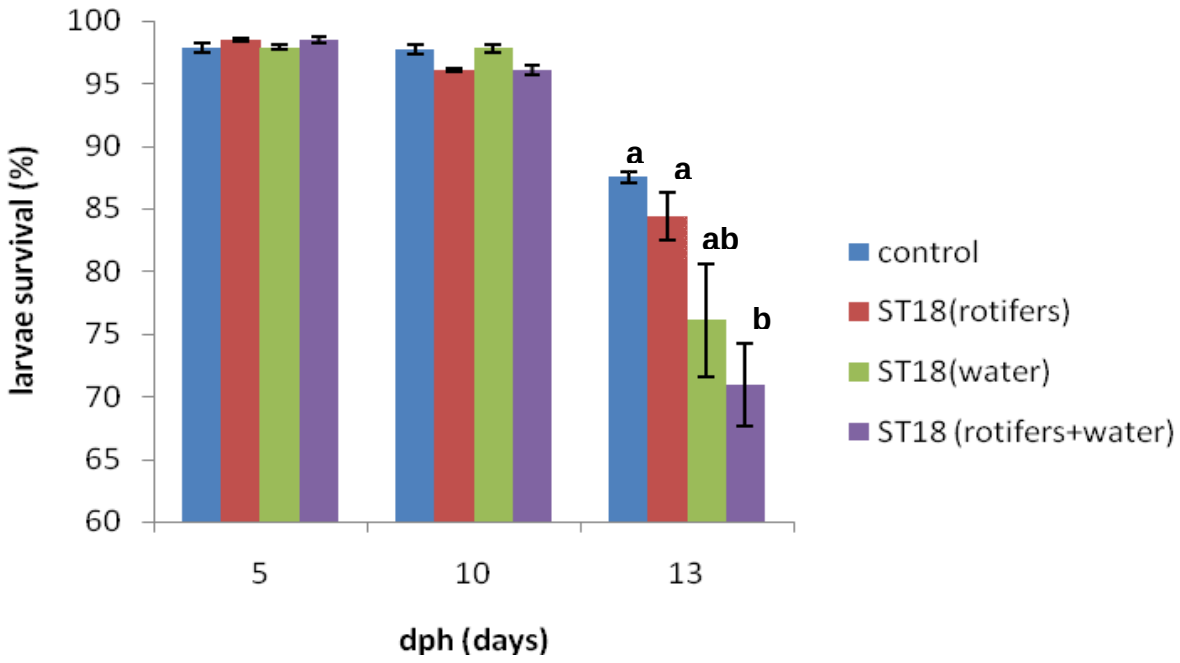

Figure 11.2 Survival percentage over the time of the Experiment 2 during first feeding. Different letters above the 13 dph results represent significant differences occurring between treatments (*P*=0.05).

Table 11.3 First feeding larvae Experiment 2 (see Chapter 10) showing outcomes for larval size and health and water chemical parameters.

Age	**2 dph**			
Treatment	**Initial state**			
Average size (mm)	4.8 ± 0.1			
Swim bladder inflation	-			
Grey gut	-			
pH	8.1 ± 0.1			
Dissolved oxygen (%)	99.1 ± 0.6			
Salinity (‰)	34.8 ± 0.1			
Temperature (°C)	15.7 ± 0.1			
Survival (%)	100			
Age	**5 dph**			
Treatment	**Control**	**ST18r + ST18 w**	**ST18r**	**ST18w**
Average size (mm)	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.1
Swim bladder inflation	-	-	-	-
Grey gut	-	-	-	-
pH	8.1 ± 0.1	8.1 ± 0.1	8.1 ± 0.1	8.1 ± 0.1
Dissolved oxygen (%)	99.1 ± 0.6	98.3 ± 0.6	98.8 ± 0.5	98.9 ± 0.6
Salinity (‰)	34.7 ± 0.1	34.7 ± 0.2	34.7 ± 0.1	34.7 ± 0.1
Temperature (°C)	16.2 ± 0.1	16.2 ± 0.1	16.2 ± 0.1	16.2 ± 0.1
Survival (%)	97.9 ± 0.4	98.5 ± 0.2	98.7 ± 0.1	97.9 ± 0.2
Age	**10 dph**			
Treatment	**Control**	**ST18r + ST18 w**	**ST18r**	**ST18w**
Average size (mm)	6.1 ± 0.1^{a}	5.7 ± 0.1^{b}	6.1 ± 0.1^{a}	5.1 ± 0.1^{b}
Swim bladder inflation	79.1 ± 3.7^{a}	43.3 ± 4.5^{b}	82.5 ± 3.5^{a}	36.6 ± 4.4^{b}
Grey gut	90 ± 2.7^{a}	42.5 ± 4.5^{b}	85 ± 0.1^{a}	20.8 ± 3.8^{c}
pH	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.1
Dissolved oxygen (%)	108.8 ± 0.6	107.0 ± 1.4	108.5 ± 0.6	107.3 ± 1.4
Salinity (‰)	34.9 ± 0.1	34.9 ± 0.2	34.9 ± 0.1	34.9 ± 0.1
Temperature (°C)	15.8 ± 0.1	15.8 ± 0.1	15.8 ± 0.1	15.8 ± 0.1
Survival (%)	97.7 ± 0.4	96.1 ± 0.4	98.1 ± 0.1	97.8 ± 0.3
Age	**13 dph**			
Treatment	**Control**	**ST18r + ST18 w**	**ST18r**	**ST18w**
Average size (mm)	6.9 ± 0.1^{a}	6.1 ± 0.1^{b}	6.9 ± 0.1^{a}	6.1 ± 0.1^{a}
Swim bladder inflation	98.3 ± 1.2^{a}	77.5 ± 4.8^{c}	95.8 ± 1.8^{ab}	86.7 ± 3.1^{bc}
Grey gut	97.5 ± 1.4^{a}	77.5 ± 3.2^{b}	99.2 ± 0.8^{a}	79.2 ± 3.3^{b}
pH	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.1
Dissolved oxygen (%)	107.5 ± 0.4	108.4 ± 0.5	107.7 ± 0.5	108.1 ± 0.4
Salinity (‰)	34.7 ± 0.1	34.7 ± 0.2	34.7 ± 0.1	34.7 ± 0.1
Temperature (°C)	15.8 ± 0.1	15.7 ± 0.1	15.7 ± 0.1	15.7 ± 0.1
Survival (%)	87.5 ± 0.4^{a}	70.9 ± 3.3^{b}	84.4 ± 1.9^{a}	76.1 ± 4.5^{b}

$^{a, b, c}$Where significant interactions between factors occur a one-way ANOVA analysis of all treatments is provided. Values within rows sharing a common superscript do not significantly differ (P>0.05).

A similar result was observed for other larvae quality features including swim bladder inflation and presence of grey gut (Table 11.3). Therefore, the addition of the potential probiont appears to be best done through introducing it to the larvae via bioencapsulation within rotifers.

11.4.4 *TRFLP analysis for Experiment 2*

The MDS plot derived from the TRFLP data for probiotic assessment experiment was inconclusive when all replicates were presented in an MDS due to the overwhelming similarity between groups. No changes could be seen and are therefore not presented. The only significant change found was when comparing the treatments to the incubator TRFLP baseline profiles: treatment 1, R=0.22; treatment 2, R=0.37; treatment 3, R=0.1; and treatment 4, R=0.15. It was also found that during the time of the experiment that all treatments were similar to each other on each sampling day and as a whole shifted in a similar manner resulting in the clustering of sampling days together with no differences observable. For the water samples it was found that there was no significant difference throughout the trial and even comparing 13 dph to the initial incubator water profiles there were only moderate changes (R=0.19-0.33). When assessing the total difference between the larval fish TRFLP profiles to that of the water it was found that they were significantly different at all time points sampled (global R=0.67).

Tracking strain ST18 was successfully done using TRF data in both the water and the larvae samples. For the experiments in which ST18 is applied to striped trumpeter larvae by bioencapsulation in rotifers the tracking of strain ST18 could be achieved at all sampling time points (Table 11.4) while it was not detected in the controls. In the larvae-derived TRFLP profiles it was seen that ST18 was only detected in the larvae fed enriched rotifers while the inclusion of ST18 to the water directly indicated ST18 appeared to be detectable in larvae 10 and 13 dph (Table 11.4). In addition there seems to be some suggestion that ST18 relative abundance appears to decline over time in the tank water (Table 11.4).

The similarity between replicates of treatments in both the larvae and water samples also increased over time as it did in the previous experiment and was seen to be highest at 13 dph in the ST18 addition treatments. The fact ST18 had become a major component of the microbial community in the replicates was further emphasised by the large abundance recorded in larvae samples (Table 11.4). Through this experiment not all TRFs of ST18 were detected using SIMPER based analysis and this may be due to biases occurring during the TRFLP analysis. The TRFLP profile data is a compilation of replicates of three separate restriction enzyme digestions and likely results in greater representation of certain TRFs compared to others in individual samples.

Table 11.4 Tracking of strain ST18 using TRF data during the rotifer bioencapsulation experiment applied to striped trumpeter larvae.

Treatment	Dph	Sample	Similarity%	ST18 TRFs		
				60 HhaI (f)	104 HinfI (f)	508 HaeIII (r)
				% contribution to % similarity:		
rotifers + algal feed	5	larvae	19.47	-	-	-
rotifer + ST18	5	larvae	75.53	10.99	62.33	28.75
control	2	larvae	11.11	-	-	-
control	5	larvae	34.11	-	-	-
control	6	larvae	15.55	-	-	-
control	10	larvae	17.29	-	-	-
control	13	larvae	40.55	-	-	-
control	2	water	70.17	-	-	-
control	5	water	56.53	-	-	-
control	6	water	74.98	-	-	-
control	10	water	48.87	-	-	-
control	13	water	58.22	-	-	-
ST18 (water + rotifers)	6	larvae	26.95	4.3	11.2	-
ST18 (water + rotifers)	10	larvae	32.48	11.1	27.5	-
ST18 (water + rotifers)	13	larvae	54.57	13.2	15.3	21.0
ST18 (water + rotifers)	5	water	53.51	-	-	-
ST18 (water + rotifers)	6	water	49.33	12.2	5.6	-
ST18 (water + rotifers)	10	water	51.87	9.3	2.5	16.9
ST18 (water + rotifers)	13	water	38.64	47.4	-	11.3
ST18 (rotifers)	6	larvae	24.82	6.1	5.9	17.2
ST18 (rotifers)	10	larvae	10.47	15.2	33.3	39.6
ST18 (rotifers)	13	larvae	13.61	22.2	48.2	9.2
ST18 (rotifers)	5	water	48.32	-	-	-
ST18 (rotifers)	6	water	55.37	-	-	-
ST18 (rotifers)	10	water	64.48	-	-	-
ST18 (rotifers)	13	water	39.27	-	-	-
ST18 (water)	6	larvae	14.85	-	-	-
ST18 (water)	10	larvae	24.08	-	-	17.8
ST18 (water)	13	larvae	21.87	-	-	22.2
ST18 (water)	5	water	52.10	-	-	-
ST18 (water)	6	water	52.59	10.1	7.5	13.5
ST18 (water)	10	water	39.34	1.5	5.5	-
ST18 (water)	13	water	66.85	5.3	-	-

11.5 Discussion

A combination of bacterial strains that complement each other and occupy different niches within the gut environment could result in an enhancement or prolongation of the desirable effects on the host immune response and health (Timmerman, et al., 2004; Panigrahi and Azad, 2007). The present results indicate that during the yolk-sac period the treatments with ST18 and ST7 proved to be unfavourable. The reduced DO levels observed on addition of the probionts to the tank water (Table 11.1) occurred possibly due to the bacterial inoculums being too high for the static system being investigated. The DO levels within the tank declined in the treatments in which the bacterial probionts were added (Table 11.1). Treatments 2 and 4 had the lowest DO levels of 73.4% and 68.9% of saturation respectively (Table 11.1) which coincided with lowest survival levels (58 ± 7% and 55 ± 8%). A bacterial bloom was also visible to the eye in the form of a floating mat. The water had slightly increased acidity with pH dropping from 8.1 to 7.9 (Table 11.1). The DO level was apparently below the level required by the larval fish to fulfil its minimal energy demand for growth. The inoculated bacteria also proliferated in the ozonated water possibly due to lack of other competitive bacteria. An improved balance in probiont populations may result in better outcomes for larval survival. The problem with striped trumpeter is they do best in very static conditions especially as yolk sac larvae and water exchange is difficult (Shaw 2006). Static conditions promote bacterial proliferation especially with the addition of nutrient rich rotifers.

The sole addition of strain ST7 was found to be disadvantageous to the culturing of striped trumpeter yolk sac larvae, while the addition of strain ST18 was shown to be less harmful. Strain ST7 is a member of the genus *Aliivibrio* and is related to *Vibrio* spp. that have been tested as probiotics in earlier studies (Gatesoupe, 1999; Makridis et al., 2001; Vine et al., 2006) but there is some criticism of their use as probiotics due to the possibility of them becoming virulent after prolonged application. The negative impact of strain ST7 was partially mitigated by co-addition of ST18 suggesting an interaction could have been occurring between the strains. This interaction could be in the form of strain ST18 producing an antimicrobial compound that inhibited strain ST7. The ability of strain ST18 to inhibit *Vibrio* strains due to a diffusible antimicrobial compound was observed in Chapter 10. It is possible that this antimicrobial compound was also actively produced during the current experiments. Whether production is a consequence of mechanisms such as quorum-sensing, in which a minimum population is needed before antimicrobial production is triggered, requires further investigation. Strain ST18 belongs to the genus *Pseudoalteromonas*, which has been frequently reported to produce a range of antimicrobial compounds (Bowman, 2007). The genus *Pseudoalteromonas* is common in marine environments and has been seen to comprise the microbiota of rotifers and *Artemia* (McIntosh et al., 2008, Bowman, 2007). It has been previously reported before that both antibacterial and antifouling activities are present in marine *Pseudoalteromonas* (Holmstrom et al., 1999). It is therefore it is possible that ST18 produces a probiotic effect through its release of water soluble compounds that inhibit or influence the growth of various bacterial species, including various pathogenic *Vibrio* spp.

In first-feeding larvae it was seen that the addition of the probiont via enriched rotifers was the most promising mode of addition. When ST18 was added to both the water and through rotifers it resulted in decreased survival, possibly due the number of bacteria present being too high. This is surprising as water was exchanged daily (300% exchange) and other studies have shown that the addition of probionts directly to the water have no detrimental effect (Makridis et al., 2001, Planas et al., 2006, McIntosh et al., 2008). These differing results may

be due to the bacterial species being used and also differences among fish species. Striped trumpeter has previously been shown to be sensitive to bacterial infections and perform best in bacteria reduced conditions (Battaglene et al 2006; Battaglene and Cobcroft 2007). Thus the introduction of strain ST18 to the larvae bioencapsulated in rotifers may have resulted in the introduction of comparatively smaller numbers of bacteria that did not compromise the growth of the developing larvae due to excessive oxygen removal.

It was encouraging that it was possible to track the probionts through the use of TRFLP analysis. By tracking distinct 16S rRNA-derived TRFs, strain ST18 was specifically detected in treatments where it was added by both bioencapsulated and by direct addition. Thus by successfully tracking strain ST18 greater confidence is provided for its biological impacts in the experiments. The TRFLP data also interestingly indicated that strain ST18 did not change the bacterial community of the larvae substantially (as suggested by ANOSIM data). This is the first study to the author's knowledge where TRFLP has been used for this type of application. In an aquaculture setting TRFLP has been used previously to assess the difference between the composition of bacteria associated with whiting mucus and the surrounding water (Smith et al., 2007). Other studies have also used TRFLP to help explain temporal bacterial changes in seawater (Hewson et al., 2003). No previous studies have used TRFLP to actively track a known bacterial isolate within an experiment environment before. It may be plausible to conclude that ST18 may release substances that do not substantially reduce the number of most bacteria present but may inhibit specific species from increasing to too high a population and/or inhibit their ability to produce pathogenic effects, for example proteolytic degradation of secreted enzymes or toxins.

It has been reported widely that one of the main modes of action and beneficial effects of probiotics in aquaculture organisms is enhancement of nutrition of host species through the production of supplemental digestive enzymes and higher growth and feeding efficiency, prevention of intestinal disorders and pre-digestion of anti-nutritional factors present in the ingredients (Verschuere et al., 2000). However, in aquaculture, probiotics can be administered either as a food supplement or as an additive to the water (Moriarty, 1998). In the current study we administered strain ST18 both in live food and water separately and live food with water in the same environment, thus, it was clearly determined where probiotics colonised and worked effectively in terms of growth, and survival in different environments (by live food and/or water). At the doses tested the most effective way to administer probionts appears to be via live food due to colonisation observed within larvae. This is in agreement with Suzer et al. (2008) who found similar results when administering probionts to gilthead sea bream (*Sparus aurata*) via addition to water or lives feeds, and saw that the best results were obtaining using live feed treatments. It has also been seen that through addition of probionts to the water or live feeds, the resulting colonization of the larvae gut may not be the amount expected. Planas (2006) found that when a *Roseobacter* sp. probiont was introduced to turbot larvae directly or through enriched rotifers, it did not colonise the gut in high numbers but was found in substantial numbers in the water. It was also seen in this study that the presence of ST18 was detected on both the water and the larvae. These experiments did not quantify the exact amount present but through occurrence of the TRF signal and the fact that ST18 TRFs were more readily detected in the water thus were possibly present in the water at higher numbers than in the larval gut.

Some of the proposed mechanisms for the probiont activity include greater survival, growth, viability or adhesion to mucosal surfaces of one species in the presence of another species, the production of different enzymes or other proteins, the creation of a probiotic niche and additive/synergistic effects of strain specific properties (Vine et al 2006). It must be taken

into account that prior inactivation of probiotic bacteria does not necessarily result in the loss of adhesion to intestinal mucus, although it may depend on the bacterial strain and the inactivation method used. Other studies have postulated probiotic modes of action to be dependent on interactions between probionts and pathogens in the digestive tract, such as competition for space or nutrients, or production by the probiotic of growth-inhibiting metabolites (Balcazar et al., 2006). Therefore, the possibility of an increased adhesion of one of the assayed bacteria in the presence of the other cannot be ruled out.

11.6 Conclusions

This study showed that the addition of *Pseudoalteromonas* sp. ST18 at the yolk sac larvae stage was not significantly different from that of the control and that during first feeding the best mode of addition of ST18 was through enriched rotifers. Through the use of TRFLP strain ST18 could be tracked and identified *in situ*. The experimental set up for these experiments resulted in the questions posed in the introduction being successfully answered, however many new questions have arisen during the course of the study. These unknown factors potentially set out future directions for research.

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12 FEEDING BY STRIPED TRUMPETER, *LATRIS LINEATA*, LARVAE, IN RESPONSE TO CHANGES IN PREY AND LARVAL DENSITY, ALGAL-INDUCED TURBIDITY AND TANK COLOUR

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12.1 Abstract

Maximising the contrast between potential prey items and the background against which they are viewed is particularly important for larvae that are reliant upon vision for feeding. This study investigated the effect of background environment on prey intake by striped trumpeter larvae that had either experience, or no experience, of feeding in a specific visual environment. Initial experiments investigated the effect of prey and larval density on rotifer prey intake and feeding rates over time. Rotifer intake increased linearly during the first 1.5 h post-feeding when fed at a rotifer density of 10 ml^{-1}. All subsequent experiments were therefore completed using prey densities ≤ 5 ml^{-1} and feeding periods of not greater than 2 h in order to ensure that prey consumption was not constrained by digestive tract capacity. Background colour significantly influenced prey intake with larvae consuming 5 times more rotifers in blue compared with black aquaria. However, larvae used in this experiment came from a blue culture tank and therefore had experience of feeding in a blue tank. A second experiment investigated the hypothesis that the change in tank colour resulted in the poor feeding in black tanks. Results showed that larvae fed best in the visual environment of which they had prior experience, and poorly in a new unfamiliar environment. The influence of changing visual environment was tested further using larvae with and without experience of feeding in an algal-induced green water environment. Larvae with experience of feeding in green water consumed significantly more rotifers in green water than larvae from a clear water environment. Larvae from a clear water environment fed equally well when shifted to either a clear or green water environment. However, larvae from a green water environment had significantly reduced feeding rates once shifted to a clear water environment. Striped trumpeter larvae appear to develop a “search image” which is highly specific to the environment in which they are feeding. Quickly changing the contrast between prey and background, and the subsequent visual image available to the larva, resulted in reduced prey intake. The ability to strongly influence feeding rates via changes to the visual environment of larvae in culture highlights the importance of optimising factors that contribute to visual discrimination of potential prey items. In particular, larval rearing systems should be designed such that transfer of larvae between systems minimises any change to the visual environment.

12.2 Introduction

Marine finfish larvae, including striped trumpeter *Latris lineata*, often hatch at a small size and early developmental state and depend primarily on vision, a light dependent sensory function, for successful feeding (Blaxter, 1986; Cobcroft and Pankhurst, 2003, 2006 in review). As a result, environmental conditions during the transition from endogenous nutrient reserves to exogenous prey capture, in particular the characteristics of the light environment, are critical for early survival and growth (Aksnes and Giske, 1993). In a culture environment, factors such as light intensity and photoperiod are often controlled with optimal light intensity ranges for feeding described for many species (Blaxter, 1968; Pankhurst and Hilder, 1998; Cobcroft et al., 2001; Downing and Litvak, 2001). However, even at light intensities sufficient for feeding, the visual capabilities of small fish impose a constraint on their ability to choose prey (Li et al., 1985; Kotrschal et al., 1990).

Striped trumpeter are being investigated as a potential aquaculture species. Control of broodstock maturation and spawning has been successfully achieved (Morehead et al., 2000); however, despite knowledge of sensory development (Cobcroft and Pankhurst, 2003), nutritional requirements (Bransden et al., 2005a; Bransden et al., 2005b) and preferences for light intensities and swim bladder inflation (Pankhurst and Hilder, 1998; Cobcroft et al., 2001; Trotter et al., 2003a; Trotter et al., 2003b), larvae often undergo large mortality events and survival to juveniles remains low and highly variable. Mortality events are often attributed to pathogenic bacteria (Battaglene et al., 2006; Battaglene et al., 2006 in review); however, periods of low prey intake (personal observation) may also affect survival.

For young larvae with poor foraging behaviour and a visual system which provides photopic acuity only just sufficient for planktonic feeding (Kotrschal et al., 1990), an environment which maximises prey contrast will likely provide larvae with the maximum chance to feed successfully. Altering tank colour and therefore the relative contrast between prey and background has significantly affected the growth and survival of a number of species (Hinshaw, 1985; Ostrowski, 1989; Chatain et al., 1991). However, the effect of tank colour on growth and survival of larvae is species-specific and in most cases few colours have been tested. For example, larval striped bass, *Morone saxatilis*, grew better in white versus black tanks (Martin-Robichaud and Peterson, 1998) whereas walleye, *Stizostedion vitreum*, larvae performed better with a dark background (Corrazza and Nickum, 1985). The effect of tank colour on growth and survival of striped trumpeter larvae is unknown. Turbidity, particularly the addition of micro algae cells (green water) in culture tanks has also resulted in improved growth and survival of larvae (Boehlert and Morgan, 1985; Naas et al., 1992; Reitan et al., 1993; Tamaru et al., 1994; Utne-Palm, 1999) with improved contrast between prey and background suggested as a possible mechanism (Boehlert and Morgan, 1985; Miner and Stein, 1993). Improved feeding by striped trumpeter larvae has been observed in green water. However, experience of feeding in one environment may have affected subsequent feeding in another (Cobcroft et al., 2001). Furthermore, evidence from production trials suggests that striped trumpeter larvae do not feed well following transfer to a new culture environment, towards which they are unfamiliar (personal observation). Thus, this study investigated the effects of tank colour, turbidity and experience of specific culture environments on the rotifer consumption by striped trumpeter larvae. Specifically the aims were: 1.) To investigate the effect of algal cell induced turbidity on prey consumption. 2.) To examine the effect of tank colour on prey consumption. 3.) To investigate the role of prior experience of feeding in a specific environment i.e., turbidity (clear or green water) and tank colour (black or blue tanks), on subsequent feeding proficiency in environments to which

larvae are experienced or unfamiliar. The effect of changing the contrast between prey and background was therefore investigated.

In order to design short duration feeding experiments that address specific questions it is first necessary to understand how larval density, prey density and feeding time, affect prey consumption. For instance, a feeding period sufficient to satiate larvae will likely mask the effect of any treatment (Vandenbyllaardt et al., 1991). Also, as capacity of the digestive tract is neared larvae can become more selective in their prey choices (Gill and Hart, 1998) and potential feeding rates are limited by the digestive processing rate (Aksnes and Giske, 1993). Therefore, the influence of larval density, rotifer density and feeding time on the consumption of rotifers by striped trumpeter larvae was also investigated.

12.3 Methods

12.3.1 *Larval rearing*

Eggs and sperm were hand striped from striped trumpeter broodstock housed at the Marine Research Laboratories, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania. Eggs from each female were fertilised with sperm from 2 or 3 males, disinfected with ozone at 1 ppm for 1 min and incubated in a 250 l upwelling incubator. Eggs were incubated at 14 ºC and a salinity ranging from 33.5 to 35.0 ppt. Each experiment used eggs from a single but different female. Eggs hatched 5 days post-fertilisation at which time water temperature was gradually increased over a 2-day period to between 14 and 16 ºC, matching the temperature of the culture systems into which the larvae were transferred (Table 12.1). Culture systems had a photoperiod of 16h: 8h, L: D with lights fading on at 0900 h. Larvae were fed rotifers *Brachionus plicatilis* at a density of 5 or 10 ml^{-1} once daily at 0900 h, or twice daily at 0900 h and 1700 h, depending on the experiment (Table 12.1). Rotifers were enriched with Algamac 2000 (Aquafarma Bio-Marine, Inc.) for 12 h at 23 ºC prior to feeding to larvae. Seawater was ozonated and held at >600 Oxidative Reductive Potential (ORP) for 10 minutes before being passed through a UV sterilization unit and charcoal filter. Culture tanks received filtered seawater at a flow of 112.5 l h^{-1} for either 24 or 8h (Table 12.1). Water surface skimmers were added to tanks from 8 to 13 dph to remove surface films during the swim bladder inflation window (Trotter et al., 2005). Morphometric assessments of 20 larvae from each culture tank were made on the day of each feeding experiment. Larvae for assessment were syphoned from each tank, anaesthetised in 0.06% 2-phenoxyethanol (2PE), and the standard length of each larva was measured under a dissecting microscope. Where applicable, at the end of each experiment, all larvae were counted and survival calculated.

Feeding performance of striped trumpeter larvae was assessed either by directly sampling larvae from the culture tanks, or in short duration feeding trials in which larvae were transferred from the culture tank into 3-l static water, test aquaria under a range of environmental conditions and larvae and prey densities.

Table 12.1 Culture tank parameters, larval stocking density, prey density and age at feeding onset of the various cohorts of larvae used in the feeding experiments. NTU – Nephelometric Turbidity Units; dph – days post-hatching.

Experiment	No. of tanks	Tank volume (l)	**Tank colour**	Turbidity (NTU)	Stocking density (larvae l^{-1})	Flow (h^{-1})	Rearing temp. (ºC ± SE)	Feeding onset (dph)	Prey density (rotifers ml^{-1})
1	2	1000	blue	0	30	24	16.4 ± 0.1	6	[a] 10
1	1	1000	blue	2.5	30	8	16.3 ± 0.1	6	10
2	24	300	black	0	[b] 1.25 - 40	8	15.1 ± 0.1	6	10
3	1	1000	blue	0.8	20	8	14.9 ± 0.1	6	10
4	4	300	black	0	20	24	14.4 ± 0.1	7	10
4	1	1000	blue	0.8	20	24	14.9 ± 0.1	6	10
5	1	1000	blue	0	30	24	16.4 ± 0.1	6	10
5	1	1000	blue	2.5	30	8	16.3 ± 0.1	6	10
6	2	300	black	3	7.5	8	16.5 ± 0.1	6	5
6	2	300	black	0	7.5	8	16.4 ± 0.1	6	5

[a] Were fed twice daily at 0900 h and 1700 h, all other tanks fed once daily at 0900 h.

[b] Range of stocking densities; 1.25, 2.5, 5, 10, 20 and 40 larvae l^{-1} with 4 replicates per treatment.

12.4 Feeding rate assessment

Prey consumption by larvae in culture tanks was assessed by first checking that the digestive system of 20 larvae sampled from each tank prior to lights on (time zero) was free of recently consumed prey. Rotifers within the culture tanks were flushed out overnight, reducing the chances of non-visual feeding during the night. Larvae sampled prior to lights on contained mastax and trophi of previously consumed rotifers; however, no freshly consumed rotifers were present in the digestive tract of any larvae. Where large numbers of tanks were being assessed simultaneously, addition of rotifers to each successive tank was delayed for two minutes. Offsetting the time of addition of rotifers allowed time for syphoning 20 larvae from each tank at the end of the prescribed feeding period, thus ensuring that larvae within each tank had exactly the same feeding period (Table 12.2). All sampled larvae were anesthetised in 2PE, fixed in 10% neutral buffered formalin, and then transferred to sea water prior to placement onto a glass histology slide for measurement of standard length. A cover slip was then placed over each larva and the number of rotifers in the digestive tract counted. Anaesthesia and or fixation of striped trumpeter larvae did not result in gut evacuation, as evidenced by the retention in the mid-hindgut of the remnants of prey (rotifer mastax and trophi) ingested on the previous day.

Assessment of prey consumption by larvae in 3-l test aquaria was similar. Following confirmation that digestive tracts of ‘time zero’ larvae were clear of previously ingested prey, larvae from the culture tanks were transferred into each of the required number of aquaria. Larvae were left for 30 min to acclimate to the new culture environment. Following acclimation, rotifers were added and larvae were allowed to feed for the prescribed feeding period (Table 12.2).

Table 12.2 The experimental conditions, tank colour, turbidity, prior culture environment, prey density, larval density, and feeding durations used in the 6 prey consumption experiments. Experiments 1 and 2 assessed prey consumption in culture tanks. Experiments 3 - 6 involved transferring larvae from their culture tank into 3 l test aquaria prior to prey consumption experiments. NTU – nephelometric turbidity units; dph – days post-hatching; CW – clear water; GW – green water.

Experiment	Age (dph)	Test aquaria size (l)	Test aquaria colour	Turbidity (NTU)	Prior culture environment (Tank colour and green or clear water)	Prey density (rotifers ml^{-1})	Larval density (l^{-1})	Prey consumption assessed after (min)	Replicates
1	6, 9, 11	1000	blue	0, 2.5	blue	10	30*	30, 60, 90, 120	2 (CW), 1 (GW)
2	15	300	black	0	black	10	1.25, 2.5, 5, 10, 20, 40*	36, 72, 108, 144	4
3	9	3	black, blue	0.8	blue (GW)	1.25, 2.5, 5, 10	5, 20	120	4
4	14	3	black, blue	0	black (CW) or blue (GW)	5	10	90	4
5	10	3	blue	0, 5, 10, 20	blue (CW or GW)	1	7.5	90	3
6	8, 15	3	black	0, 5	black (CW or GW)	1	5	120	4

* density of larvae added to tanks at the start of the experiment

12.4.1 *Experiment 1 – Effect of feeding duration on rotifer consumption in green or clear water*

Experiment 1 examined the number of rotifers consumed at 30 min intervals by larvae held in either clear or green water 1000-l blue culture tanks (Table 12.1). One culture tank had algae *Nannochloropsis oculata* (green water) added each morning at 0900 to achieve a turbidity of 2.5 Nephelometric Turbidity Units (NTU) and remained static during the light period. A further two culture tanks had continuous flow-through of clear water (filtered to 1 μm without algal cell addition). The feeding rates of larvae within culture tanks were assessed 6, 9 and 11 dph after the addition of rotifers (Table 12.2).

12.4.2 *Experiment 2 – Effect of larval density on rotifer consumption in clear water*

Experiment 2 investigated the effect of larval density on feeding rates in black 300-l hemispherical culture tanks under clear water conditions. There were 24-tanks each containing larvae at one of 6 densities, 1.25, 2.5, 5, 10, 20 and 40 larvae l^{-1} with four replicate tanks per prey density (Table 12.1). Prey consumption was assessed 15 dph on 20 larvae sampled from each tank 36, 72, 108 and 144 min after the addition of rotifers (Table 12.2).

12.4.3 *Experiment 3 – Effect of larval density, prey density, and aquaria colour on prey consumption*

Experiment 3 examined the effect of larval and prey density on rotifer consumption by larvae in 3-l test aquaria. Treatments were two larval densities, 5 and 20 l^{-1}, and four rotifer densities, 1.25, 2.5, 5 and 10 rotifers ml^{-1}, in a fully orthogonal design. There were 4 replicate aquaria for each treatment, two of which were blue and two black in colour (Table 12.2). Larvae came from a 1000-l blue culture tank fed rotifers in green water (0.8 NTU) (Table 12.1).

12.4.4 *Experiment 4 – Effect of experience and aquaria colour on rotifer consumption*

Experiment 4 examined in more detail the effect of aquarium colour on prey consumption rates. Larvae reared in clear water culture conditions in a 1000-l blue culture tank and a 300-l black culture tank were used (Table 12.1). A short duration feeding experiment was conducted in 3-l aquaria in clear water with 20 larvae per aquarium. The experiment was set up as a fully orthogonal design with 2 prior culture environments, black and blue tanks, and two new culture environments, black and blue aquaria. Larvae were transferred from a blue culture tank to either a blue (familiar) or black (unfamiliar) aquarium or from a black culture tank to a black (familiar) or blue (unfamiliar) aquarium. Larvae were fed rotifers in clear water (Table 12.2).

12.4.5 *Experiment 5 – Effect of prior experience of feeding in a clear or green water environment on subsequent rotifer consumption in clear or green water*

Experiment 5 assessed the effect of prior experience of feeding in a clear or green water environment (Table 12.1) on subsequent rotifer consumption in clear water and increasing levels of algal cell induced turbidity. Turbidity levels tested were 0, 5, 10 and 20 NTU.

There were 3 replicate blue 3-l aquaria per combination of prior culture environment and turbidity, each containing 20 larvae. Rotifers were added to each aquarium and larvae were allowed to feed for 1.5 h (Table 12.2).

12.4.6 *Experiment 6 – Effect of prior experience of feeding in a clear or green water environment on subsequent rotifer consumption in water containing a range of live and re-suspended algal species*

Experiment 6 examined the effect of different algal species and prior culture history on rotifer consumption 8 and 15 dph. Larvae from a 300-l black, clear water culture tank and larvae from a 300-l black, green water (*N. oculata*, 3 NTU) culture tank (Table 12.1) were transferred to black 3-l test aquaria each containing one of six treatments. Treatments were: clear water, live *N. oculata* cells; live *Isochrysis galbana* cells and 3 resuspensions of Instant Algae® (Reed Mariculture Inc. US) cell pastes; *Tetraselmis suecica, N. oculata and P. lutheri*, each having four replicates. All algal treatments were diluted to a turbidity level of 5 NTU using filtered and ozonated sea water (Table 12.2).

12.4.7 *Statistical analyses*

All data were evaluated for homogeneity of variance via residual plots and Levene's Test. Where appropriate data was log transformed prior to analysis. Dependent on the experimental design, feeding rate data were analysed using a two, three or four factor ANOVA with tank or aquarium included as a nested factor. A regression analysis examined the effect of rotifer density on consumption rates in Experiment 3. The percentage of larvae feeding was analysed by first transforming data using an arcsin√ transformation and then running ANOVA's where appropriate. Differences between the mean lengths of larvae coming from different rearing cultures were tested using a one factor ANOVA. Significance was accepted at $P = 0.05$. Where significant differences occurred a Tukey's post-hoc test was performed. All statistical analyses were completed using SPSS version 10.0.

12.5 Results

12.5.1 *Experiment 1 – Effect of feeding duration on rotifer consumption in green or clear water*

Larvae reared in a green water environment were significantly larger than larvae reared in clear water on 9 and 11 dph (Table 12.3; Experiment 1). Only 15% of larvae 6 dph from either a clear or green water environment were feeding 2 h post-rotifer addition (Figure 12.1). Neither the proportion of larvae feeding nor rotifer consumption 6 dph was affected by turbidity or time post-rotifer addition (Table 12.4; Experiment 1). The number of rotifers consumed by larvae 9 dph was significantly affected by time post-rotifer addition but not by turbidity. The proportion of larvae feeding was significantly affected by turbidity with a mean of 0.73 ± 0.04 ($n = 8$) and 0.87 ± 0.06 ($n = 4$) of larvae feeding in clear and green water, respectively (Figure 12.1; Table 12.4; Experiment 1). By 11 dph both turbidity and time post-rotifer addition significantly affected rotifer intake. On average larvae in green water consumed significantly more rotifers than larvae in clear water and rotifer intake increased with increasing time post-rotifer addition. The proportion of larvae feeding in clear and green water was and 0.81 ± 0.08 ($n=4$) and 0.87 ± 0.04 ($n=4$), respectively. There was no indication that feeding rates declined during the 2 h feeding period. Maximum consumption occurred during the final 30 min feeding period for larvae 9 and 11 dph (Figure 12.1).

Table 12.3 Mean length of larvae used in each experiment with results of one-factor ANOVA showing differences between larvae coming from different culture environments. Asterisks indicate significant difference between the length of clear and green water-reared larvae.

		Length (mm, mean ± se)					
Experiment	dph	green water	clear water	*df* effect	*df* error	*F* value	*P* value
1	6	5.18 ± 0.02	5.25 ± 0.01	1	153	16.013	< 0.000*
1	9	5.67 ± 0.04	5.41 ± 0.02	1	220	32.262	< 0.000*
1	11	6.11 ± 0.03	5.48 ± 0.03	1	198	216.548	< 0.000*
2	15	-------	[a] 6.4 - 5.8	-	-	-	-
3	9	5.63 ± 0.04	-------	-	-	-	-
4	14	6.84 ± 0.04	6.36 ± 0.05	1	31	63.344	< 0.000*
5	10	6.17 ± 0.04	5.55 ± 0.04	1	38	104.837	< 0.000*
6	8	5.29 ± 0.01	5.22 ± 0.01	1	41	18.047	< 0.000*
6	15	7.76 ± 0.05	7.42 ± 0.05	1	78	22.22	< 0.000*

[a] Size of larvae was dependent on the larval density treatment see Battaglene et al. (2006 in review)

12.5.2 *Experiment 2 - Effect of larval density on rotifer consumption in clear water*

Larval density had no effect on prey consumption by larvae in 300 l clear water culture tanks during the first 144 min post-feeding (Table 12.4; Experiment 2). Rotifer consumption increased with time post-rotifer addition (Figure 12.2a). Consumption of rotifers during the first three 36 min time intervals was not significantly different; however, rotifer consumption significantly decreased during the final 36 min feeding period (Figure 12.2b; Table 12.4; Experiment 2). The percentage of larvae feeding was significantly affected by sampling time and larval density (Table 12.4; Experiment 2). The proportion of larvae feeding increased from 0.83 ± 0.02 (n = 24) 36 min post-rotifer addition to between 0.94 and 0.96 at any other sampling time. Post-hoc testing revealed no significant differences in the proportion of larvae feeding at each larval density.

Table 12.4 Results of two-factor ANOVA's with rotifer intake, proportion of larvae feeding and number of rotifers consumed during each feeding period as dependent variables, for larvae 6, 9 and 11 dph in Experiment 1 and larvae 15 dph in Experiment 2. Asterisks indicate significant P values.

dph	Effect	*df* effect	*df* error	*F* value	*P* value	
	Experiment 1.					
	Prey consumption					
6	Time	3	4	3.474	0.128	
	Turbidity	1	4	0.062	0.814	
	Time × Turbidity	3	4	0.911	0.510	
	Tank (Time * Turbidity)	4	198	0.911	0.459	
9	Time	3	4	8.732	0.030	*
	Turbidity	1	4	7.432	0.051	
	Time × Turbidity	3	4	0.539	0.680	
	Tank (Time × Turbidity)	4	165	2.166	0.075	
11	Time	3	152	24.001	0.000	*
	Turbidity	1	152	9.757	0.002	*
	Time × Turbidity	3	152	0.157	0.925	
	Proportion Feeding					
6	Time	3	4	3.679	0.120	
	Turbidity	1	4	0.010	0.925	
	Time × Turbidity	3	4	0.758	0.573	
9	Time	3	4	5.814	0.061	
	Turbidity	1	4	9.464	0.034	*
	Time × Turbidity	3	4	1.536	0.335	
	Prey consumption - time interval					
6	Time	3	4	0.761	0.572	
	Turbidity	1	4	0.000	1.000	
	Time × Turbidity	3	4	1.526	0.337	
9	Time	3	4	0.710	0.595	
	Turbidity	1	4	1.448	0.295	
	Time × Turbidity	3	4	0.269	0.846	
	Experiment 2.					
	Prey consumption					
15	Time	3	72	112.241	0.000	*
	Larval density	5	72	1.230	0.304	
	Time × Larval density	15	72	0.931	0.535	
	Tank (Time × Turbidity)	72	1824	2.552	0.000	*
	Proportion Feeding					
	Time	3	72	14.980	0.000	*
	Larval density	5	72	2.495	0.039	*
	Time × Larval density	15	72	0.567	0.891	
	Prey consumption - time interval					
	Time	3	72	6.081	0.001	*
	Larval density	5	72	0.171	0.973	
	Time × Larval density	15	72	2.075	0.021	*

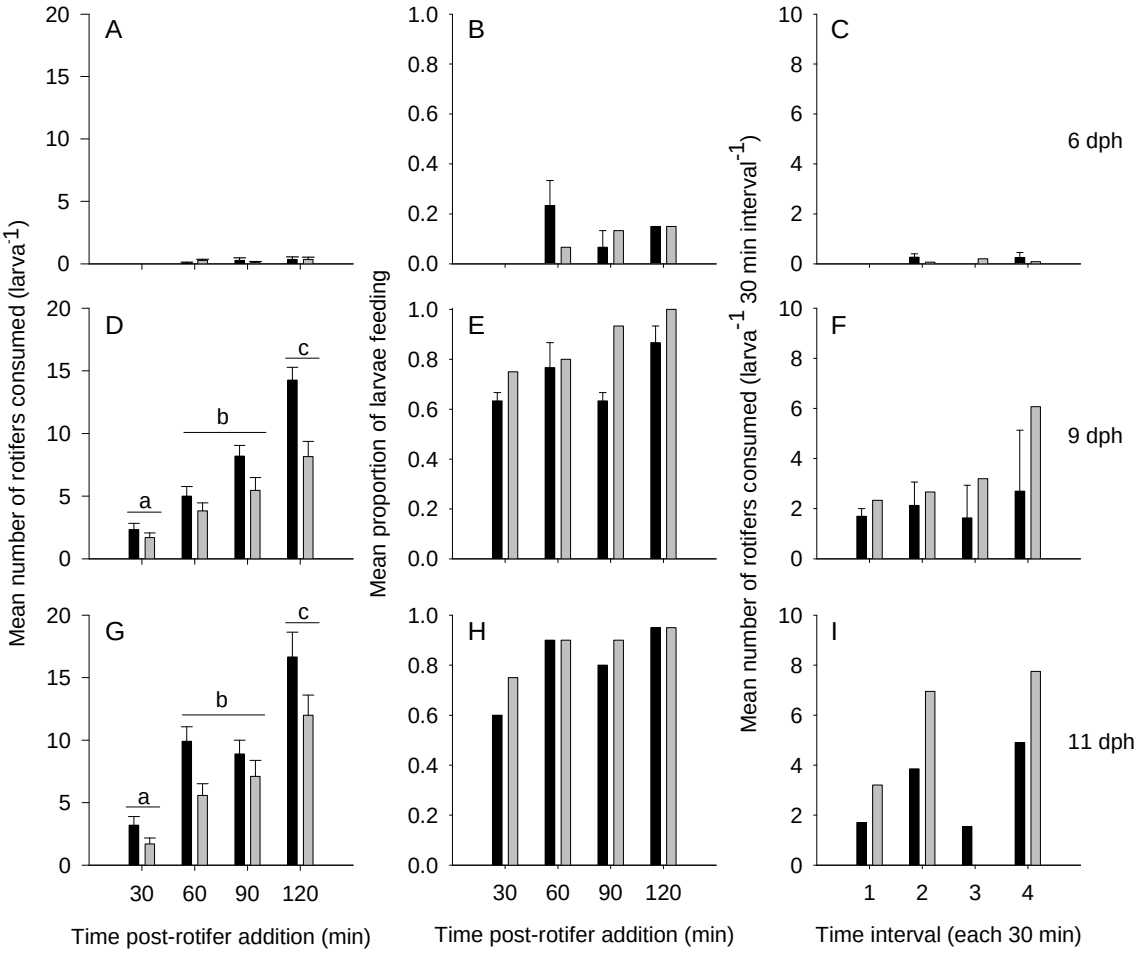

Figure 12.1 Experiment 1. Larvae 6 (A,B,C), 9 (D,E,F) and 11 (G,H,I) dph were fed rotifers in either clear water (▭) or green water (▬) and sampled every 30 minutes for 2 h. A, D, G) Mean (± SE) rotifer intake (clear water, n = 40 except 11 dph where n = 20; green water, n = 20). B, E, H) Mean (± SE) proportion of larvae feeding (clear water, n = 2 except 11 dph where n = 1; green water n = 1). C, F, I) Mean (± SE) intake of rotifers during each 30 min period (clear water, n = 2 except 11 dph where n = 1; green water, n = 1), calculated by excluding the average number of rotifers consumed in the preceding time periods. Means within graphs sharing common superscripts are not significantly different (P > 0.05).

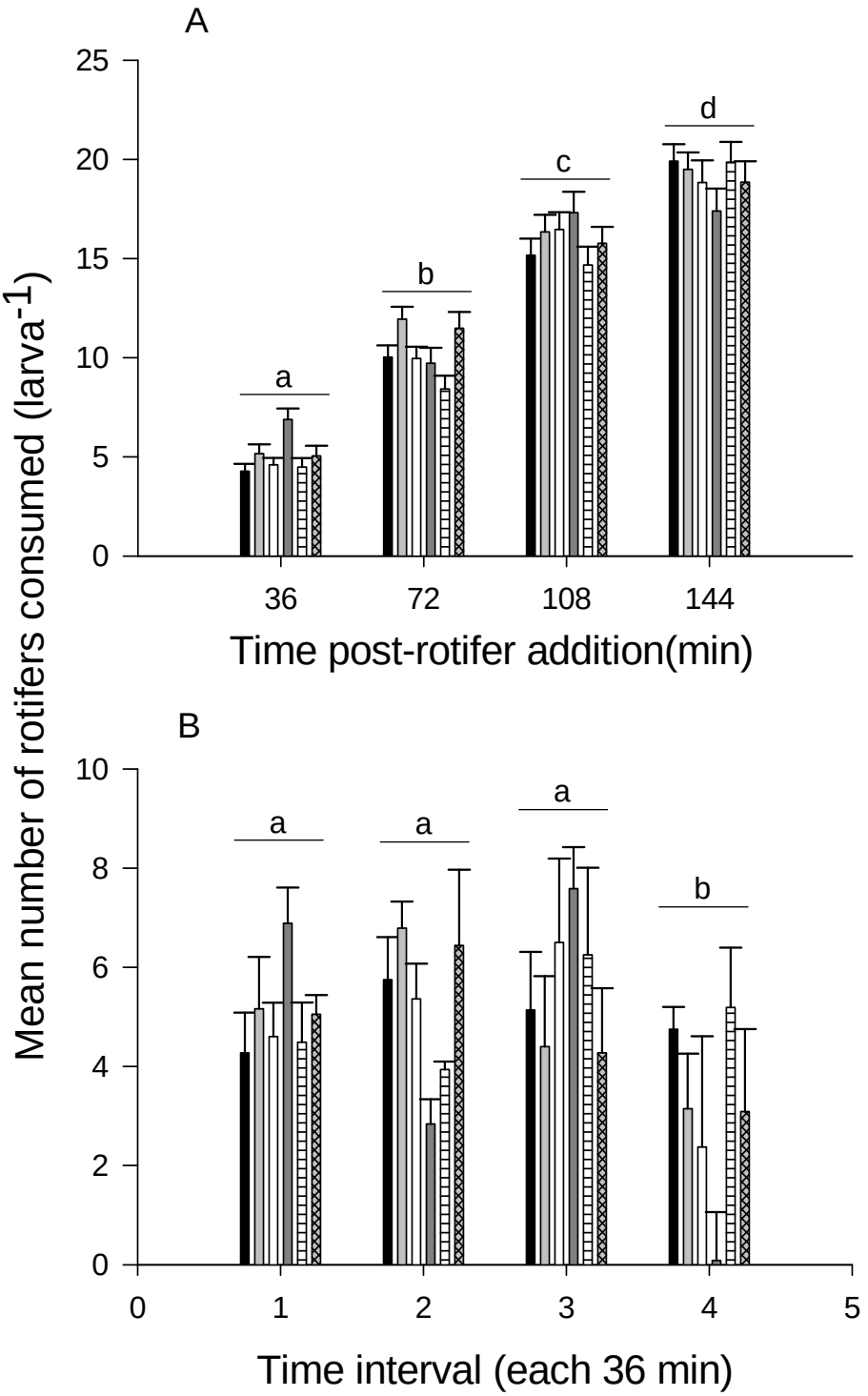

Figure 12.2 Experiment 2. Larvae 15 dph held at densities of 1.25 (■), 2.5 (▭), 5 (□), 10 (▬), 20 (▬) and 40 (▬) larvae l^{-1} were fed rotifers at a density of 10 ml^{-1} and sampled every 36 min for 144 min. A) Mean (± SE, n = 20) rotifer intake. B) Mean (± SE, n = 4) intake of rotifers during each 36 min period, calculated by excluding the average number of rotifers consumed in the preceding time periods. Means sharing common superscripts are not significantly different (P > 0.05).

12.5.3 *Experiment 3 – Effect of larval density, prey density, and aquaria colour on prey consumption*

Aquarium colour had a significant effect on rotifer consumption; however, prey density and larval density had no significant effect (Figure 12.3; Table 12.5; Experiment 3). Larvae feeding in blue aquaria consumed 10.3 ± 0.3 rotifers $2h^{-1}$(mean ± se, n = 213) compared with 2.0 ± 0.4 rotifers $2h^{-1}$ (n = 241) in black aquaria. Excluding black tanks from the analysis did not alter the effects of prey and larval density with neither significantly affecting rotifer intake. However, running a regression analysis using mean prey intake for larvae from each tank resulted in a significant positive regression between increasing prey density and increasing prey intake; prey intake = 0.3535 × prey density + 4.8088 (P = 0.040; r^2 = 0.056) (Table 12.5; Experiment 3). The proportion of larvae feeding in each aquarium was significantly affected only by aquarium colour and not by prey or larval density (Figure 12.3, Table 12.5; Experiment 3). The proportion of larvae feeding was 0.29 ± 0.07 (n = 4) and 0.92 ± 0.02 (n = 4) in black and blue aquaria, respectively.

12.5.4 *Experiment 4 – Effect of experience and aquaria colour on rotifer consumption*

The larvae transferred from a black culture tank into test aquaria were significantly smaller than those transferred from a blue culture tank (Table 12.3; Experiment 4). The interaction between culture tank colour and test aquarium colour had a significant effect on the number of rotifers consumed and the proportion of larvae feeding within aquaria (Figure 12.4; Table 12.5; Experiment 4). Greatest consumption, 15.8 ± 0.9 rotifers 90 min^{-1}(n = 80), occurred where larvae from a blue culture tank were transferred to blue aquaria. Only 0.4 ± 0.1 (n = 79) rotifers 90 min^{-1} were consumed by larvae transferred from a blue culture tank to a black aquarium. Changing the colour of tanks significantly reduced subsequent rotifer consumption (Figure 12.4; Table 12.5; Experiment 4).

Table 12.5. Results of three-factor ANOVA's of Experiment 3 and two-factor ANOVA's of Experiment 4. Asterisks indicate significant P values.

Effect	*df* effect	*df* error	*F* value	*P* value	
Experiment 3.					
Prey consumption					
Larval density…(1)	1	16	1.441	0.244	
Prey density…(2)	3	16	2.315	0.108	
Aquaria colour…(3)	1	16	80.642	0.000	*
1 × 2	3	16	2.189	0.122	
1 × 3	1	16	0.569	0.460	
2 × 3	3	16	0.640	0.598	
1 × 2 × 3	3	16	2.065	0.138	
Tank (1 × 2 × 3)	16	422	3.626	0.000	*
Proportion Feeding					
Larval density…(1)	1	16	3.543	0.078	
Prey density…(2)	3	16	1.287	0.313	
Aquaria colour…(3)	1	16	62.963	0.000	*
1 × 2	3	16	0.571	0.642	
1 × 3	1	16	0.149	0.704	
2 × 3	3	16	1.062	0.393	
1 × 2 × 3	3	16	1.158	0.356	
Anova and Regresion - Prey density					
Prey density	3	28	0.600	0.620	
Regression	1	2	23.334	0.040	*
Deviations from regression	2	28	0.071	0.932	
Experiment 4.					
Prey consumption					
History (culture tank colour)	1	12	1.015	0.334	
Aquaria colour	1	12	13.272	0.003	*
History × Aquaria colour	1	12	31.831	0.000	*
Tank (History × Aquaria colour)	12	302	6.535	0.000	*
Proportion Feeding					
History (culture tank colour)	1	12	1.553	0.236	
Aquaria colour	1	12	25.680	0.000	*
History × Aquaria colour	1	12	33.601	0.000	*

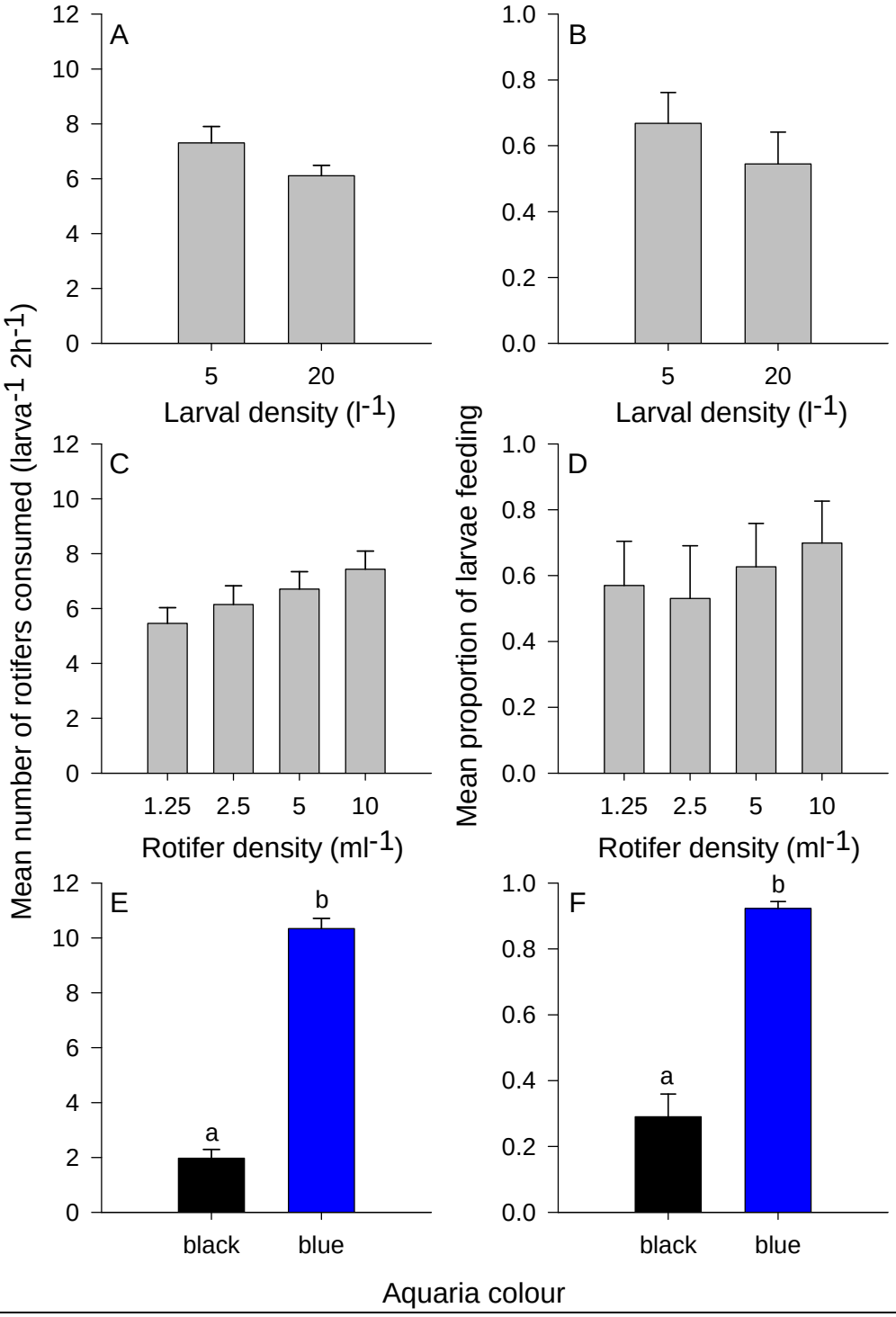

Figure 12.3 Experiment 3. Larvae 9 dph reared in blue tanks were distributed to black or blue aquaria at two densities 5 and 20 larvae l^{-1} and fed rotifers at 1.25, 2.5, 5, 10 or 20 ml^{-1} for 2 h. A, C, E) Mean intake of rotifers. B, D, F) Mean proportion of larvae feeding. Figure shows results of each main effect; A, B) Larval density; C, D) Rotifer density; E, F) Aquaria colour. Means ± SE sharing common superscripts within graphs are not significantly different ($P > 0.05$).

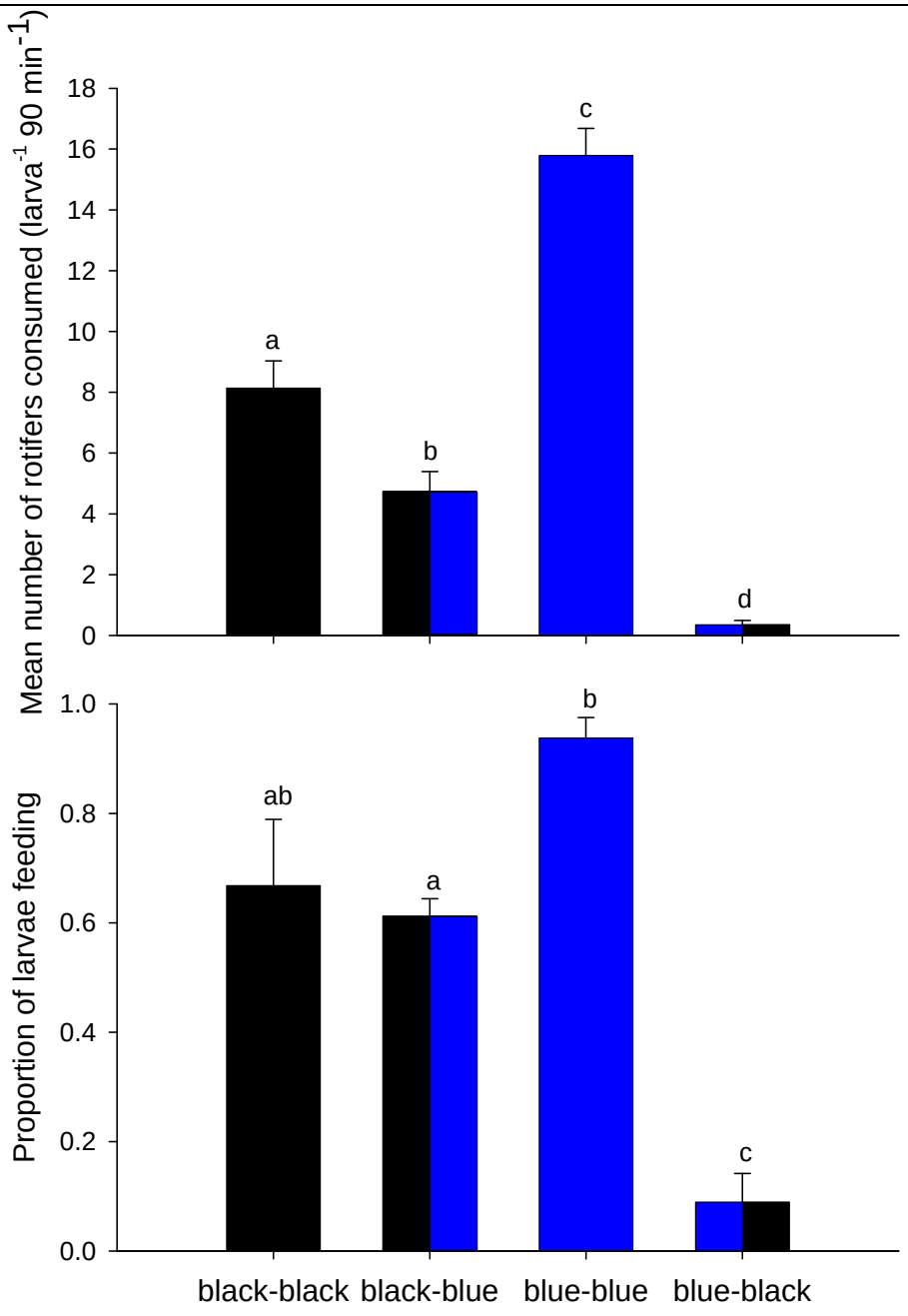

Figure 12.4 Experiment 4. Larvae 14 dph reared in either a black or blue culture tank were subsequently fed rotifers at a density of 5 ml^{-1} in either a black or blue aquarium. A) Mean (± SE, n = 20) rotifer intake. B) Mean (± SE, n = 4) proportion of larvae feeding. Means sharing common superscripts are not significantly different (P > 0.05).

12.5.5 *Experiment 5 – Effect of prior experience of feeding in a clear or green water environment on subsequent rotifer consumption in clear or green water*

Larvae reared in a green water environment were significantly larger than those from a clear water environment (Table 12.3; Experiment 5). Culture environment, turbidity and the interaction between culture environment and turbidity had a significant effect on the number of rotifers consumed by larvae (Figure 12.5; Table 12.6; Experiment 5). In general, prey intake improved with increasing turbidity and larvae from a green water environment consumed more prey than those from a clear water environment. The proportion of larvae feeding was also affected by prior culture environment and turbidity. On average significantly more larvae from a green water environment fed in comparison to those from clear water and more larvae fed in turbid water compared with clear water (Figure 12.5; Table 12.6; Experiment 5).

12.5.6 *Experiment 6 – Effect of prior experience of feeding in a clear or green water environment on subsequent rotifer consumption in water containing a range of live and re-suspended algal species*

Larvae transferred from a green water environment to aquaria were significantly larger than those transferred from a clear water environment (Table 12.3; Experiment 6). Consumption of rotifers by larvae 8 dph was significantly affected by algal treatment but not by prior culture history or their interaction (Figure 12.6; Table 12.6; Experiment 6). Best consumption rates occurred in *N. oculata* (live cells and resuspended algal cell paste) and live *I. galbana*. Consumption of rotifers by larvae 15 dph was significantly affected by both algal treatment and prior culture environment but not their interaction. Best consumption rates again occurred in *N. oculata* (resuspended algal cell paste). Larvae 15 dph reared in a clear water environment consumed significantly more rotifers than larvae reared in a green water environment (Figure 12.6; Table 12.6; Experiment 6).

Table 12.6 Results of two-factor ANOVA's with rotifer intake and proportion of larvae feeding as dependant variables for Experiments 5 and 6. Asterisks indicate significant P values.

Effect	*df* effect	*df* error	*F* value	*P* value	
Experiment 5.					
Prey consumption					
History	1	16	16.706	0.001	*
Turbidity	3	16	18.449	0.000	*
History × Turbidity	3	16	4.689	0.015	*
Tank (History * Turbidity)	16	317	0.650	0.842	
Proportion Feeding					
History	1	16	5.584	0.031	*
Turbidity	3	16	12.201	0.000	*
History × Turbidity	3	16	0.377	0.771	
Experiment 6.					
8 dph					
Prey consumption					
History	1	36	1.612	0.114	
Algal species	5	36	8.635	0.000	*
History × Algal species	5	36	0.430	0.825	
Tank (History × Algal species)	36	901	5.266	0.000	*
Proportion Feeding					
History	1	36	0.002	0.968	
Algal species	5	36	5.746	0.001	*
History × Algal species	5	36	0.510	0.767	
15 dph					
Prey consumption					
History	1	36	12.339	0.001	*
Algal species	5	36	3.714	0.008	*
History × Algal species	5	36	0.924	0.474	
Tank (History × Algal species)	36	911	5.561	0.000	*
Proportion Feeding					
History	1	36	5.642	0.023	*
Algal species	5	36	2.149	0.082	
History × Algal species	5	36	0.254	0.935	

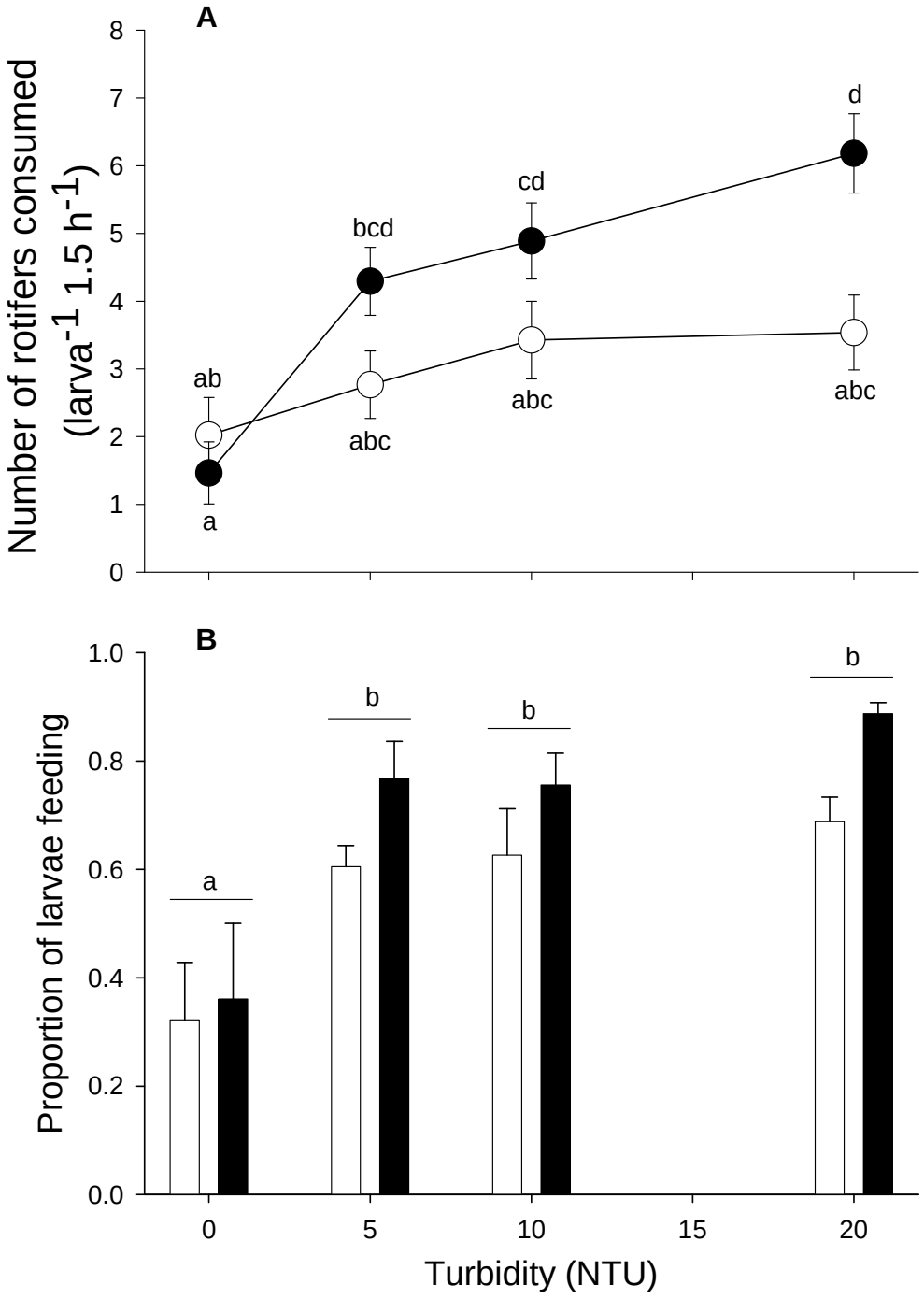

Figure 12.5 Experiment 5. Larvae 10 dph reared in either green water (■) or clear water (□) culture tanks were subsequently fed rotifers at a density of 1 ml^{-1} in aquaria with either, clear water or algal cell induced turbid water of 5, 10 or 20 Nephelometric Turbidity Units - NTU. A) Mean (± SE, n = 15) rotifer intake. B) Mean (± SE, n = 3) proportion of larvae feeding. Means sharing common superscripts are not significantly different (P > 0.05).

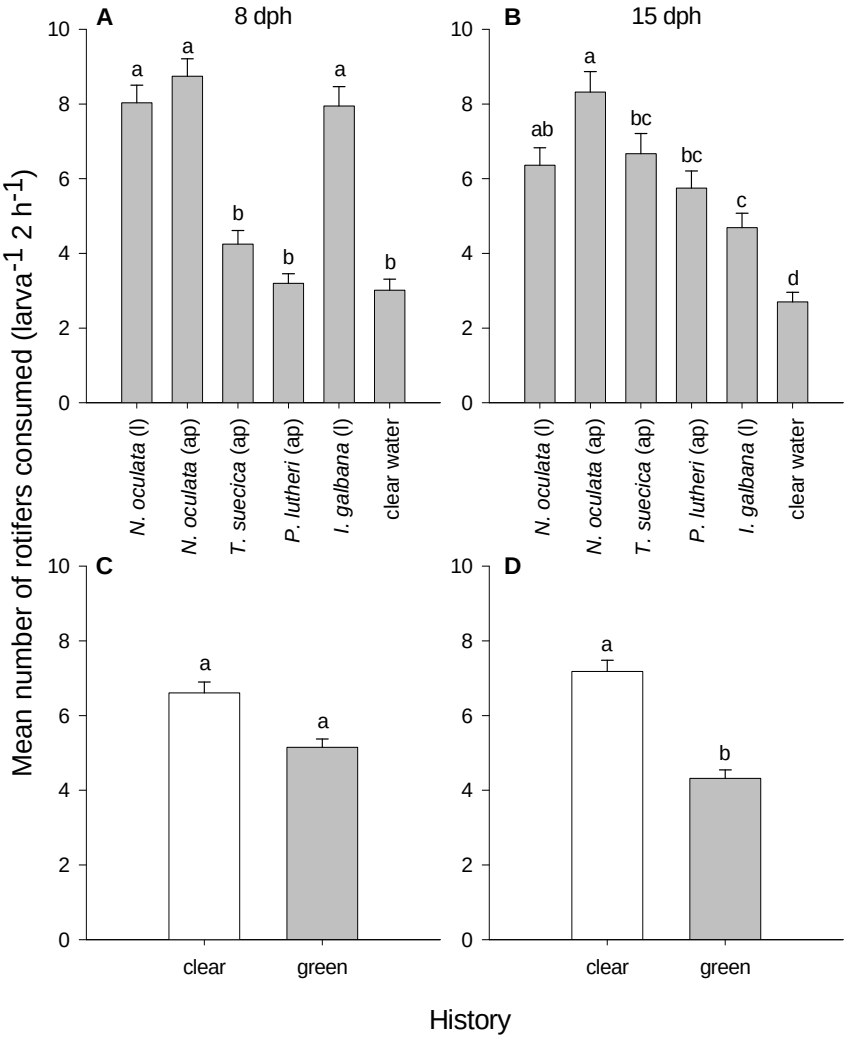

Figure 12.6 Experiment 6. Larvae 8 (A, C) and 15 (B, D) dph reared in either green or clear water culture tanks were subsequently fed rotifers at a density of 1 ml^{-1} in 3-l aquaria. Aquaria contained green water produced with a range of algal species all at a turbidity of 5 Nephelometric Turbidity Units - NTU. Some algal species were live (l) and some were resuspended algal pastes (ap). A, B) Mean (± SE, n = 20) rotifer intake in each algal species. C, D) Mean (± SE, n = 60) rotifer intake for larvae from a clear or green water environment. Means sharing common superscripts are not significantly different (P > 0.05).

12.6 Discussion

In the current study, the effect of aquarium colour on prey intake was far stronger than the effect of either prey or larval density. Tank colour has been found to significantly affect the growth of larvae, with a dark background better for walleye (Corazza and Nickum, 1985), dover sole (Dendrinos et al., 1984), dolphin fish (Ostrowski, 1989), striped bass (Martin-

Robichaud and Peterson, 1998) and yellow perch larvae (Hinshaw, 1985), while a light background was better for gilthead sea bream (Chatain and Ounais-Guschemann, 1991), eurasian perch (Tamazouzt et al., 2000) and haddock larvae (Downing and Litvak, 1999). Differences in feeding and development in different tank colours has also been seen for larval striped bass, *Morone saxatilis*, where swim bladder inflation success was higher in black compared with clear tanks, and larvae were more likely to be feeding in black compared with white tanks, irrespective of whether they had inflated swim bladders or not (Martin-Robichaud and Peterson, 1998). Furthermore, there is evidence that the use of green water masks the effect of tank colour for grouper larvae, *Epinephelus suillus* (Duray, 1996). However, given the strong effect of tank colour in Experiment 3, where an algal cell induced turbidity level of 0.8 NTU was used, it seems that a higher level of turbidity would be needed to negate the effect of tank colour for striped trumpeter larvae. The species-specific differences in tank colour preference may be related to some species displaying a phototactic response to tank reflections resulting in "walling" and non-feeding (Corazza and Nickum, 1981). Better survival in dark-coloured tanks may also be due to insufficient contrast between prey and background in white tanks (Chatain and Ounais-Guschemann, 1991) or a result of black tanks better reproducing natural lighting conditions because light reflecting from tank walls and bottom is minimised (Naas et al., 1996). Background colour may have a significant effect on the physiological response to stress and therefore influence growth and survival (Rodrigues and Sumpter, 1984; Gilham and Baker, 1985; Rotllant et al., 2003). In the current study, both colour of culture tanks and the colour of the test aquaria into which larvae were transferred, significantly affected rotifer intake. When larvae were transferred to a new culture environment towards which they were unfamiliar, feeding rates declined. Larvae transferred to blue aquaria, the same colour as their prior culture environment, consumed 5 times as many rotifers and 60% more larvae fed in comparison to larvae transferred from blue tanks to black test aquaria. Both turbidity and background colour will affect the contrast between prey and background and therefore the ability of larvae to identify potential prey items.

Transfer of larvae to new culture tanks can stress larvae, resulting in decreased feeding rates in the short-term. In the current study, larvae had 30 min to acclimate to their new culture environment prior to prey addition. Comparison of feeding rates between transferred and un-transferred larvae did not reveal differences. For instance, larvae reared in blue culture tanks in Experiment 1 consumed 15 rotifers $2h^{-1}$ whereas larvae transferred from a blue tank to blue aquaria in Experiment 4 consumed 15.8 rotifers $2h^{-1}$. Thus, rather than transfer stress, it seems that predation success of striped trumpeter larvae was reduced following a change in tank colour and resultant change in contrast between prey and background. One possible explanation is that striped trumpeter larvae develop a search image for prey in specific environments. According to Tinbergen (1960) and Dawkins (1971) a search image is 'a perceptual change in the ability of a predator to detect familiar cryptic prey'. Typically development of a search image will result in a preference for the prey type fitting the developed search image. However, the presence of a search image can only be confirmed once other forms of learning have been excluded as possibilities (Dawkins, 1971). In the current experiments, there was no prey choice so learning to handle one specific prey type over another, actively exhibiting a preference or avoidance for a specific prey type independent of the larva's ability to see the different types, and learning specialised hunting techniques, can be excluded as possible learned components. Also, given that prior culture history experiments involved a change in tank size, water dynamics and prey distribution characteristics, the possibilities of learning to; look in a particular type of place, visit a particular place or alter search path strategies to increase the chances of encountering prey, are unlikely. By excluding each of the aforementioned learned possibilities, it is likely that

striped trumpeter larvae form a search image, which is specific to the light environment and background tank colour against which potential prey items are viewed. Quickly changing the background environment would therefore render the developed search image unusable.

Experiments which involved larvae feeding in either an environment to which they were experienced or unfamiliar, support the hypothesis that larval predation is affected by a change in the visual environment. Cobcroft et al. (2001) found that feeding by striped trumpeter larvae is most likely affected by their prior culture environment, with larvae performing better in an environment towards which they had experience. In the current study, larvae from the same cohort with and without experience of feeding in a green water environment were placed in increasing levels of turbid water (Experiment 5). Larvae reared in green water fed better in green water compared with larvae reared in clear water, and prey intake of green water reared larvae was reduced in clear water. However, larvae reared in clear water did not feed any better in clear water than larvae reared in green water. Thus, the ability of larvae to adjust to and subsequently feed in a new environment was dependent on the environment toward which larvae were experienced. Larvae from green water were larger and therefore may have been more capable of feeding than the smaller clear water reared larvae, regardless of environment. Improved growth and survival in green water is likely the result of a number of factors including improved nutrition (Moffatt, 1981; Reitan et al., 1993; Tamaru et al., 1994). However, given the short duration of prey intake experiments it is unlikely that longer-term effects of algal addition, such as improved nutrition, are influencing prey intake. Rather, increased feeding rates in turbid water may be the result of improved contrast between prey and background (Boehlert and Morgan, 1985; Miner and Stein, 1993) or chemical stimulation of feeding (Lazo et al., 2000). Furthermore, behaviour responses of larvae are elicited by chemicals isolated from prey species that the larvae have experience of but not from chemicals isolated from prey species to which larvae are unfamiliar (Dempsey, 1978). Similarly, green water may elicit a feeding response for experienced larvae. Improved feeding by green water reared larvae is in agreement with larvae from Experiment 1, but not Experiment 6. It seems that green water larvae from Experiment 6 were compromised in some manner as in all previous trials with striped trumpeter larvae and in identical trials with greenback flounder (Shaw et al., 2006, Chapter 2) larvae with experience outperformed those without.

Not only did prior experience of green water affect subsequent feeding but prior tank colour also affected subsequent feeding. Larvae reared in black tanks fed better when transferred to blue aquaria than did larvae reared in blue tanks transferred to black aquaria. Furthermore, larvae reared in green water (*N. oculata*) did not feed well when transferred to different species of algae even though turbidity levels remained the same. Larvae reared in clear water were much better at feeding in different species of algae, all of which they were previously unfamiliar with. Search image formation may limit the ability of larvae to quickly change feeding strategies following a change in visual environment. Given that even a change in algal species was sufficient to decrease prey intake, it seems that any developed search image is highly specific. A highly specific search image is consistent with the assumption that a search image will be adopted only under conditions of high encounter and discarded when encounter rates become low or very high (Tinbergen, 1960; Dawkins, 1971; Krebs, 1973). As such it follows that larvae that have a well developed search image will adapt less quickly to a visual environment change than larvae which either have a less developed, or are not using a search image, in there predation sequence. Following this line of reasoning, I suggest that striped trumpeter have a more strongly developed search image when feeding in green water or blue tanks in comparison to larvae reared in clear water or black tanks. It also suggests that larvae cannot quickly (within minutes) change their search strategy to

accommodate a new environment. Over the longer term striped trumpeter are capable of successfully negotiating a permanent change in environment. Larvae that were initially fed in blue tanks had significantly reduced feeding once transferred to a black rearing tank; however, prey intake and growth of larvae resumed after several days (unpublished data).

All cohorts of striped trumpeter larvae reared in green water were larger than larvae reared in clear water after 6 dph. Similarly, growth and survival of many larval species is improved in turbid environments induced by algal cell addition or suspended inorganic particles (Boehlert and Morgan, 1985; Naas et al., 1992; Reitan et al., 1993; Lazo et al., 2000; Cobcroft et al., 2001), including striped trumpeter (Shaw, 2006, Chapter 6). Improved growth and survival in green water has been attributed to a number of factors including; improved nutrition and health of rotifers (Reitan et al., 1993; Tamaru et al., 1994), direct nutritional input from algal cells (Moffatt, 1981; Vasquez-Yeomans et al., 1990), improved distribution of larvae or rotifers in tanks (Vandenbyllaardt et al., 1991; Bristow et al., 1996; Rieger and Summerfelt, 1997), and via reductions in cannibalism (Hecht and Pienaar, 1993). Differences in the size of larvae used in short duration feeding experiments could confound the effects of the experimental treatments if there is a significant correlation between increasing larval length and prey intake. In the current study, striped trumpeter larvae do not show large increases in prey intake with age once larvae have successfully transferred from endogenous to exogenous prey (Figure 12.1 and Figure 12.6). Furthermore, the magnitude of the length increase from 8 to 15 dph in Experiment 6 is far greater than the difference in length between larvae of the same age reared in different culture environments. Therefore, it is unlikely that the results of short duration feeding experiments are affected by differences in larval size arising from prior culture conditions.

Differences in short-term feeding rates between larval groups can be obscured if larvae have time to reach satiation. In the current study, prey intake by larvae 9 and 11 dph during successive 30 min feeding intervals did not decline during the 2 h feeding period, suggesting that they had not reached satiation. However, older larvae 15 dph, showed a decline in feeding rate after 108 minutes of feeding. The decline in feeding rate observed for larvae 15 dph but not for younger larvae is reflective of improved feeding capacity resulting from increased locomotor ability (Wanzenbock and Schiemer, 1989) and sensory capacity (Pankhurst, 1994; Cobcroft and Pankhurst, 2003) which occurs with age. Experiments 1 and 2 used a high prey density of 10 rotifers ml^{-1} so it is reasonable to expect that rotifer intake in all subsequent experiments, where larvae were fed at lower prey densities and time periods of 2 h or less, was not constrained by capacity of the larval digestive tract.

The number of rotifers consumed by striped trumpeter larvae 9 dph increased as prey density increased within the range of 1.25 – 10 rotifers ml^{-1}. However, the relationship across the range of prey densities tested was relatively weak, indicating that striped trumpeter larvae are capable of feeding even at the lowest prey density tested. Similarly, greenback flounder, *Rhombosolea tapirina*, larvae, had increased short-term rotifer intake with increasing rotifer density up to a density of 0.1 rotifers ml^{-1}. At densities above 0.1 rotifers ml^{-1} prey intake in greenback flounder did not increase (Shaw et al., 2006). Growth and survival of other marine larval species has also been reported to increase with increasing prey density (Parra and Yuféra, 2000) until an asymptote is reached (Rabe and Brown, 2000). Further increases in prey density above the asymptote likely has detrimental effects resulting from increases in bacteria proliferation (Houde, 1975) and increased speeds of food passage through the digestive system (Werner and Blaxter, 1980). The level at which prey intake shifts from being density-dependent to density-independent varies between fish species (Houde and Schekter, 1980) and is reliant on prey type and culture conditions (Dou et al., 2000; Morales-

Ventura et al., 2004; Shaw et al., 2006). At prey densities above the density-dependent threshold, differences in growth can still be observed owing to changes in energy expenditure per consumed prey item and prey assimilation efficiency (Parra and Yuféra, 2000). For instance, the attack rate of cod increased and the time spent swimming decreased with increasing prey density (Munk, 1995). Thus differences in growth can be observed despite similar rates of rotifer intake.

Decreasing the density of larvae within culture tanks has the effect of increasing the absolute number of prey available per larva and has resulted in increases in growth and survival of larvae (El-Sayed, 2002). However, larval densities of 1.25 – 40 larvae l^{-1} in Experiment 2 or 5, and 20 larvae l^{-1} in Experiment 3, had no effect on rotifer intake during the first 144 min post-rotifer addition. At a density of 40 larvae l^{-1} the number of rotifers available per larva was 250 rotifers day^{-1}. Assuming that larvae can feed throughout the light period i.e., 16 h, and the maximum consumption in any experiment was approximately 8 rotifers h^{-1}, then a maximum of 128 rotifers $larvae^{-1}$ day^{-1} would be consumed. Such a feeding rate would result in a density of 4.88 rotifers ml^{-1} remaining at the end of the photophase. Thus, even at the highest larval density tested, rotifer density did not appear to limit consumption. However, although larval density had no affect on prey intake, the growth of striped trumpeter larvae was affected by larval density. Larvae reared at a density of 1.25 and 2.5 larvae l^{-1} for 15 days were larger and had higher rates of swim bladder inflation 87.5 ± 10 % compared to larvae reared at 10, 20 and 40 larvae l^{-1} (Battaglene et al., 2006 in review). Thus larval density can have an effect on growth that is independent of prey intake. Poor growth at higher larval densities may result from an increase in the energy expenditure per consumed rotifer, or behavioural interactions among larvae. At high larval densities it is possible that larval predation sequences are more often interrupted, thus, decreasing predation success. Similarly, when prey densities are very high larvae can become confused with too many prey items in the visual field (Laurel et al., 2001; Temple et al., 2004).

In conclusion, prey intake by striped trumpeter larvae is strongly influenced by the background against which potential prey items are viewed and by experience of a specific culture environment. In green water or blue tanks it is likely that larvae formed a highly specific ‘search image’. Formation of a search image resulted in poor feeding once larvae were transferred to a new visual environment, indicating that the search strategy did not change quickly. Prey intake was very similar across the range of prey and larval densities tested. Differences in growth, despite similar prey intake, likely results from differences in energetic processes associated with feeding at different prey and larval densities and in environments which provide differing degrees of contrast between prey and background.

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13 EFFECTS OF TURBULENCE AND TURBIDITY ON GROWTH, SURVIVAL AND ROTIFER INTAKE OF STRIPED TRUMPETER, *LATRIS LINEATA*, LARVAE

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13.1 Abstract

Both turbulence and turbidity can influence predator-prey interactions and therefore have longer-term affects on growth and survival of larvae. Much research has concentrated on the effects of turbulence and turbidity on short-term processes such as foraging behaviour, or long-term effects on growth and survival. Few studies have investigated simultaneously short and long-term effects or the interaction of turbulence and turbidity on larval performance in culture. Thus, the effects of turbulence induced by aeration and turbidity induced by algal cell addition, on growth, survival and rotifer intake of striped trumpeter, *Latris lineata*, larvae were investigated. Experiments were conducted in replicate 300 l black hemispherical tanks with turbulence created via aeration from the centre bottom of each tank. Turbulence levels higher than 200 ml min^{-1} aeration resulted in significantly reduced growth, survival and rotifer intake of larvae from first feeding to 14 days-post hatching (dph), and no larvae survived under static conditions after 12 days. Prey intake increased in larvae 10 - 14 dph in comparison with larvae 6 and 8 dph and prey intake in older larvae 14 dph increased at the higher turbulence level of 800 ml min^{-1} in comparison to younger larvae. Furthermore, larvae 10 dph were more capable of coping with an increased aeration level of 400 ml min^{-1} than larvae receiving aeration at 400 ml min^{-1} from 6 dph. There was no interaction between turbulence and turbidity on growth, survival or prey intake. Larvae reared in turbid (green water) out performed those in clear water. Improvement in growth, survival and feed intake was evident for larvae reared in green water provided by either live algal cells, or concentrated algal paste of *Nannochloropsis oculata*. Green water reared larvae, despite being of the same size 10 dph as clear water reared larvae, were better equipped to cope with an increase in aeration from 200 to 400 ml min^{-1} than larvae reared in clear water. Combining the best level of aeration, 200 ml min^{-1}, with green water, resulted in the highest growth and survival of striped trumpeter achieved to date. It remains to be tested whether a turbulence level below 200 ml min^{-1} would provide for further improvement to growth and survival. Further investigation into increasing turbulence with age to gain maximal growth rates requires more attention primarily in regard to timing and strength of turbulent increases.

Key Words: Aeration, green water, feeding, striped trumpeter, turbulence, turbidity

13.2 Introduction

Most marine larvae including striped trumpeter, *Latris lineata*, are primarily dependant on vision for successful feeding during early larval development (Houde and Schekter, 1980; Blaxter, 1986; Pankhurst, 1994; Cobcroft and Pankhurst, 2003). However, visual acuity during this early larval period is very under developed, resulting from a small retina and resultant small lens and short focal length (Kotrschal *et al.*, 1990; Pankhurst, 1994). Thus, in order for larvae to successfully shift from endogenous nutrient reserves to exogenous prey, factors critical to a successful predator-prey interaction must be optimal. Critical factors include prey type, density and size (Werner, 1974; O'Brien, 1979; Cobcroft *et al.*, 2001); however, commonly other factors such as light (Blaxter, 1986; Mills *et al.*, 1986), turbidity (Cobcroft *et al.*, 2001) and turbulence (Rothschild and Osborn, 1988) play an important role.

Water turbulence increases the relative velocities between predator and prey and therefore increases encounter rates (Rothschild and Osborn, 1988; MacKenzie and Kiorboe, 1995). However, prey encounter is only the first component of a typical predation sequence (encounter, pursuit, attack and capture) and although turbulence increases encounter rates, it can have negative effects on pursuit, attack and capture success (MacKenzie et al., 1994; MacKenzie and Kiorboe, 2000). Notionally, there is a point at which the benefits of increased encounter rate are offset by decreased pursuit and capture success. Theoretical studies have predicted a dome-shaped relationship between turbulence and larval fish ingestion rates (MacKenzie *et al.*, 1994), with the level of turbulence that results in maximum ingestion rates increasing with larval length, primarily as a result of size-related improvement in visual range and swimming ability (Fiksen *et al.*, 1998). Finding a dome-shaped relationship between feed intake and turbulence in field or laboratory studies has proven more difficult (MacKenzie, 2000) with studies showing both positive (Sundby and Fossum, 1990; Dower et al., 1998) and negative (Lough and Mountain, 1996) responses, possibly the result of experimental methodologies not providing fine enough resolution (MacKenzie, 2000). Furthermore, most studies have investigated the effects of turbulence on short term processes such as encounter rates, pursuit success, or prey intake, while few studies (Barahona-Fernandes, 1978; Battaglene and Talbot, 1993; Ellis et al., 1997) have investigated the effect of turbulence on longer term growth and survival of larvae. Studies that have investigated the interaction between turbulence and light intensity (Utne and Stiansen, 2002), or turbulence and turbidity (Utne-Palm, 2004), have looked at effects only over small time frames. For instance, the effect of turbulence and turbidity on prey attack rate and swimming activity of Atlantic herring was investigated (Utne-Palm, 2004); however, no indication of the long term effects on growth and survival are available. The effects of turbulence on the short term processes of encounter, pursuit and capture success, may well be different to the effect of turbulence on longer term growth and survival (MacKenzie, 2000). To our knowledge, only Toledo et al. (2002) has investigated simultaneously the effects of turbulence on both short term processes i.e., feed intake, and longer term outcomes i.e., growth and survival of larvae, and in this case growth was only assessed 2 days post-hatching (dph).

Turbidity (often green water) has been shown to improve prey consumption, growth and survival of marine larvae [e.g. Pacific herring, *Clupea harengus pallasi* (Boehlert and Morgan, 1985), Atlantic halibut, *Hippoglossus hippoglossus* (*Naas et al.*, 1992), turbot, *Scophthalmus maximus* (Reitan *et al.*, 1993), cobia, *Rachycentron canadum*, (Faulk and Holt, 2005) and striped mullet, *Mugil cephalus* (Tamaru et al., 1994)]. Algal-induced turbidity also significantly increased the percentage of striped trumpeter larvae feeding on *Artemia* (Cobcroft et al., 2001). However, turbidity is not beneficial to all species of larvae and typically has negative effects on juveniles and adult fishes (Vinyard and O'Brien, 1976;

Barrett et al., 1992; Utne, 1997). Furthermore, the amount of turbidity is important with some larvae showing negative effects at high turbidity (Boehlert and Morgan, 1985). Thus, the beneficial effects of turbidity are often only evident at relatively low turbidities during the larval phase. For example, greenback flounder, *Rhombosolea tapirina*, larvae, showed improved prey consumption in algal cell induced turbid conditions when feeding on rotifers, but not when feeding on *Artemia* (Shaw et al., 2005 in press).

Turbidity within a culture situation is typically provided by live algal cells; however, suspended sediment and clay particles have also been used (Vinyard and O'Brien, 1976; Boehlert and Morgan, 1985; Reitan et al., 1993; Tamaru et al., 1994; Utne, 1997; Lazo et al., 2000). More recently highly concentrated algal pastes have become commercially available. These commercial products offer an inexpensive method of green water culture; however, there are key differences between live algal cells and concentrated dead cells. Differences include, bacterial diversity, stability in the water column, dropout of cells in low turbulent environments and differences in light scattering resulting from different sized and shapes of cells even when of the same species (personal observation). These differences have the potential to affect the foraging behavior, growth and survival of larvae. A comparison between live and concentrated algal cells provides an opportunity to separate out some of the confounding factors in the use of green water and determine if turbidity is a major factor influencing overall larval performance.

Striped trumpeter are a candidate aquaculture species currently under investigation at the Marine Research Laboratories, University of Tasmania (Pankhurst and Hilder, 1998; Cobcroft and Pankhurst, 2003; Morehead and Hart, 2003). Larval rearing has been highly variable and, unexplained, episodic mortality remains a problem during early larval rearing despite a better understanding of nutritional requirements (Bransden et al., 2004; Bransden et al., 2005a; Battaglene et al., 2005 in press). Turbidity has been shown to improve *Artemia* consumption in short duration experiments during early larval rearing (Cobcroft *et al.*, 2001) but the longer term effects are unknown. Furthermore, the effects of turbulence and the interaction of turbulence and turbidity on growth, survival and development of cultured striped trumpeter larvae have not previously been investigated. The aims of the study were to: 1) investigate the effects of a range of turbulence levels during early larval rearing and 2) investigate the effects of various combinations of turbulence and turbidity on growth, survival and prey intake of striped trumpeter larvae.

13.3 Materials and methods

13.3.1 *Larval rearing*

Eggs and sperm were hand stripped from striped trumpeter broodstock held at the Marine Research Laboratories, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Hobart, under controlled conditions and fed a mixed diet including formulated feeds, fresh fish and squid as described by Bransden et al ., (2007). A single but different female provided the eggs for each experiment with sperm from four (Experiment 1) and three (Experiment 2) males used to fertilise eggs. The fetilisation rates were 96% and 99% for Experiment 1 and 2, respectively. Eggs were disinfected with ozone at 1 ppm and incubated in a 250 l upwelling incubator at 14.6 ± 0.1 ºC (mean ± se) and 14.2 ± 0.1 ºC and a salinity ranging between 33.58 to 34.18 and 33.90 to 34.84 in Experiments 1 and 2, respectively. Eggs hatched 5 days post-fertilisation at which point the temperature was increased 0.5 ºC per 12 h, reaching 16ºC prior to transfer to the experiment system.

Larvae were transferred 4 and 2 dph in Experiments 1 and 2 respectively, to the experiment system consisting of 24 black hemispherical tanks each containing 300 l of water. Each tank was stocked at a density of 5 larvae l^{-1} and the entire system was maintained under static conditions (no air and no water flow) until first feeding at 6 dph. Thereafter, a 390 µm central outlet screen was fitted, through which water, rotifers and algae could pass when water flow was turned on. Water temperature was maintained at 16.2 ± 0.1 ºC (mean ± se, n = 288) and 15.7 ± 0.1 ºC (n = 310) and salinity 34.4 ± 0.1 and 34.5 ± 0.1, during Experiments 1 and 2, respectively. Oxygen saturation remained above 89% during both experiments. Photoperiod was maintained at 16h : 8h, L:D with lights fading on at 900 h. Mean light intensity at the water surface was 4.3 ± 0.2 µmol s^{-1} m^{-2}. Temperature, light and other early larval rearing conditions follow current best hatchery practices and earlier studies (Cobcroft et al., 2001; Trotter et al., 2003a; Trotter et al., 2003b; Bransden et al., 2004).

Larvae in both experiments were fed rotifers (*Brachionus plicatilis*) at a density of 5 rotifers ml^{-1}, once per day, at approximately 0920 h. Rotifers were enriched with AlgaMac 2000 (Aquafauna Bio-Marine, Inc.) for 12 h at 23 ºC. Immediately prior to the addition of rotifers, the water flow was turned off and the central outlet screen removed and replaced with a rubber stopper that contained an air line (Figure 13.1). This process was reversed at 0100 h (lights off), providing 8 h during the dark phase in which rotifers and algae were flushed from each tank at a water flow rate of 112.5 l h^{-1} (3 exchanges in 8 h). Surface skimmers were added to tanks from 8 to 13 dph to remove surface films (Trotter et al., 2005).

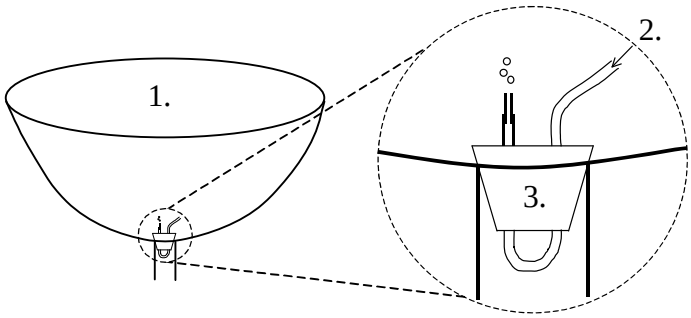

Figure 13.1 Air delivery system used in both Experiments; 1.) 300 l black hemispherical tank, 2.) 4 mm air line, 3.) Rubber stopper placed into central drain through which air was delivered during the photophase.

13.3.2 *Experiment 1*

Experiment 1 examined the effect of turbulence (aeration) on growth, survival, condition and feed intake of striped trumpeter larvae from first feeding (6 dph) to 14 dph, encompassing the swim bladder inflation window (Trotter et al., 2005) in clear water. From 6 dph each tank had aeration provided at one of 6 levels, static (no air), 200 ml min^{-1}, 400 ml min^{-1}, 800 ml min^{-1}, 1600 ml min^{-1}, and 3200 ml min^{-1}. Aeration was measured by placing a water filled inverted volumetric cylinder over the central bubble stream and timing how long it took to displace the 200 ml of water. Aeration was only provided during the photophase when there was no water flow. There were four tanks per treatment. Feed intake was assessed 6, 8, 10, 12 and 14 dph. Prior to lights going on, 20 larvae were removed from each tank (time zero) to check that the digestive tract was free of prey. At 0920 h (lights fully on), rotifers were added to two tanks at a time, with two minutes between the addition of rotifers to each successive pair of tanks. Staggering the addition of prey to each tank allowed time for siphoning 24 larvae from each tank immediately following the two-hour feeding period and

thus ensured that larvae in each tank had the same feeding period. All sampled larvae were anaesthetised in 2-phenoxyethanol and then fixed in 10% neutral buffered formalin. Preserved larvae were placed onto a glass slide and individually measured using a eye piece graticule fitted to a dissecting microscope, assessed for swim bladder inflation and larval condition. Assessment of larval condition was made by assigning larvae a rating from 1 to 3. A larva with a rating of 3 being a normal looking larva, whereas a ranking of 1 indicated poor larval condition including: pulmonary oedema, jaw or skeletal deformity, and/or unabsorbed yolk sac and oil droplets. Larvae receiving a condition score of 2 contained one of the above conditions at a low level of severity. After larval condition was assessed a cover slip was then placed over the larva and the number of rotifers in the stomach counted. Anaesthesia of striped trumpeter larvae does not result in gut evacuation, as evidenced by the retention in the mid-hindgut of the remnants of prey (rotifer mastax and trophy) ingested on the previous day. However, fixation of larvae using formalin results in the loss over time (days) of gas from inflated swim bladders (Trotter, 2003). Thus, swim bladder inflation was assessed using freshly (same day) anaesthetised and fixed larvae to avoid the problem of swim bladder deflation. Each tank was drained and larvae collected on a net 14 dph to estimate final survival. Survival estimates were adjusted to account for larvae sampled from tanks during experiments. Larval viability (V) at the end of the experiment was calculated using: $V = S \times SBI$ (where S is the percentage of larvae surviving and SBI is the percentage of larvae with an inflated swim bladder (Battaglene and Talbot, 1993).

13.3.3 *Experiment 2*

Experiment 2 assessed the effect of algal-induced turbidity and turbulence on growth, survival, feed intake and health of striped trumpeter larvae from 6 to 16 dph. There were three turbidity treatments: clear water (without algal cell addition) at a nephalophidic turbidity unit (NTU) of <0.01, and two green water turbidity treatments, one produced using live *Nannochloropsis oculata* at a turbidity of 3 NTU and the other using concentrated *N. oculata* algal paste (Reed Mariculture Inc., California) re-suspended at 3 NTU. Evidence from Experiment 1 indicated that prey consumption rates at higher turbulence levels improved as larvae developed, thus each turbidity treatment was run with one of two aeration levels; either 200 ml min^{-1} for the duration of the experiment, or 200 ml min^{-1} from 6 dph increasing to 400 ml min^{-1} at 10 dph and for the duration of the experiment. There were 4 replicate tanks of each treatment. Aeration and water flow was set up in the same manner as Experiment 1 with turbulence during the photophase and water flow during the scotophase. Each tank had a 70 l reservoir into which the required amount of algae, algal paste or filtered water (clear water) was added daily. All 70 l tanks were filled at approximately 1200 h for addition to experiment tanks the following morning at 0845 h, thus allowing enough time for equilibration to the temperature of the culture tanks. The amount of algae and algae paste required to achieve a turbidity of 3 NTU in each experiment tank was assessed daily using a HACH 2100 (Hach Company, Loveland, Colorado, USA) portable turbidity meter. Feeding rates were assessed 8, 10, 12, 14 and 16 dph as per Experiment 1. The incidence of larvae swimming into tank edges, known as “wall-nosing” (Bristow and Summerfelt, 1994), hereon termed “walling”, was assessed by ranking its severity from 1 to 4 using the criteria: 1.) No walling, 2.) < 10% of larvae walling, 3.) from 10 to 50 % of larvae walling and 4.) > 50% of larvae walling. Walling was assessed 9, 11, 13 and 15 dph at 1100 h. Standard length, larval condition, and swim bladder inflation were assessed on 20 freshly anaesthetised larvae from each tank 16 dph as per Experiment 1. In addition, larvae can have a condition of the gut, termed “grey gut syndrome” which was assessed 16 dph. Grey gut syndrome can be observed in whole larvae as the loss of transparency and discolouration of the central to posterior area of the mid gut and likely

results from either; extensive vacuolation of enterocytes or bacterial enteritis in the walls of the intestine (Bransden et al., 2005b). The presence or absence of grey gut was recorded for each larva sampled. Survival and viability was assessed 16 dph as per Experiment 1.

13.3.4 *Statistical analysis*

Statistical analyses were conducted using SPSS version 10.0. All data were evaluated for homogeneity of variance using Levene's Test and residual plots; where necessary data were log transformed. All percentage data was arcsin√ transformed prior to analysis. Differences in length, survival, condition, viability, swim bladder inflation, grey gut syndrome and walling behaviour were assessed from individual tank means using a one factor (turbulence) ANOVA for Experiment 1, or two factor (turbulence, turbidity) ANOVA for Experiment 2. Feed intake rates and the percentage of larvae feeding were assessed using a 2 factor (turbulence, age) ANOVA (Experiment 1), or using a 3 factor (turbidity, turbulence, age) ANOVA (Experiment 2) with tank nested in both analyses. Due to high mortality in the static treatment from Experiment 1, data from this treatment were excluded from feeding rates, condition and length analyses. Where significant effects were found, a Tukey's post-hoc test was performed with significance assessed at the $P = 0.05$ level.

13.4 Results

13.4.1 *Experiment 1*

No larvae survived to 14 dph in static tanks. Growth and survival of larvae in the remaining treatments increased with decreasing turbulence (Figure 13.2 & Figure 13.3). At 14 dph larvae reared at the lowest aeration level, 200 ml min^{-1}, were significantly larger, 6.60 ± 0.03 mm (n = 119), than those in all other treatments (Figure 13.2b; $F = 19.168$; d.f. 4, 15; $P < 0.001$). Mean survival 14 dph was also highest, 30.5 ± 3.4% (n = 4), at an aeration level of 200 ml min^{-1} and significantly different to survival in 1600 and 3200 ml min^{-1} treatments (Figure 13.3; $F = 9.030$; d.f. 5, 18; $P < 0.001$). Length measures of fixed larvae were on average 3.5% shorter than length measures of fresh anaesthetised larvae of the same age (data not shown).

Swim bladder inflation in larvae 14 dph was very low in all treatments, with a maximum of 11.3 ± 4.1 % in the 400 ml min^{-1} treatment. Mean swim bladder inflation was below 5% in all other treatments (Figure 13.3). Larval viability reflected the low level of SBI with less than 2.5% of all larvae viable. The proportion of larvae 14 dph that received a condition score of three was not affected by aeration level ($F = 0.362$; d.f. 4,15; $P = 0.832$) with 86.5 ± 5.2 % of larvae rated a three and in good condition (Figure 13.3).

Prey intake was significantly affected by age ($F = 318.215$; d.f. 4,100; $P < 0.001$) and turbulence ($F = 5.777$; d.f. 4,106; $P < 0.001$) with no significant interaction between age and turbulence ($F = 1.414$; d.f. 16, 84; $P = 0.155$). First-feeding intake 6 dph was significantly lower, with maximum consumption of 0.16 ± 0.05 rotifers $2h^{-1}$, compared with any other sampling day. Larvae 8 dph consumed more prey than larvae 6 dph but consumption was significantly lower than consumption on 10, 12 and 14 dph. There was no difference in mean prey intake over the period 10 –14 dph (Figure 13.4). Maximum consumption occurred at an aeration of 200 and 400 ml min^{-1} with a significant decrease in prey intake evident at 1600 and 3200 ml min^{-1}. The highest feeding rates occurred at 400 ml min^{-1} in larvae 6 dph, at 200 ml min^{-1} in larvae 8, 10 and 12 dph and at 800 ml min^{-1} in larvae 14 dph.

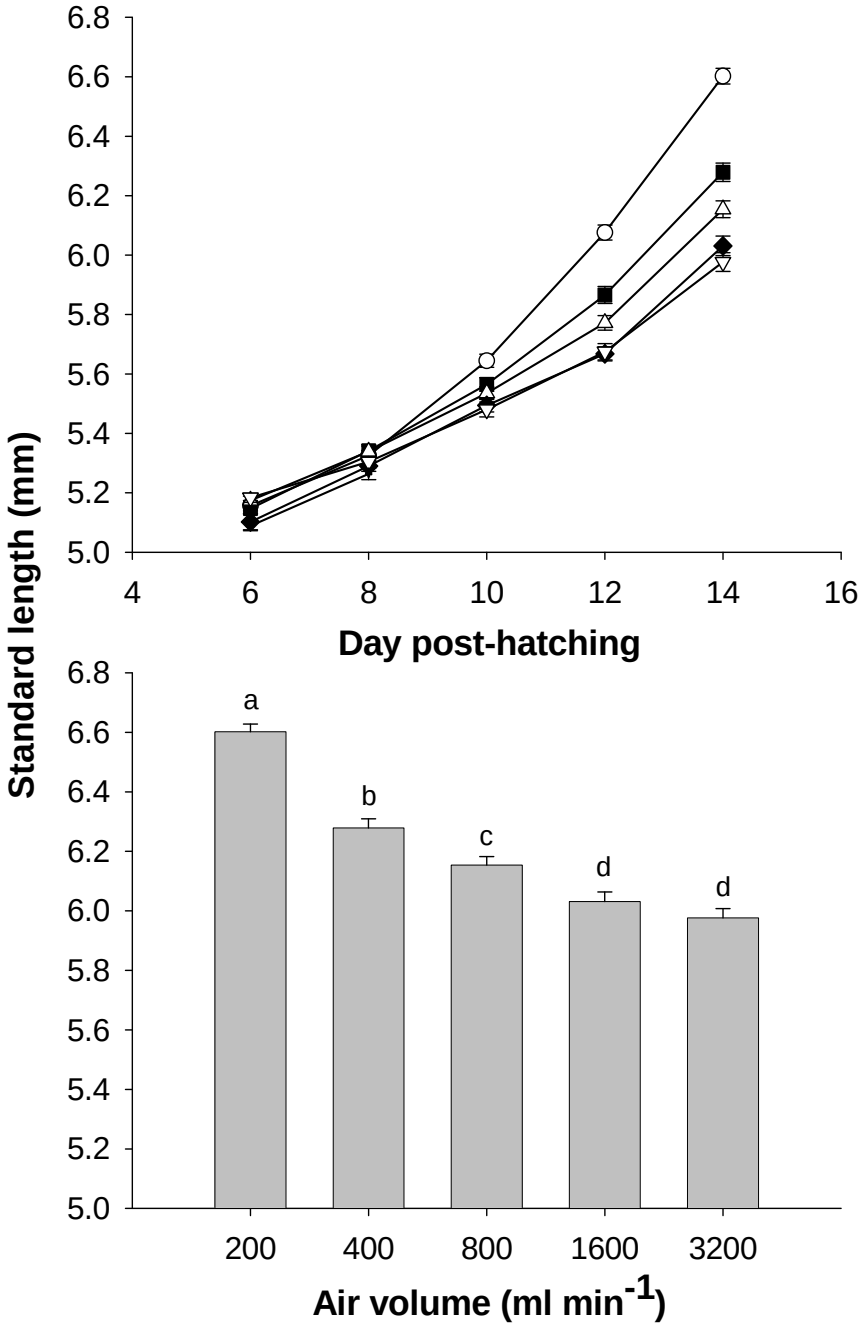

Figure 13.2 A) Growth of larvae in each turbulence treatment of Experiment 1; 200 (○), 400 (■), 800 (△), 1600 (◆) and 3200 (▽) ml min^{-1}. No larvae survived to 14 dph in static tanks. Points are mean ± SE. The number of larvae sampled varied due to decreasing survival with age and treatment but n > 44 in all cases. B) Final mean standard length (± SE) of larvae 14 dph from Experiment 1. Bars sharing common superscripts are not significantly different.

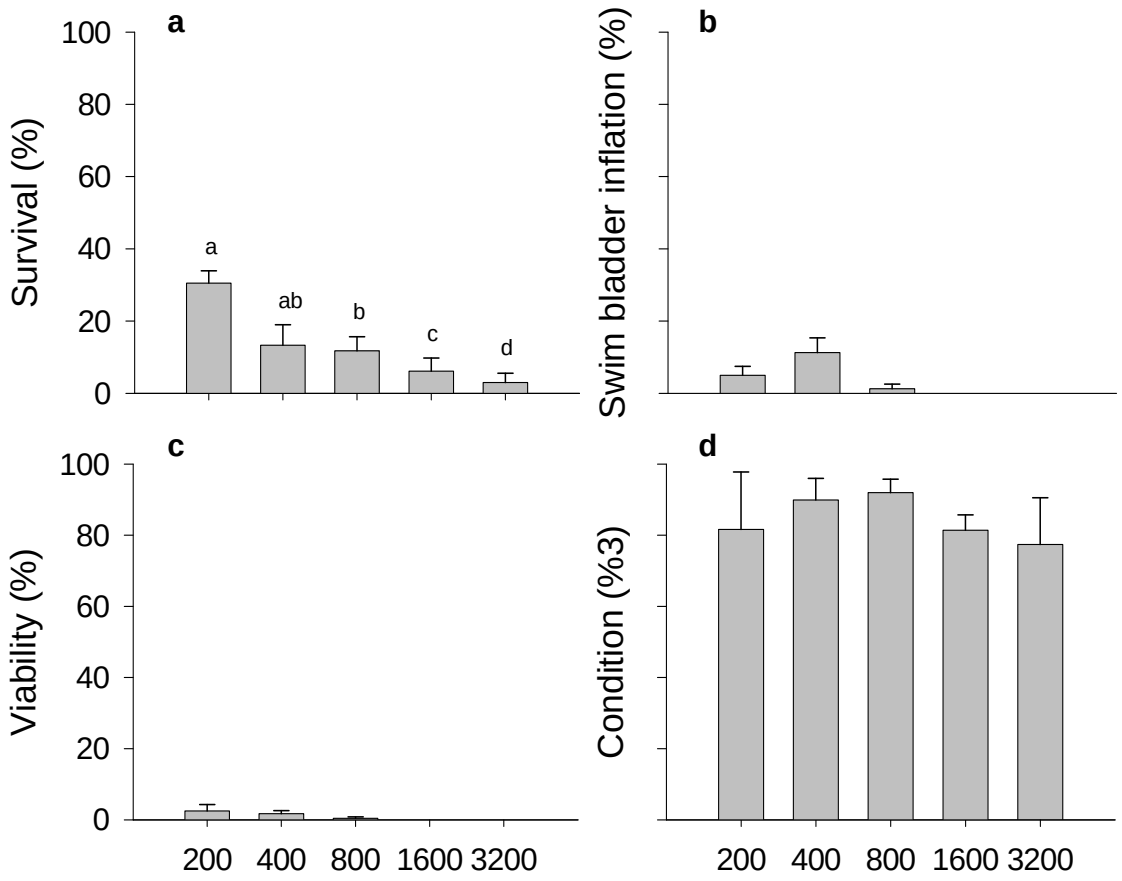

Figure 13.3 Mean a) survival, b) swim bladder inflation, c) viability and, d) percentage of larvae receiving a condition score of three (± SE, n = 4) in each treatment 14 dph from Experiment 1. Bars sharing common superscripts are not significantly different. Static treatment had zero survival after 12 dph.

The percentage of larvae feeding was significantly affected by age ($F = 179.787$; d.f. 4, 81; $P < 0.001$) and turbulence ($F = 2.819$; d.f. 5, 81; $P = 0.021$) but not the interaction between age and turbulence ($F = 0.962$; d.f. 18, 81; $P = 0.511$). However, turbulence affected the percentage of larvae feeding only when data from 6 dph were included, because a low 6.2 ± 1.8% of larvae fed. Excluding data from larvae 6 dph, greater than 79% of larvae fed at all aeration levels with a maximum of 94.8 ± 1.6% of larvae feeding 14 dph.

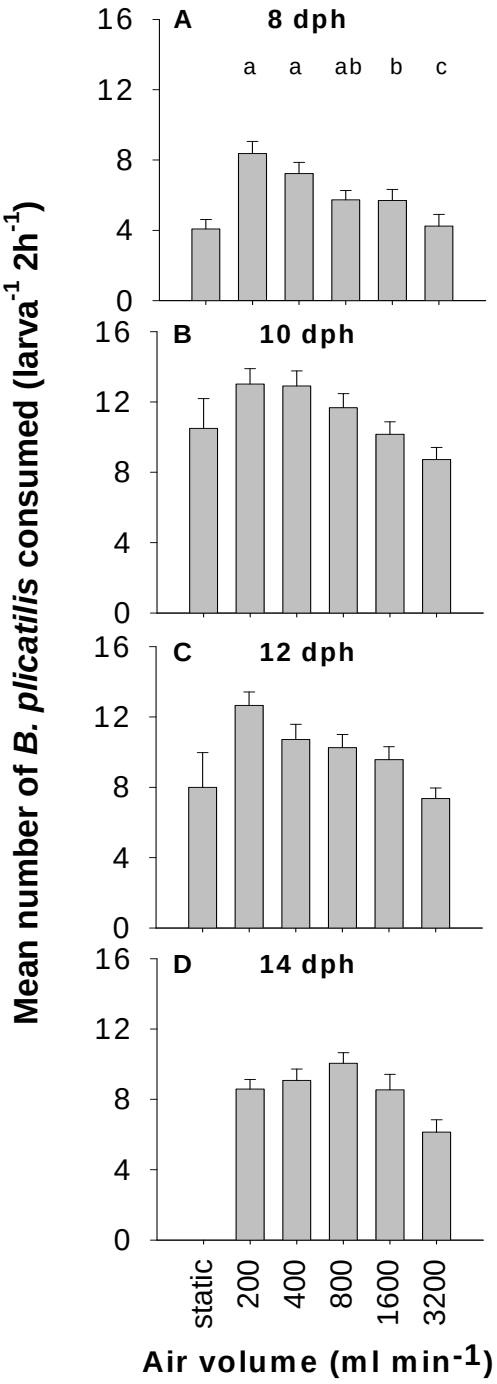

Figure 13.4 Mean (± SE) rotifer intake by larvae 8, 10, 12 and 14 dph from Experiment 1. The number of larvae sampled varied due to decreasing survival with age and treatment but n > 43 except for the static treatment where n ranged from 11 to 29. There was no significant interaction between turbulence and age, thus superscripts indicate significant differences in the main effect, turbidity, when data is grouped for age.

13.4.2 *Experiment 2*

Length of larvae 16 dph was significantly affected by turbidity ($F = 222.028$; d.f. 2,18; $P < 0.001$) and the interaction between turbidity and turbulence ($F = 5.738$; d.f. 2,18; $P < 0.001$) but not by turbulence ($F = 3.168$; d.f. 1,18; $P = 0.092$). Larvae reared in live algal cells were significantly larger in length than larvae reared in either clear water or algae paste,

irrespective of aeration rate. Larvae reared in algae paste were significantly larger than those reared in clear water (Figure 13.5).

Survival of larvae to 16 dph was significantly affected by turbidity but not by turbulence or the interaction between turbidity and turbulence (Table 13.1; Figure 13.6). Both algae paste and live algal treatments had significantly higher survival than clear water treatments (Figure 13.6a; Table 13.1). The best survival, 89.9 ± 5.2% (n = 4), occurred in live algae with low aeration.

Swim bladder inflation was significantly affected by turbidity but not turbulence or the interaction between turbidity and turbulence (Table 1; Figure 13.6). There were no differences in SBI rates between any green water treatments; the highest SBI rate was 96.25 ± 3.8% (n = 4) in live algal tanks; however, SBI in clear water treatments was significantly lower than SBI in any green water treatment (Table 13.1; Figure 13.6). Mean SBI in clear water tanks with high aeration was 53.8 ± 7.1% (n = 4). The combination of high survival and SBI rates in all green water treatments resulted in a significantly higher percentage of viable larvae (Table 13.1; Figure 13.6). Greater than 70% of larvae from any green water treatment were viable, whereas less than 30% of clear water reared larvae were viable at the end of the experiment. Viability was significantly affected by turbidity but not turbulence or the interaction between turbulence and turbidity (Table 13.1; Figure 13.6). The incidence of larvae walling was significantly affected by turbidity but not turbulence and there was no significant interaction effect (Table 13.1; Figure 13.6). During the period 9 to 15 dph, 10 to 50% of larvae were walling in clear water treatments whereas the mean incidence of walling in any green water treatment was below 10%.

The percentage of larvae with grey gut syndrome was significantly affected by turbidity but not turbulence or the interaction between turbidity and turbulence. The incidence of grey gut was higher in all green water treatments (37.5 – 63.8%) in comparison to clear water treatments (2.5 - 5.0%) (Table 13.1; Figure 13.6). There was a significant interaction between turbidity and turbulence on the condition rating of larvae (Table 13.1; Figure 13.6). However, post-hoc testing was unable to reveal any significant differences among treatments. The percentage of larvae 16 dph that received a condition rating of three was above 86% in all treatments.

Consumption of rotifers was significantly affected by age ($F = 46.811$; d.f. 4, 90; $P < 0.001$), turbidity ($F = 36.452$; d.f. 2, 90; $P < 0.001$), turbulence ($F = 5.436$; d.f. 1, 90; $P = 0.022$) and all two-way interactions (Figure 13.7). The consumption of rotifers increased after 8 dph but there was no trend for increasing consumption over the period 10 to 16 dph. Larvae reared in any green water treatment generally consumed more rotifers than larvae reared in either clear water treatment. Increasing turbulence decreased the rotifer consumption of larvae in clear water 10 and 16 dph, but had no effect on larvae in green water treatments. The percentage of fish feeding in each treatment on any day averaged 99.3 ± 0.3% (n = 120).

Table 13.1 The effect of turbidity and turbulence (two-way ANOVA) on survival, swim bladder inflation, viability, grey gut syndrome, walling and condition of larvae 16 dph from Experiment 2.

Effect	*df* effect	*df* error	*F* value	*P* value
Survival				
Turbidity	2	18	25.524	0.000*
Turbulence	1	18	0.000	0.986
Turbidity × Turbulence	2	18	2.115	0.150
Swim bladder inflation				
Turbidity	2	18	14.388	0.000*
Turbulence	1	18	1.828	0.193
Turbidity × Turbulence	2	18	0.210	0.813
Viability				
Turbidity	2	18	58.201	0.000*
Turbulence	1	18	0.801	0.382
Turbidity × Turbulence	2	18	1.413	0.269
Grey gut syndrome				
Turbidity	2	18	40.132	0.000*
Turbulence	1	18	2.977	0.102
Turbidity × Turbulence	2	18	0.382	0.724
Condition				
Turbidity	2	18	0.188	0.830
Turbulence	1	18	4.737	0.043*
Turbidity × Turbulence	2	18	5.012	0.019*
Walling				
Turbidity	2	18	241.378	0.000*
Turbulence	1	18	3.978	0.062
Turbidity × Turbulence	2	18	1.541	0.241

* Significant

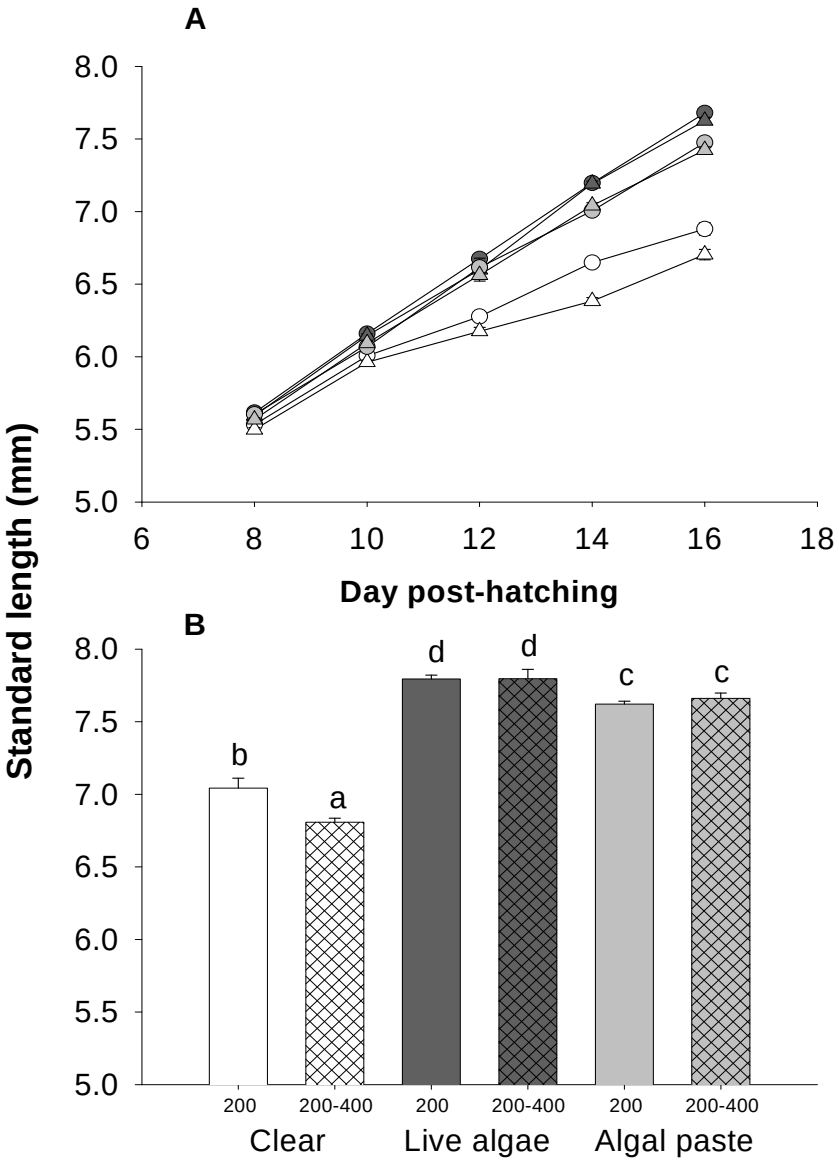

Figure 13.5 Larvae were reared in either clear water (clear symbols and bars), live algae (dark grey symbols and bars) or re-suspended algal paste (light grey symbols and bars) at an aeration level of either 200 ml min^{-1} (circles and solid bars) or at 200 ml min^{-1} increased to 400 ml min^{-1} 10 dph (triangles and cross-hatched bars). A) Growth of larvae from Experiment 2: Points are mean standard length ± SE (n = 80) of fixed larvae. B) Final mean standard length ± SE (n = 80) of fresh anaesthetised larvae 16 dph from Experiment 2. Bars sharing common superscripts are not significantly different.

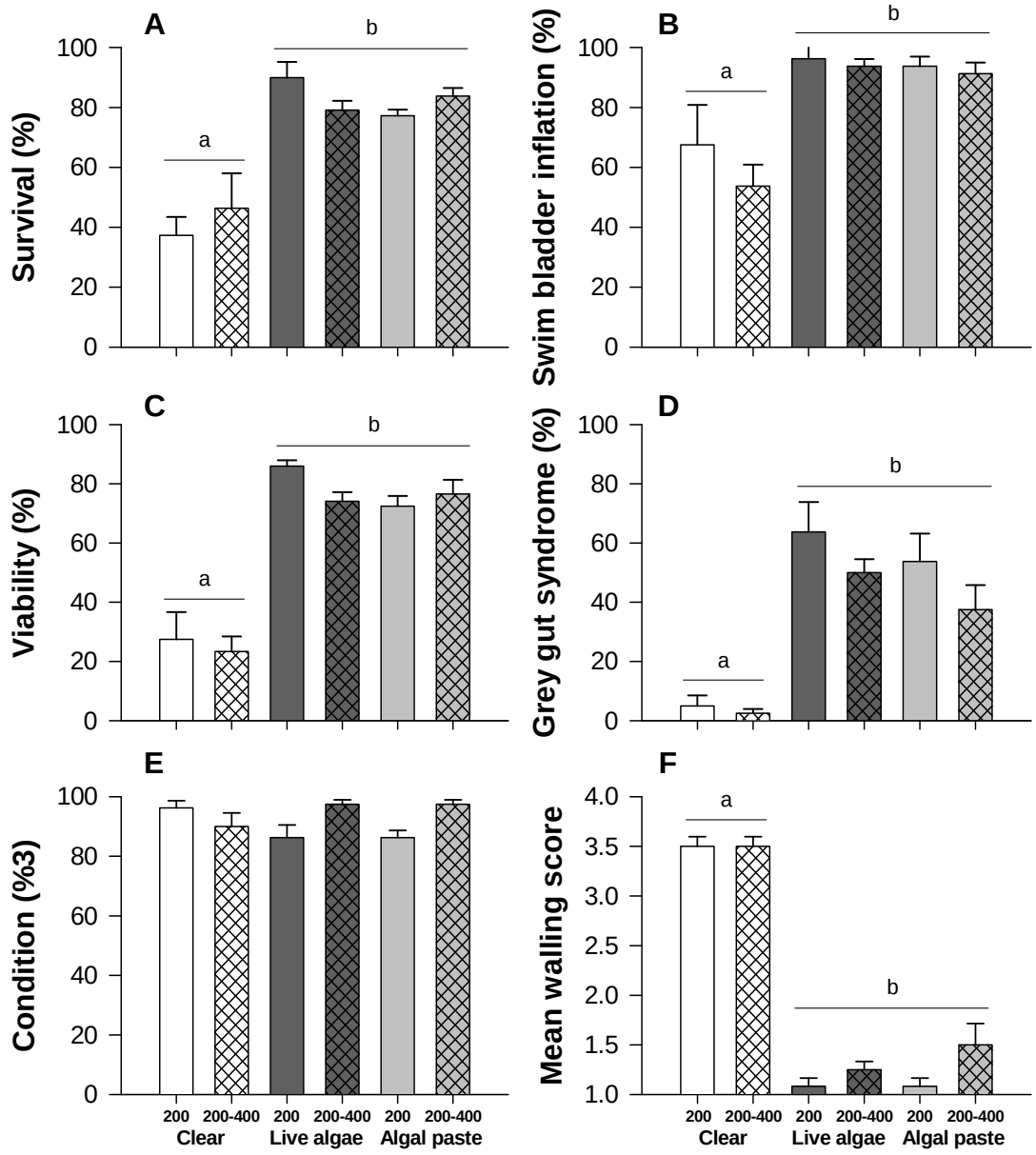

Figure 13.6 Larvae were reared in either clear water (clear bars), live algae (dark grey bars) or re-suspended algal paste (light grey bars) at an aeration level of either 200 ml min^{-1} (solid bars) or at 200 ml min^{-1} increased to 400 ml min^{-1} 10 dph (cross-hatched bars). Mean A) survival, B) swim bladder inflation, C) viability, D) grey gut syndrome E) percentage of larvae receiving a condition score of three and F) walling score of larvae (± SE, n = 4) in each treatment 16 dph from Experiment 2. Bars sharing common superscripts are not significantly different.

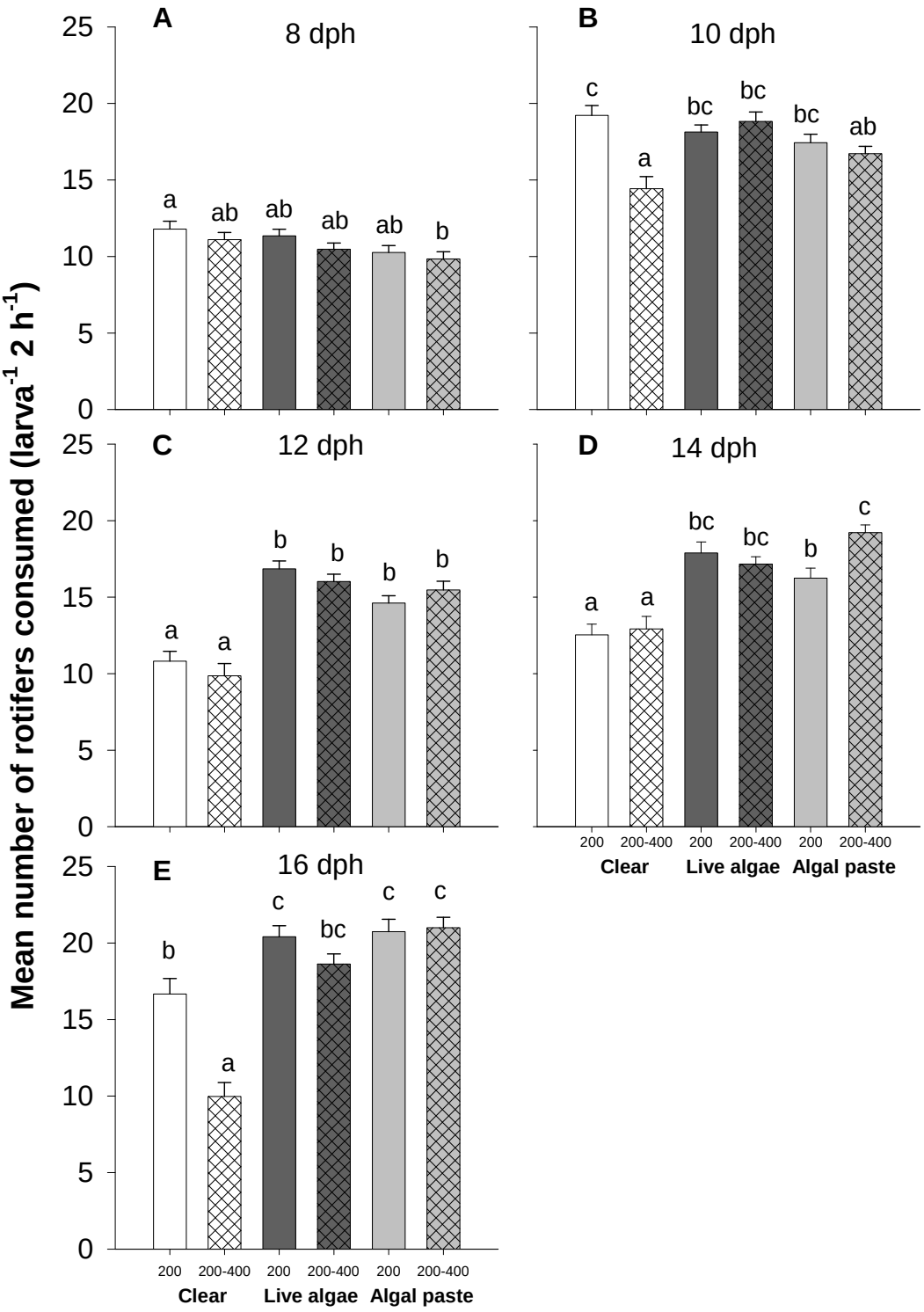

Figure 13.7 Larvae were reared in either clear water (clear bars), live algae (dark grey bars) or re-suspended algal paste (light grey bars) at an aeration level of either 200 ml min^{-1} (solid bars) or at 200 ml min^{-1} increased to 400 ml min^{-1} 10 dph (cross-hatched bars). Mean feed intake (± SE, n = 80) by larvae 8, 10, 12, 14 and 16 dph from Experiment 2. Bars sharing common superscripts are not significantly different.

13.5 Discussion

The optimal level of aeration is species-specific and influenced by the type of tank and aeration system used. Striped trumpeter larvae reared at a turbulence of 200 ml min^{-1} had improved growth and survival in comparison to larvae reared at higher turbulence levels, or in static conditions. Both field and laboratory studies have found turbulence to influence feeding or aspects of the foraging sequence (MacKenzie and Leggett, 1991; MacKenzie et al., 1994; Muelbert et al., 1994; Dower et al., 1998; Utne-Palm, 2004). For example, a laboratory study showed that survival of larval nassau grouper, *Epinephelus striatus,* improved at low turbulence levels compared with no turbulence or higher turbulence levels (Ellis *et al.*, 1997). Similarly, blue whiting, *Micromesistius poutassou,* larvae in the field consumed more and larger prey at low rather than high turbulence levels (Hillgruber and Kloppmann, 2000). Field studies have shown that turbulence can alter prey selection of radiated shanny, *Ulvaria subbifurcata*, such that fewer but larger prey are consumed on days of high turbulence resulting in higher levels of gut fullness (Dower et al., 1998). Comparing optimal aeration levels between different laboratories is difficult due to differences in tank design, size, shape and aeration delivery systems; adjusting aeration levels into common units (ml min^{-1} l^{-1}) provides a method of comparison. The best aeration for first feeding larvae in the current study was 200 ml min^{-1} (0.67 ml min^{-1} l^{-1}), for first feeding grouper, *Epinephelus coioides*, 0.62 ml min^{-1} l^{-1} (Toledo et al., 2002), for first feeding Nassau Grouper, 0.3 ml min^{-1} l^{-1} (Ellis et al., 1997), for seven band grouper larvae, *Epinephelus septemfasciatus*, 0.2 ml min^{-1} l^{-1} (Shiotani et al., 2005) and for sea bass, *Dicentrarchus labrax* L., 0 to 30 dph, 0.27 – 0.45 ml min^{-1} l^{-1} (Barahona-Fernandes, 1978). For sea bass the best aeration level was based on highest survival as growth was not affected (Barahona-Fernandes, 1978). For grouper, growth was only assessed 2 dph but was already improved at the lowest aeration levels tested, 0.62 and 1.25 ml min^{-1} l^{-1}, and survival was significantly higher at 0.62 ml min^{-1} l^{-1}, 6 dph (Toledo et al., 2002). An aeration rate of <0.83 ml min^{-1} l^{-1} increased the SBI rate of Australian bass compared with larvae reared at >16.66 ml min^{-1} l^{-1} but had no effect on survival or growth (Battaglene and Talbot, 1993).

As turbulence increased in Experiment 1, there was a significant trend for decreased growth and survival consistent with the decrease in prey consumption rates. The divergence in growth between turbulence treatments was first seen between 8 and 10 dph. Absorption of the yolk sac and oil droplet is complete at this time and larvae rely on exogenous feeding (Trotter et al., 2003b). Since prey density per water volume was constant between treatments, and in fitting with theoretical and laboratory studies, decreased feeding rates of young larvae were likely a result of diminished pursuit and capture success at the higher turbulence levels (Hillgruber and Kloppmann, 2000). However, decreased growth and survival at higher turbulence levels may have also resulted from physical injury of larvae upon contact with tank walls and the central bubble stream and screen.

Given that pursuit success decreases with increasing turbulence (MacKenzie and Kiorboe, 2000) and maximum growth and survival was seen at the lowest turbulence level tested, further improvements in growth at an aeration level between 0 and 200 ml min^{-1} cannot be discounted. Indeed, lower levels were found to be beneficial for sea bass and nassau grouper, albeit in different sized tanks (Barahona-Fernandes, 1978; Ellis et al., 1997). Thus, within the aeration range 0 to 200 ml min^{-1} it is probable that an age or size-specific set-point will be reached where improvements in foraging behaviour via increased encounter rates will be offset by the concomitant decline in pursuit and capture success, and foraging success will decline. Finer scale control of turbulence than was available in our study would be required to test this hypothesis.

No striped trumpeter larvae survived under static conditions and this is consistent with findings on larval nassau grouper (Ellis et al., 1997). Larvae of both species became trapped at the water surface, possibly in an attempt to capture rotifers that had accumulated at the surface, and died. Other possible causes of mortalities in static tanks include low dissolved oxygen (DO) and changes to bacterial communities. DO concentrations within static tanks in our study were not lower than tanks receiving aeration. However, crowding of larvae and rotifers at the surface and decreased movement of larvae, may have caused undetected localized decreases in dissolved oxygen levels sufficient for inhibited respiration and/or dehydration (Ellis *et al.*, 1997). Barahona-Fernandes (1978), found that total bacteria counts in tanks with low aeration, 0.5 ml min^{-1}, were 10 times higher than in tanks with high aeration and may have contributed to the low survival of sea bass. Bacterial levels were not monitored in the current study. It should be noted that during the yolk-sac phase better survival of striped trumpeter has been linked with static tanks, and yolk-sac larvae in the current study were held under static water conditions (Trotter et al., 2005). Thus, the potential negative effects of reduced DO and increased bacterial levels, as discussed, are unlikely to be issues until the addition of rotifers and first feeding.

As the larvae increased in age there was a general trend for improvement in prey intake at higher turbulence levels. Improvements in prey consumption occurred even though larvae held at higher turbulence levels were smaller in length due to sub-optimal feeding at high turbulence levels early in development. In general, the foraging behaviour of larvae improves with larval size, concomitant with improvements in visual capabilities, locomotor ability and overall foraging success. It follows therefore that any dome shape relationship between increasing turbulence and prey intake would shift to the right with increasing larval size, with the resultant optimal turbulence level increasing with larval size (Fiksen et al., 1998). Hence, maximal prey consumption rates would result where water turbulence is increased throughout the larval period at a rate proportional to the larva's increased ability to feed. Whether increased prey consumption would correspond with improvements in growth would be dependant on the increased energetic costs associated with feeding in more turbulent waters.

Increasing the aeration in Experiment 2 did not increase the growth of larvae reared in any environment. Following on from Experiment 1, which gave evidence for the possibility of increased growth potential by increasing turbulence with age, one would expect those larvae shifted to a higher turbulence level 10 dph would do better than those remaining at low turbulence. In support of this argument, turbulence resulted in a significant increase in attack position rate for small cod larvae when fed at low prey density (< 35 l^{-1}) but not at high prey density (> 35 l^{-1}) (MacKenzie and Kiorboe, 1995). Alternatively, any improvements to overall prey intake following the increase in turbulence may have been offset by increased energetic costs associated with pursuit and capture of prey.

Under clear water conditions, increasing the turbulence 10 dph significantly decreased larval growth in comparison to clear water larvae on low turbulence and all green water treatments. The reduction in growth was reflected in a decrease in prey consumption and occurred even though larvae in all treatments were of the same length when the turbulence was increased, 10 dph. Two possible reasons for decreased growth in clearwater at higher aeration are that clear water reared larvae are not as well conditioned as green water reared larvae to deal with an increase in turbulence or they had poorer larval health from walling or less suitable bacterial communities. Despite the reduced prey intake and growth, clear water larvae with increasing aeration in Experiment 2 did not have the mortality rate of larvae reared at 400 ml min^{-1} from 6 dph in Experiment 1. Thus, larval foraging ability had improved sufficiently

during the 4 days on low aeration to continue growing once introduced to a higher turbulence level.

Larvae reared in green water outperformed those in clear water in agreement with many other studies (Naas *et al.*, 1992; Reitan *et al.*, 1993; Tamaru *et al.*, 1994). One facet of the turbid environment that likely improves growth is an increase in the ability of larvae to detect prey, resulting from increased contrast between prey and background (Boehlert and Morgan, 1985; Hinshaw, 1985; Miner and Stein, 1993). Such increases in detection capability, coupled with the relatively high prey densities used in our study, especially in comparison with average oceanic prey densities, likely saturated any positive effect of increased encounter rate arising from turbulence.

Green water is known to have direct and indirect effects on larval nutrient intake and health of live prey (Moffatt, 1981; Vasquez-Yeomans *et al.*, 1990; Reitan *et al.*, 1993; Tamaru *et al.*, 1994). Evidence for altered nutrient intake by green water reared larvae in this study can be seen from the occurrence of grey gut syndrome. Grey gut syndrome is commonly observed in striped trumpeter larvae likely resulting from either; extensive vacuolation of enterocytes, indicative of lipid accumulation in intestinal cells (Bransden et al., 2005b) or bacterial enteritis characterised by sloughing of gut epithelial cells (Cobcroft *et al.*, 2004). The latter of these two mechanisms often results in poor prey digestion and ultimately larval mortality. In the current experiment no mortality was associated with the higher incidence of grey gut syndrome observed for larvae reared in green water treatments. Instead, it is likely that the appearance of grey gut syndrome reflects the difference in fatty acid profiles of the live prey in clear and green water treatments, albeit probably small owing to enrichment prior to feed out, and also the higher prey consumption by larvae in green water resulting in a higher lipid diet.

Green water has also been reported to alter larval distribution within tanks (Vandenbyllaardt et al., 1991; Naas et al., 1992; Rieger and Summerfelt, 1997) and significantly reduced the proportion of larvae walling in the current study. A reduction in walling, likely resulting from a reduction in light reflecting from tank walls (Bristow *et al.*, 1996), increases the time during which larvae are actively foraging for prey. It may also protect them from physical damage associated with touching hard surfaces. Given that a high percentage of larvae were walling in clear water and therefore not feeding, the incidence of walling likely had a strong influence on both the decreased short term feed intake and reduced growth of clear water reared larvae.

The window during which striped trumpeter larvae are able to inflate their swim bladder extends over four days at 16 ^{0}C when larvae are 5.7 to 6.1 mm in length (Trotter et al., 2005). Surface skimmers are required during the swim bladder inflation window to remove surface films that prevent larvae inflating their swim bladders (Chatain and Ounais-Guschemann, 1990; Trotter et al., 2003a; Trotter et al., 2003b). However, despite the use of surface skimmers and similar culture conditions, the proportion of larvae with inflated swim bladders differed significantly between Experiments 1 and 2. Comparison of the clear water 200 ml min^{-1} treatment in both Experiments 1 and 2 indicates very little difference between growth and survival; however, a 60% improvement in inflation by larvae of Experiment 2. The cause for poor inflation in the first experiment is unclear and may simply reflect differences in the quality of eggs between experiments. However, excessive aeration can cause a barrier to larvae accessing the surface (Abdul-Elah et al., 1983; Battaglene and Talbot 1993). Poor swim bladder inflation usually results from an inability to actively ingest air at the surface and/or transfer air via a pneumatic gut to the swim bladder (Doroshev et al.,

1981; Rieger and Summerfelt, 1998). Inability to successfully inflate the swim bladder may result from bacterial infections causing blockage of the pneumatic duct (Marty *et al.*, 1995), genetically related performance (Hadley et al 1987) or physical retardation of inflation via surface films which prevent larvae accessing air (Chatain and Ounais-Guschemann, 1990; Kitajima et al., 1994; Trotter et al, 1995). Improved swim bladder inflation was also observed in both green water treatments.. The improved growth and survival of larvae in green water suggests that larvae were in better health than those reared in clear water and thus may be better equipped to penetrate the water surface and successfully inflate their swim bladder (Trotter et al., 2003b). Green water tanks also had different surface films possibly making it easier for larvae to gain surface access.

Turbidity provided by either live algal cells, or concentrated algal paste, significantly improved growth, survival, viability, feed intake and swim bladder inflation rates, of striped trumpeter larvae in comparison with larvae reared in a clear water environment. Larvae reared in live algal cells had a mean final length significantly larger than those grown in an algal-paste induced green water environment. However, there was no difference in survival, swim bladder inflation, feed intake, overall condition or grey gut syndrome of larvae reared in either of the green water treatments. The improvements in growth in live algae may be related to differences in the microbial environment or nutritional profiles of rotifers between the two green water treatments.

Striped trumpeter larvae benefited from a low level of turbulence in comparison to higher or static conditions in clear water. Turbidity provided by the addition of either live algae or concentrated algal paste to tanks further improved larval growth and survival such that the turbulent regimes tested no longer had a significant positive affect on growth or survival. Growth may be improved by providing turbulence at a level which is commensurable with the foraging ability of the larvae, and it is likely that under conditions where prey densities are low, turbulence will be an important factor in determining growth and survival of young larvae. Further investigation into increasing turbulence with age to gain maximal growth rates requires more attention, primarily in regard to the timing and strength of turbulent increases.

13.6 Acknowledgements

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14 WEANING STRATEGIES FOR STRIPED TRUMPETER (*LATRIS LINEATA*) POST-LARVAE CULTURE

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14.1 Abstract

The striped trumpeter (*Latris lineata*) is a native fish being developed for aquaculture in Tasmania, Australia. Striped trumpeter have a long post-larval stage and rearing has been reliant on the long-term use of *Artemia*. Two experiments were conducted to investigate weaning strategies. The first experiment used 52 days post hatch (dph) post-larvae previously reared on *Artemia* from 16 to 52 dph enriched with either low or high ascorbic acid. The treatments were *Artemia*, a microdiet or co-feeding. The post-larvae were reared until 67 dph. The *Artemia* treatment yielded significantly higher mean survival, followed by co-feeding and the microdiet. Feeding *Artemia* yielded significantly heavier post-larvae and the microdiet produced significantly smaller post-larvae. A second experiment used 41 dph post-larvae and investigated the effect of co-feeding duration prior to feeding with *Artemia* and microdiet as controls. Co-feeding periods of 5, 10 and 15 days were tested. Co-feeding for 5 days and microdiet feeding yielded significantly poorer survival compared to *Artemia*. *Artemia* feeding yielded significantly heavier post-larvae. In both experiments, the diets did not have a significant effect on jaw morphology. These experiments are the first to examine weaning strategies for striped trumpeter post-larvae and suggest co-feeding post-larvae from 40 dph and feeding microdiet exclusively at 50 dph.

14.2 Introduction

One of the significant challenges facing the growth of marine fish farming is the unreliable production of juveniles as seed for stocking into the culture systems (Battaglene and Fielder 1997; Planas and Cunha 1999; Carter 2006). Refinement of larviculture techniques can facilitate the improvement of hatchery production. Currently, the larviculture of many marine fish species is reliant on the use of live feeds. Rotifers and *Artemia* are the two most commonly used live feeds (Sorgeloos et al., 2001; Olsen et al., 2004). However, live feeds are not ideal because their production requires additional investment in algal production systems and on-going labour costs (Le Ruyet et al., 1993; Kam and Leung 2002). The nutrient content of *Artemia* of different strains is highly variable and difficult to manipulate (Sorgeloos et al., 2001). Furthermore, the tendency of *Artemia* to retroconvert the important fatty acid, docosahexaenoic acid (DHA 22:6 n-3), to eicosapentaenoic acid (EPA 20:5 n-3) can be detrimental to marine fish development, which requires a specific balance of DHA:EPA for normal development (Navarro et al., 1999; Sargent et al., 1999; Izquierdo et al., 2000).

The use of formulated diets for larviculture offers cost savings, by reducing or eliminating the production of live feeds, as well as a customisable medium for delivering a consistent

nutritional profile during larval feeding (Cahu and Zambonino Infante 2001). Improvements in production techniques and improved understanding of larval nutrient requirements have lead to the development of microdiets which are physically and nutritionally appropriate for larviculture of marine fish larvae (Cahu and Zambonino Infante 2001; Fletcher et al., 2007). Earlier concerns about nutrient content and digestibility of microdiets have largely been overcome (Cahu et al., 2003a; Yufera and Darias 2007). Microdiets simplify the logistics of storage and eliminate the need for additional culture systems (Le Ruyet et al., 1993; Cahu and Zambonino Infante 2001, Kam and Leung 2002).

Weaning is the process of transitioning from live feeds to formulated inert diets and can be done gradually via co-feeding or abruptly. Co-feeding larval fish involves introducing a microdiet to fish larvae by feeding it together with live feeds (Rosenlund et al., 1997). The use of a co-feeding strategy has yielded variable results. In some fish such as barramundi (*Lates calcarifer*) (Curnow et al., 2006) and shi drum (*Umbrina cirrosa*) (Papadakis et al., 2008) weaning onto microdiets can be achieved after co-feeding with rotifers, bypassing the *Artemia* feeding stage. In contrast, co-feeding with *Artemia* is still necessary for increased survival and growth for other species such as Atlantic cod (*Gadus morhua*) (Fletcher et al., 2007). High survival and growth during weaning is achieved by determining the point during larviculture at which larvae are able to ingest and digest microdiets and the means by which inert diets are introduced (Vega-Orellana et al., 2006).

Striped trumpeter (*Latris lineata*) a deep sea fish endemic to South-eastern Australia and New Zealand, is under development as an aquaculture species in Tasmania (Tracey and Lyle 2007; Battaglene and Cobcroft 2007). The demand for striped trumpeter is high because of its excellent white-flesh properties and its rich content of the long-chain polyunsaturated fatty acids found to be beneficial for human health (Nichols et al., 2005). However, the fishery for striped trumpeter has declined to less than 30 m t per annum and aquaculture of this species is the only way to assure sustainable commercial supplies. Striped trumpeter has a protracted post-larval development or “paperfish” stage and has proven difficult to culture (Battaglene and Cobcroft 2007). Despite recent advances, hatchery produced juveniles have exhibited a high incidence of malformations (Trotter et al., 2001; Cobcroft et al., 2001; Battaglene and Cobcroft 2007). Research has identified the required content of long-chain fatty acids and selected vitamins in live feeds (Bransden et al., 2004a, b and 2005a, b; Brown et al., 2005a, b). Historically, striped trumpeter post-larvae were reared using enriched *Artemia* until 100 dph. Earlier weaning onto microdiets can reduce the use of *Artemia*, and possibly improve larval survival and growth and reduce the incidence of malformations by providing post-larvae with a better and more consistent supply of nutrients; particularly ascorbic acid (AA) (Callan et al., 2003; Fletcher et al., 2007; Kestemont et al., 2007).

The aim of the study was to determine: i) if the *Artemia* feeding stage could be shortened ii) the optimal period of co-feeding live and microdiets and iii) if feeding solely with microdiets was possible before 50 dph. We also tested the effect of different levels of ascorbic acid (AA) in *Artemia* enrichment on the incidence and severity of jaw deformities.

14.3 Materials and methods

14.3.1 *Rearing of post-larvae*

Striped trumpeter eggs were obtained from captive broodstock held at the Marine Research Laboratories, Tasmanian Aquaculture Fisheries Institute, Tasmania. Eggs were incubated at 14.0 ± 0.8 ^{o}C and larvae reared to first feeding using standard hatchery protocols (Battaglene

and Cobcroft 2007). Larvae were fed rotifers (10/mL) enriched with AlgaMac™ 2000 (Aquafauna Biomarine, Hawthorne, CA, USA) from 6 to 18 dph. Post-larvae used in experiment one were fed with *Artemia* enriched with two types of emulsions, one yielded *Artemia* with low AA (636 ± 18 μg AA/g mean ± SE) and the other yielded *Artemia* with high AA (7904 ± 710μg AA/g) from 16 to 52 dph (Brown et al., 2005b). AA content of *Artemia* was determined using the methods described by Brown et al., (2005a). Post-larvae used in experiment two were fed *Artemia* enriched with AlgaMac™ 3050 (AM3050, Aquafauna Biomarine, Hawthorne, CA, USA).

14.3.2 *Feeds*

Artemia (1 x 10^6 animals) were fed with 0.1 g/L of AlgaMac™ 3050 (AM3050; Aquafauna Biomarine, Hawthorne, CA, USA) in 70-L conical vessels for 12 h at 26-28 ^oC. Hereafter, mention of *Artemia* used for the experiments refers to animals prepared using this method. A mix of two sizes of Gemma Micro™ (Skretting, Stavanger, Norway), 150 and 300, were used. In experiment one, a 30:70 % mix of Gemma Micro™ 150 and 300 was used. For experiment two, a 50:50 % mix of Gemma Micro™ 150 and 300 was used. *Artemia* was fed twice per day at 0900 and 1600. Microdiets were dispensed using a computer controlled programmable feeder system (Department of Fisheries, Western Australia http://www.fish.wa.gov.au/amf/). Feeders were calibrated to dispense the rations over different periods of time depending on treatments. Feeders were loaded daily at 0900, and 1600 for the microdiet-fed treatments and dispensed food over 24 h. For the co-feeding treatments the feeders were loaded only at 0900 and 1600 to coincide with *Artemia* feeding and dispensed microdiet only over 14 h.

14.3.3 *Experimental system*

The experimental system consisted of black 300 L hemispherical tanks with a central screened outlet, constantly illuminated by halogen bulbs emitting 11.2 ± 1.7 μmol s^{-1} m^2. Each tank was supplied daily with 900 % (112.5 L/h) recirculating seawater and 100 % flow-through (12.5 L/h). Seawater was ozonated following sand filtration, foam fractionation, cartridge filtration, and was passed through ultraviolet and charcoal filtration and temperature control (Smith et al., 2006). Water quality was measured daily at 0900 and reported as mean ± SE. In experiment one, temperature was 16.1 ± 0.1 C, pH 8.1 ± 0.1, DO 99.8 ± 0.1 % of saturation, and salinity 33.9 ± 0.0 g/L. In experiment 2, temperature was 16.0 ± 0.1 C, pH 8.10 ± 0.1, DO 99.5 ± 0.1 % of saturation, and salinity 33.9 ± 0.0 g/L. All of the tanks were siphoned manually twice daily, once in the morning and again in the afternoon to remove uneaten food, mortalities, faeces and organic debris to prevent fouling.

14.3.4 *Experiment one: Performance Comparison of Post-larval Fed UsingArtemia, Co-feeding and a Microdiet.*

At 52 dph, 250 post-larvae were stocked into each of 12 tanks. Six tanks were randomly stocked with post-larvae reared using *Artemia* high in AA, and six tanks with post-larvae reared using *Artemia* low in AA. Six tanks for each prior feeding treatment were then randomly assigned with one of three feeding treatments namely, *Artemia*, co- feeding, and the commercial microdiet. *Artemia*-fed groups were fed at a density of 0.5 *Artemia*/mL (0.6 g dry matter [DM] day^{-1}) at 0900 and 1600 daily. From 55 dph until 67 dph, *Artemia* density was increased to 0.75 *Artemia*/mL (0.9 g DM). The co-feeding group was fed with *Artemia* at a density of 0.25 *Artemia*/mL (0.3 g DM) from 52 dph and increased to 0.375 *Artemia*/mL (0.5 g DM) from 55 dph to 67 dph. In addition, co-feeding treatments received 8 g/day of the

microdiet at 0.2 g every 20 min. The microdiet- fed treatment received 16 g/day at the same rate.

14.3.5 *Experiment two: Duration of the Co-feeding Period on Post-larval Performance*

At 41 dph, 245 post-larvae were stocked into each of 20 tanks. There were three co-feeding treatments of different durations (5, 10 and 15 days) before sole feeding with microdiets. *Artemia* and microdiet-fed treatments served as control treatments. There were four replicate tanks for each treatment. Co-feeding groups were fed with two rations of 0.25 *Artemia*/mL (0.3 g DM) at 0900 and 1600 daily and 4 g/day of microdiet at 0.1 g every 20 min. *Artemia*-fed groups were fed with two rations of 0.5 *Artemia*/mL (0.6 g DM) at 0900 and 1600 daily. The microdiet-fed groups were fed with 8 g rations at 0.1 g every 20 min.

14.3.6 *Larval sampling*

Dead post-larvae collected during siphoning were counted. On sampling days, post-larvae were removed by siphon from the tanks and transferred to 250 mL containers containing anaesthetic (1 % 2-phenoxyethanol). In experiment one, 20 post-larvae were randomly taken from each replicate tank at the start of the experiment and at 60 dph and 67 dph. In experiment two, 40 post-larvae were taken from the rearing tank as an initial sample and 30 post-larvae were taken from each replicate tank at 55 dph. Post-larvae were measured using a dissecting microscope and a calibrated graticule for total length (TL, nearest 0.1 mm), and the incidence and severity of jaw malformations were scored using a scale of 0 (normal) to 3 (severely malformed) (Cobcroft et al., 2001). Any other deformities or signs of starvation including poor condition or shrunken abdomens were noted. Post-larvae were transferred onto preweighed glass microfibre filters, washed with 0.5 mol ammonium formate solution to remove salt (Brown et al., 1998) and stored in liquid nitrogen. Post-larvae in filters were freeze dried to constant weight and weighed to the nearest mg to determine dry weight (DW).

14.3.7 *Statistical analyses*

Results are reported as mean ± SE. Data were tested for normality and homogeneity and percentage data were arcsine-transformed prior to analysis. Significant differences in larval survival and growth among treatments were determined by performing two-way analysis of variance (ANOVA) for experiment one, using the previous rearing with *Artemia*, high or low in AA, and feeding treatments as fixed factors. One-way ANOVA was used for experiment two, with feeding treatment as the fixed factor. Tukey's-b multiple comparison of means was used to separate treatments when significant differences were found. Statistical significance was accepted at $P < 0.05$. Statistical analyses were performed using SPSS version 13.0 statistical software.

14.4 Results

14.4.1 *Survival*

In experiment one, all of the feeding treatments yielded high survival (96.2 ± 0.8 %, n = 12). Feeding treatment had a significant effect on larval survival but there was no significant interaction between feeding treatment and prior rearing with *Artemia* with high or low AA

content. Survival of post-larvae at the end of the experiment originating from the cohort fed with *Artemia* low in AA (97.0 ± 0.9 %, n = 6) was significantly higher than those fed with *Artemia* high in AA (95.5 ± 1.3 %, n = 6) (df = 1, F = 8.7, $P < 0.05$). Post-larvae fed solely with *Artemia* exhibited significantly higher survival (98.6 ± 0.3 %, n = 4) than co-feeding (97.0 ± 0.7 %) which was significantly higher than post-larvae fed with microdiet alone (93.1 ± 0.8 %) (df = 2, F = 45.4, $P < 0.01$). Heavy bacterial films developed in tanks fed microdiets towards the end of the experiment.

In experiment two, feeding treatment had a more pronounced influence on larval survival. Survival of post-larvae fed with *Artemia* (83.1 ± 3.1 % n = 4) was not significantly different from those co-fed for 10 days (74.7 ± 5.6 % n = 4) or 15 days (79.4 ± 2.9 %) but significantly better than those co-fed for 5 days (52.6 ± 5.7 % n = 4) or fed solely with microdiet (35.1 ± 4.0 % n = 4), which had similar survival (df = 4, F = 19.6, $P < 0.05$). Overall, survival increased with increased duration of co-feeding (Figure 14.1).

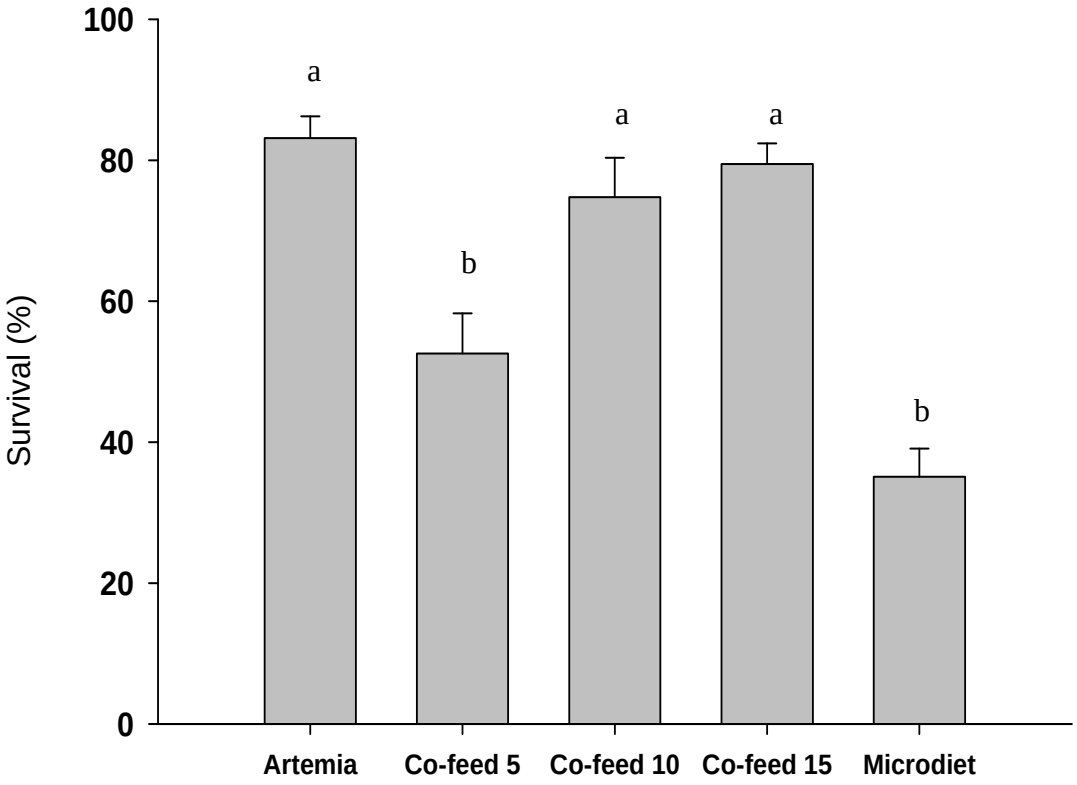

Figure 14.1 Mean larval survival at 55 dph (mean ± SE). Significant difference between groups ($P<0.05$) are indicated by different letters. (Experiment 2).

14.4.2 *Growth*

Post-larvae sampled from both experiments did not exhibit any physical signs of starvation. In experiment 1, fish previously reared with *Artemia* low in AA were significantly heavier compared to post-larvae originating from the cohort reared with *Artemia* high in AA at the start of the experiment at 52 dph (df = 1, F = 28.9, $P < 0.05$). This difference remained significant at 60 dph (df = 1, F = 14.0, $P < 0.05$) and at 67 dph (df = 1, F = 4.6, $P < 0.05$)

(Table 14.1). At the start of the experiment, there was no significant difference in the DW of the post-larvae among feeding treatments. At 60 dph, post-larvae from the *Artemia*-fed groups were significantly heavier than post-larvae from the co-feeding and microdiet treatments and post-larvae from the co-feeding treatments were heavier than post-larvae fed microdiet ($df = 2$, $F = 15.0$, $P < 0.05$). At 67 dph, there was no significant difference between post-larvae fed with *Artemia* and co-feeding. Post-larvae fed solely with microdiet remained significantly smaller ($df = 2$, $F = 11.6$, $P < 0.05$) (Table 14.1). Similar but less pronounced trends were observed in larval TL (Table 14.1). The one exception was for *Artemia*-fed post-larvae at 67 dph which were significantly longer than co-fed post-larvae ($df = 2$, $F = 13.6$, $P < 0.05$).

Table 14.1 Mean DW and mean TL of larva at 52, 60 and 67 dph. DW are expressed in mg ± SE and TL in mm ± SE. Significant differences between treatments within each day and treatment grouping ($P < 0.05$) are represented by different letters.

Measure		Mean dry weight/larva (mg)[a]		
Age		52 dph	60 dph	67 dph
Treatment	n			
Low ascorbic acid	6	8.51 ± 0.11 a	12.83 ± 0.32 a	17.99 ± 0.50 a
High ascorbic acid	6	7.53 ± 0.20 b	11.50 ± 0.44 b	16.67 ± 0.69 b
Artemia	4	8.24 ± 0.18 x	13.31 ± 0.34 x	19.03 ± 0.68 x
Co-feeding	4	7.97 ± 0.28 x	12.24 ± 0.42 y	17.55 ± 0.44 x
Microdiet	4	7.85 ± 0.33 x	10.93 ± 0.43 z	15.42 ± 0.55 y
Measure		Mean total length (mm) [a]		
Age		52 dph	60 dph	67 dph
Treatment	n			
Low ascorbic acid	6	18.29 ± 0.13 a	20.61 ± 0.14 a	23.86 ± 0.18 a
High ascorbic acid	6	17.61 ± 0.14 b	19.97 ± 0.17 b	23.27 ± 0.21 b
Artemia	4	18.06 ± 0.15 x	21.06 ± 0.18 x	24.35 ± 0.25 x
Co-feeding	4	17.81 ± 0.19 x	20.28 ± 0.18 y	23.66 ± 0.24 y
Microdiet	4	17.97 ± 0.16 x	19.52 ± 0.17 z	22.70 ± 0.19 z

[a]Mean TL was calculated from 20 larvae from each replicate tank. DW was calculated from pooling and freeze drying the 20 larvae used for measurements.

Post-larvae used in experiment 2 weighed 6.4 ± 0.2 mg and measured 17.0 ± 0.1 mm (n = 40) at the beginning of the experiment. Significant differences were found in the DW of the post-larvae at the end of the experiment. Post-larvae fed with *Artemia* were significantly heavier compared to all of the other feeding treatments. Post-larvae fed solely with microdiet were significantly heavier than those co-fed for 15 days (df = 4, F = 22.8, P < 0.05). Similar results were found for post-larval TL and the *Artemia*-fed group had the longest post-larvae (Table 14.2). Post-larvae fed solely with microdiet were significantly longer compared to post-larvae from the co-feeding treatments which yielded post-larvae of similar lengths (df = 4, F = 33.1, P < 0.05) (Table 14.2).

Table 14.2 Mean DW and mean total length TL of larva at 55 dph. DW are expressed in mg ± SE and TL in mm ± SE. Significant differences between treatments within growth measurements (P<0.05) are represented by different letters.

Treatment	n	Mean dry weight/larva (mg)[a]	Mean total length/larva (mm)[a]
Artemia	4	11.10 ± 0.20 a	20.99 ± 0.10 a
Co-feed (5 d)	4	8.76 ± 0.25 bc	19.79 ± 0.06 c
Co-feed (10 d)	4	8.76 ± 0.20 bc	19.53 ± 0.11 c
Co-feed (15 d)	4	8.56 ± 0.21 c	19.45 ± 0.11 c
Microdiet	4	9.56 ± 0.22 b	20.37 ± 0.13 b

[a]Mean TL was calculated from 30 larvae from each replicate tank. DW was calculated from pooling and freeze drying the 30 larvae used for measurements.

14.4.3 *Jaw malformations*

In both experiments, feeding treatment did not have any significant effect on the incidence and severity of jaw malformations. Neither did previous feeding with *Artemia* low or high in AA. In experiment one, the proportion of post-larvae with normal jaws in the population progressively decreased with larval age. There was a significantly higher proportion of post-larvae with normal jaws at 52 dph (85.0 ± 2.8 %) compared to 60 and 67 dph. There was a lower proportion of normal jaws at 67 dph (56.9 ± 5.4 %) compared to 60 dph (75.0 ± 3.0 %) (df = 2, F = 18.9, P < 0.05) (Figure 14.2). In experiment 2, the proportion of post-larvae with normal jaws in the initial sample of 40 post-larvae at 41dph was 94.9 %. The mean proportion of post-larvae with normal jaws at 55 dph was 65.5 ± 2.7 %.

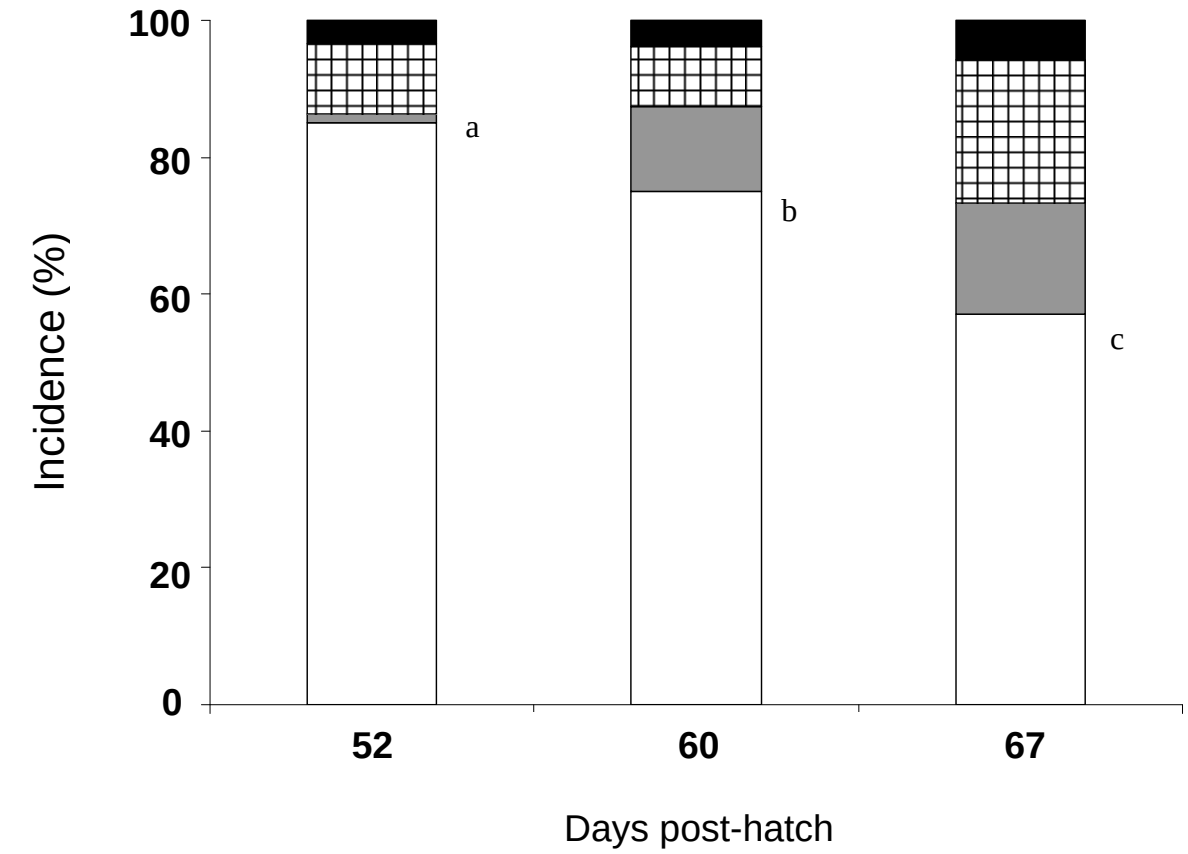

Figure 14.2 Percentage of larvae with malformation scores. Feeding with *Artemia* with low or high AA and feeding treatments had no significant influence on jaw morphology. Samples are grouped according to age (n = 12). Significant differences in the percentage of normal jaws between ages are indicated by different letters ($P < 0.05$). (Experiment 1).

14.5 Discussion

Although it is not currently possible to eliminate *Artemia* feeding in striped trumpeter our experiments show that the introduction of weaning can be greatly reduced from 100 dph. Introducing microdiets at 52 dph yielded high survival and provided a target for weaning in production tanks. Survival of post-larvae fed solely with microdiet at 41 dph was lower compared to feeding with *Artemia* and co-feeding. However, the post-larvae from the microdiet-fed treatment were larger than post-larvae from the co feeding treatments but smaller than the *Artemia*-fed post-larvae. The survival of fewer but larger fish suggests that only the fittest individuals were able to wean successfully. The larger size of 41 dph post-larvae fed solely on microdiet compared to the co-fed post-larvae indicates that microdiets provide the necessary nutrients for larval growth and development. However, feed intake is a potential confounding factor as feed rations were not adjusted to account for changing densities. It is possible that post-larvae may have grown better based on greater food availability. However, in both experiments there was intentional overfeeding of *Artemia* and microdiets to minimise the potential confounding effects of food limitation. In over feeding tanks it is important not to have water quality deteriorate. The large amount of uneaten microdiet and microbial films in the first experiment prompted us to reduce the ration in the second experiment to avoid the deterioration of water quality.

There are a number of possible causes of increased mortality and lower growth in post-larvae fed solely on microdiets. First, smaller post-larvae may not have been able to compete and capture sufficient quantities of microdiet, although the lack of physical evidence of starvation indicates this was not the case. Second, some larvae may not have been able to digest the diet or get sufficient nutritional value from the microdiets. This has been suggested to occur in Atlantic cod (Hamlin and Kling 2001) and in gilthead sea bream (Robin and Vincent 2003) larvae. Third, striped trumpeter larvae are highly susceptible to poor water quality and microbial activity associated with high organic loading from the microdiet (Battaglene et al., 2006). Results from both experiments suggest that post-larvae fed solely with *Artemia* had higher survival and growth during the post-larval growth phase. However, prolonged feeding with *Artemia* is likely to lead to depressed growth because of the deficient fatty acid profile of *Artemia* for feeding to striped trumpeter post-larvae (Bransden at al. 2005a).

Co-feeding with *Artemia* and microdiets have been found to be superior to feeding with *Artemia* or microdiet alone in barramundi species (Curnow et al., 2006), sole species (Engrola et al., 2007), and Atlantic cod species (Callan et al., 2003; Fletcher 2007). We found that co-feeding yielded higher survival and larger post-larvae compared to feeding solely with microdiet in experiment one but lower survival and growth compared to post-larvae fed solely with *Artemia*. In experiment two, the growth results for post-larvae fed solely with microdiet are confounded by low survival. Increasing the co-feeding period improved survival in post-larvae from 41 to 55 dph. However, larval growth was not influenced by the duration of co-feeding.

Maintaining water quality by siphoning tank bottoms was necessary to maintain water quality and was done twice daily. Bacterial colonies formed on the outflow pipes during experiment one and lead us to reduce microdiet input in experiment two to maintain water quality. Striped trumpeter post-larvae are highly sensitive to changes in water chemistry and suffer when water quality deteriorates (Battaglene et al., 2006; Battaglene and Cobcroft 2007). The 300 L experimental tanks have a relatively large surface volume to water volume

compared to commercial production tanks which have larger volumes and are able to accommodate higher organic load from the uneaten feed, faeces and dead post-larvae. In production tanks less siphoning is required due to the self cleaning dynamics of water flow and potentially negative effects from lowered oxygen are avoided. It is important to test the results derived from experimental tanks at production levels. Following the current study, weaning of striped trumpeter is now routinely carried out from as early as 30 dph and is completed by 50 dph with no reduction in growth or survival (A. Overweter, Marine Research Laboratories, University of Tasmania, personal communication).

The development of jaw malformations in cultured striped trumpeter remains a bottleneck during larviculture. Previous studies have shown that supplementation with AA in the feeds can reduce the incidence of malformations in fish larvae (Cahu et al., 2003b; Kestemont et al., 2007). Results from the first experiment showed that high AA levels in the *Artemia* did not reduce the incidence of jaw malformations. Neither did the various feeding treatments have any detectible effect on jaw malformation rates suggesting it is not solely nutritionally related. Cobcroft and Battaglene (2009), found that jaw malformations are associated with physical damage from larvae swimming against the tank wall and is influenced by tank colour.

When feeding microdiets to striped trumpeter it is advisable to ensure that the particles are spread evenly over the tank surface as was the case in the current study and allow them to float. This ensures that larvae are able to feed on the particles before they sink to the bottom. This is especially important for striped trumpeter post-larvae which are neustonic and show a great affinity for the water surface (Furlani and Ruwald 1999; Battaglene and Cobcroft 2007).

In conclusion, striped trumpeter as an aquaculture species holds great promise but the lack of juvenile production is hampering its commercial development. The use of microdiets over live feeds in larviculture offers potential cost savings, and ensures a consistent nutritional profile. Striped trumpeter post-larvae can be weaned onto formulated diets at 52 dph with high survival (> 90 %) but significant reductions in growth. Post-larvae can be weaned onto formulated diets at 41 dph but with lower survival (~35 %). Co-feeding improved survival of post-larvae over fish fed formulated diets and a minimum co-feeding period of 10 days is recommended.

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15 EFFECTS OF TEMPERATURE REGIME ON GROWTH AND DEVELOPMENT OF POST-LARVAL STRIPED TRUMPETER (*LATRIS LINEATA*)

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15.1 Abstract

The striped trumpeter (*Latris lineata*) is a promising new candidate for diversification of aquaculture in temperate regions of Australasia. Striped trumpeter is also of scientific interest due to an unusually prolonged post-larval phase. The research was aimed at identifying the optimal temperature for rearing post-larval striped trumpeter approaching metamorphosis. Three-hundred-day-old post hatch post-larvae (12.1 ± 0.2 g, 114.0 ± 0.5 mm, mean ± SE) were reared at 12, 14, 16 and 18 ^{o}C, over 84 d. Survival, growth and metamorphosis into juveniles were recorded every 21 d. Fish were fed to apparent satiation and reared in oxygen saturated water (95.9 ± 2.6 %). At 14 ^{o}C, fish exhibited the best growth, had significantly higher lipid content and the majority (> 90 %) of the population metamorphosed into juveniles. The performance of fish reared at 16 ^{o}C was similar to those at 14 ^{o}C but the carcass had a significantly higher protein content and a significantly smaller proportion of the population (66.2 ± 3.0 %) metamorphosed into juveniles. Growth rate models predicted that growth was maximised between 12.9 ^{o}C (thermal growth coefficient) and 14.4 ^{o}C (specific growth rate). At 12 ^{o}C, fish showed the highest food conversion efficiency and all of the fish metamorphosed into juveniles. At 18 ^{o}C, fish showed the poorest growth, metamorphosis, and protein and energy retention. This is the first study on the effects of temperature on growth and development of striped trumpeter post-larvae. The results have important implications for aquaculture and fisheries management of striped trumpeter, in particular the rearing of post-larvae in hatcheries, timing of stocking into sea cages especially prior to metamorphosis, and for wild stock recruitment models.

15.2 Introduction

Temperature is one of the most significant abiotic factors affecting fish growth (Brett, 1979). The optimum temperature may change with the size and age of the fish (Pedersen and Jobling, 1989; Bjornsson et al., 2001). Within the optimum temperature range, fish growth is maximised (Jobling, 1997; Katersky and Carter, 2007). Outside the optimum temperature range growth is depressed. Exposure to temperatures beyond the optimum can also lead to the development of deformities (Jobling, 1994; Sfakianakis et al., 2006). Temperature also influences larval development and condition (Green and Fisher, 2004; Georgakopoulou et al., 2007). From an aquaculture perspective, identifying the optimum temperature is crucial in order to maximise growth and nutrient retention, decrease wastage of feed and reduce the detrimental impact of negative effects on growth and development (Van Ham et al., 2003; Katersky and Carter, 2007; Handeland et al., 2008).

The striped trumpeter (*Latris lineata*) was selected as an alternative to Atlantic salmon (*Salmo salar*) for aquaculture in Tasmania following a review by Searle and Zacharin (1994) and has the potential to be cultured throughout the temperate regions of the Southern hemisphere. Favourable properties of the striped trumpeter include its high concentration of polyunsaturated fatty acids (PUFAs), tolerance to high stocking densities, docile nature, ability to take formulated feed and superb eating qualities (Nichols et al., 2005; Battaglene and Cobcroft, 2007).

Despite recent progress in larval rearing there is still a lack of juveniles for stocking in commercial facilities. Production of fish for stocking is further complicated by an extended neustonic, oceanic, post-larvae or 'paperfish' phase lasting up to nine months (Furlani and Ruwald, 1999; Tracey et al., 2006; Battaglene and Cobcroft, 2007). Post-larvae are particularly delicate and prone to stress leading to high rates of mortality, especially following handling or when subjected to poor water quality. Cultured post-larvae metamorphose into the juvenile form at 80 mm total length and the adult form, with its distinctive striped pattern, at 180 to 200 mm (Tracey, 2007). Juvenile striped trumpeter are more robust to handling than post-larvae and are less susceptible to diseases and can be successfully cultured in sea cages (Battaglene and Cobcroft, 2007). Previous studies have identified 12 ^{o}C to 14 ^{o}C as the optimum temperatures for egg incubation (Bermudes and Ritar, 1999; Morehead and Hart, 2003) and 16 ^{o}C for the rearing of larvae (Trotter et al., 2003). These findings suggest that optimum temperatures for rearing striped trumpeter vary with the development of the fish and relatively small increments in rearing temperature have a significant impact on performance. Culture requirements for post-larvae and juveniles had not been defined prior to the current study.

The aim of the study was to determine the optimal temperature for survival, growth and development of striped trumpeter post-larvae over the temperature range they experience in the wild. Post-larvae were reared at 12, 14, 16 and 18 ^{o}C for 84 d as they approached metamorphosis into juveniles. Post-larvae performance was assessed using temperature related growth, food conversion efficiency, development and whole body composition (Katersky and Carter, 2007).

15.3 Materials and Methods

15.3.1 *Source of animals and experimental system*

This study received approval from the Animal Ethics Committee of the University of Tasmania (A0008719). Larvae were hatched from eggs originating from captive broodstock and were subsequently reared using established hatchery protocols (Battaglene and Cobcroft, 2007). Eggs were incubated at 14.0 ^{o}C and larvae were reared at 16.4 ± 0.1 ^{o}C to 70 days-post-hatch (dph). From 70 dph to 289 dph post-larvae were held at 17.7 ± 0.1 ^{o}C. The experimental system at the Marine Research Laboratories, Tasmanian Aquaculture and Fisheries Institute consisted of sixteen 150 L round flat-bottomed tanks with marbled sides (Cobcroft and Battaglene, 2009) and black bottoms and external stand pipes. Lighting was provided by fluorescent globes emitting 2.80 µmol ± 0.09 (mean ± SE) of light, measured from the water surface at middle of each tank. Photoperiod was maintained at 16-h light: 8-h dark throughout the experiment. Seawater was filtered through one micron bag filters and circulated through heat chillers. Flow rates in all of the tanks were maintained at 108 L h^{-1} from the beginning of the experiment and increased to 144 L h^{-1} after 30 d and 162 L h^{-1} after 50 d to preserve water quality. Water quality was measured daily using an YSI 660 Multi-probe (YSI Incorporated, Ohio, USA) for temperature, pH and salinity and an YSI 220

probe (YSI Incorporated, Ohio, USA) for oxygen. Faeces and uneaten food were removed twice daily from the tanks using a siphon, once before the first feed and again in the afternoon. A batch exchange of 70 % of the tank volume was performed at 16:00 daily. Water quality measures (mean ± SE) were 8.13 ± 0.08 pH, 33.8 ± 0.2 ppt salinity and 95.9 ± 2.6 % oxygen saturation for the duration of the experiment.

15.3.2 *Experimental design*

Sixteen 300 dph fish (12.1 ± 0.2 g, 114.0 ± 0.5 mm, mean ± SE; n = 256) were stocked randomly into each replicate tank. Fish were acclimatised for a period of 10 d. During acclimation, temperatures were adjusted by a maximum of one degree day^{-1} until the assigned treatment temperatures were reached and maintained. Fish were fed a commercial extruded diet (Nutra TP™ 1.5 mm; Skretting, Australia). The composition of the diet was 937.8 g kg^{-1} dry matter (DM), 483.7 g kg^{-1} DM crude protein, 262.2 g kg^{-1} DM total lipid, 124.0 g kg^{-1} DM ash and 20.5 MJ kg^{-1} gross energy. The feed was dispensed using computerised automatic feeders with an equal amount of food being dispensed every hour throughout the day (Curnow et al., 2006). Fish were fed to satiation by observing leftover pellets and adjusting feed volumes accordingly.

The growth experiment was conducted over 84 d. Each row of four replicate tanks was randomly assigned a temperature of 12, 14, 16 or 18 ^{o}C. At the beginning of the experiment and every 21 d thereafter all of the fish were anaesthetised using Aqui-S™ (Aqui-S, New Zealand) at a dose of 1/200 v/v Aqui-S™ in seawater. Each fish was weighed (to 0.1 g), measured for total length (to 1 mm) and scored for development using the characteristic appearance of lateral stripes scored on a scale of zero to three, with zero indicating the absence of stripes and three indicating the completion of development into juveniles (Figure 15.1). After each weight check, fish were given a prophylactic dose of oxytetracycline incorporated into the food at 100 mg kg^{-1} biomass for 10 d to reduce the risk of bacterial infection. Feed intake (FI) was measured 10 d after weight checks upon the resumption of pre-handling feeding behaviour. Fish were fed every hour until satiation and the uneaten food was collected by siphoning at 4, 9 and 14 h. Recovered material was rinsed with a 0.5 mol ammonium formate solution to remove salt and sieved through a 1 mm mesh prior to drying. Samples were dried in a 60 ^{o}C oven for 48 h and weighed. At the end of the experiment, four fish were randomly sampled from each tank, autoclaved (Williams et al., 1995) and freeze dried to a constant weight prior to whole-body composition analysis. Five fish randomly sampled at the start of the experiment were used as an initial measure of whole-body composition.

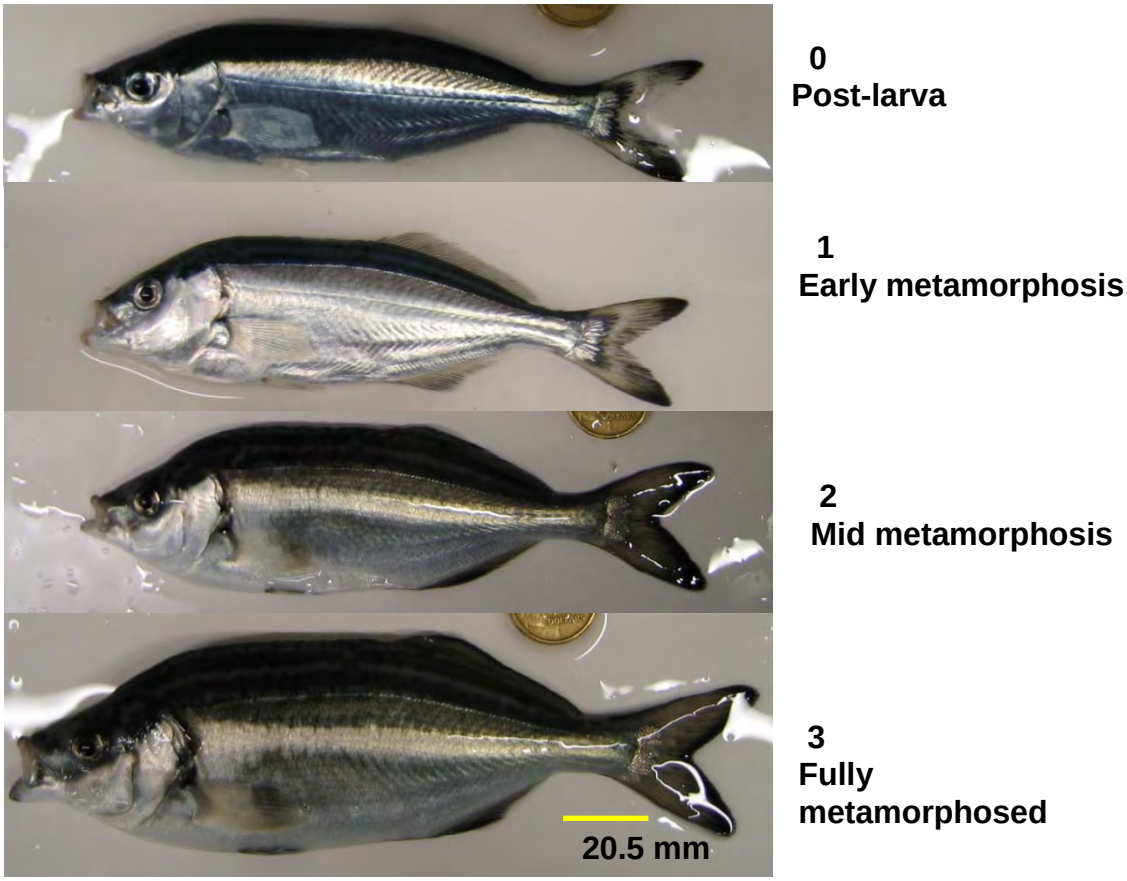

Figure 15.1 Photographs showing the progression of metamorphosis with scores used for this experiment (0 – post-larvae, 1 – early metamorphosis, 2 – mid metamorphosis 3 – fully metamorphosed). The appearance of dark stripes across the body was used to identify the stages of metamorphosis.

15.3.3 *Chemical analysis*

The dry weight of the commercial extruded diet samples and individual fish were determined by freeze drying to a constant weight. Freeze dried samples of the diet and individual fish were ground to a homogenous powder prior to chemical analysis. Standard methods were used to determine crude protein (Thermo Finnigan 1112 Series elemental analyser, *N* X 6.25); total lipid (Bligh and Dyer, 1959); and ash (combustion at 550 °C for 6 h) (AOAC, 1995). Energy content was calculated using conversion factors for protein (23.6 kJ g^{-1}) and lipid (36.2 kJ g^{-1}) (Brafield, 1985). Three replicate measurements for crude protein, total lipid and ash were performed for each fish. Energy content of the diets was determined directly using a bomb calorimeter. All composition data are presented as a mean percentage of the wet weight (WW) of each individual fish sampled from each treatment (Shearer, 1994).

15.3.4 *Calculations*

Condition factor (*k*) was calculated using the formula: $k = WW / TL^3$, where WW is wet weight (g) and TL is total length (cm) (Weatherley and Gill, 1987). Specific growth rate (SGR, % d^{-1}) was calculated from individual fish weights using the formula: $SGR = 100 \times (lnFBM - lnIBM) / d$) where FBM is final biomass (g); IBM the initial biomass (g) and d the number of days. The thermal-unit growth coefficient (TGC) was calculated using the formula: $TGC = 100 \times (FBM^{1/3} - IBM^{1/3}) / TDD$; where FBM is final biomass (g); IBM the initial biomass (g) and TDD is the total degree days; TDD was calculated by multiplying the temperature by the number of days. Coefficient of variation of WW for weight per tank (CV, %) was calculated as: 100 x *standard deviation* / *mean wet weight*. Feed intake was calculated by subtracting the dry weight of the recovered feed from the amount of feed dispensed. Food conversion efficiency (FCE, %) was calculated using the formula: *FCE* = ((*FBM-IBM*) / *TFI X 100)*; where TFI is total feed intake (g) for the period. Productive protein value (PPV, %) was calculated using the formula: *PPV* = 100 X (*Fish protein gain (g)* / *Total protein consumed (g)*). Productive energy value (PEV, %) was calculated using the formula: *PEV* = 100 X (*Fish energy gain (MJ)* / *Total energy consumed (MJ)*).

15.3.5 *Statistical analysis*

All results are reported as mean ± SE. Data were tested for normality and homogeneity using Levene's test of equality. Percent data were arcsine square-root-transformed prior to analysis. Testing tank placement across rows as a separate factor did not produce any significant differences. Analysis of wet weight and total length were therefore performed using a one-way ANOVA with temperature as the sole factor. One-way ANOVA was used to detect differences in SGR, TGC, FI, FCE, PPV and PEV. A Tukey's post-hoc multiple comparison test was used to analyse significant ANOVA results. Statistical significance was accepted at $P = 0.05$. Statistical analyses were performed using SPSS version 15.0 statistical software. Regression analysis and fitting quadratic polynomial models were performed using SigmaPlot version 9.0.

15.4 Results

15.4.1 *Temperature and survival*

The mean daily temperature over the 84 d experimental period was stable throughout the trial within treatments (12.1 ± 0.1, 14.1 ± 0.1, 16.1 ± 0.1, 17.9 ± 0.1 oC, n = 4 tanks). One replicate tank from the 12 ^{o}C treatment lost eight fish due to an overdose of anaesthetic during the 21 d measurement; feeding and other analyses were adjusted to account for this reduction in animals. Excluding accidental losses survival for the whole experiment (99.2 ± 0.1 %) was high across all treatments.

15.4.2 *Growth*

At 0 d, there were no significant differences in the mean WW and TL of fish among treatments. Initial mean WW and mean TL of fish for all treatments were 12.1 ± 0.2 g and 114 ± 0.5 mm (n = 320). At 21 d, fish at 14 ^{o}C and at 16 ^{o}C were significantly heavier compared to those at 12 ^{o}C and at 18 ^{o}C (df = 3, F = 13.8, P < 0.001). There were no significant differences found in TL among treatments. At 42 d, fish at 14 ^{o}C and at 16 ^{o}C were significantly heavier than fish at 12 ^{o}C; fish at 18 ^{o}C were the smallest (df = 3, F = 39.8, P < 0.001). Fish at 12 ^{o}C were significantly shorter compared to other treatments (df = 3, F = 8.1, P = 0.03). At 63 d, fish at 14 ^{o}C and at16 ^{o}C were significantly heavier than fish at 12 ^{o}C and at 18 ^{o}C, which were similar (df = 3, F = 17.1, P < 0.001). Fish at 12 ^{o}C were significantly shorter (df = 3, F = 10.4, P = 0.001). At 84 d, fish at 14 ^{o}C were significantly heavier than those at 12 ^{o}C and at 18 ^{o}C, the smallest fish were found in the 18 ^{o}C treatment (df = 3, F = 10.9, P = 0.001). Fish at 12 ^{o}C were significantly shorter than fish at 14 ^{o}C and 18 ^{o}C which had similar lengths (df = 3, F = 4.1, P = 0.03) (Figure 15.2; Table 15.1). There were no significant differences in condition factor among treatments on sampling days (data not presented).

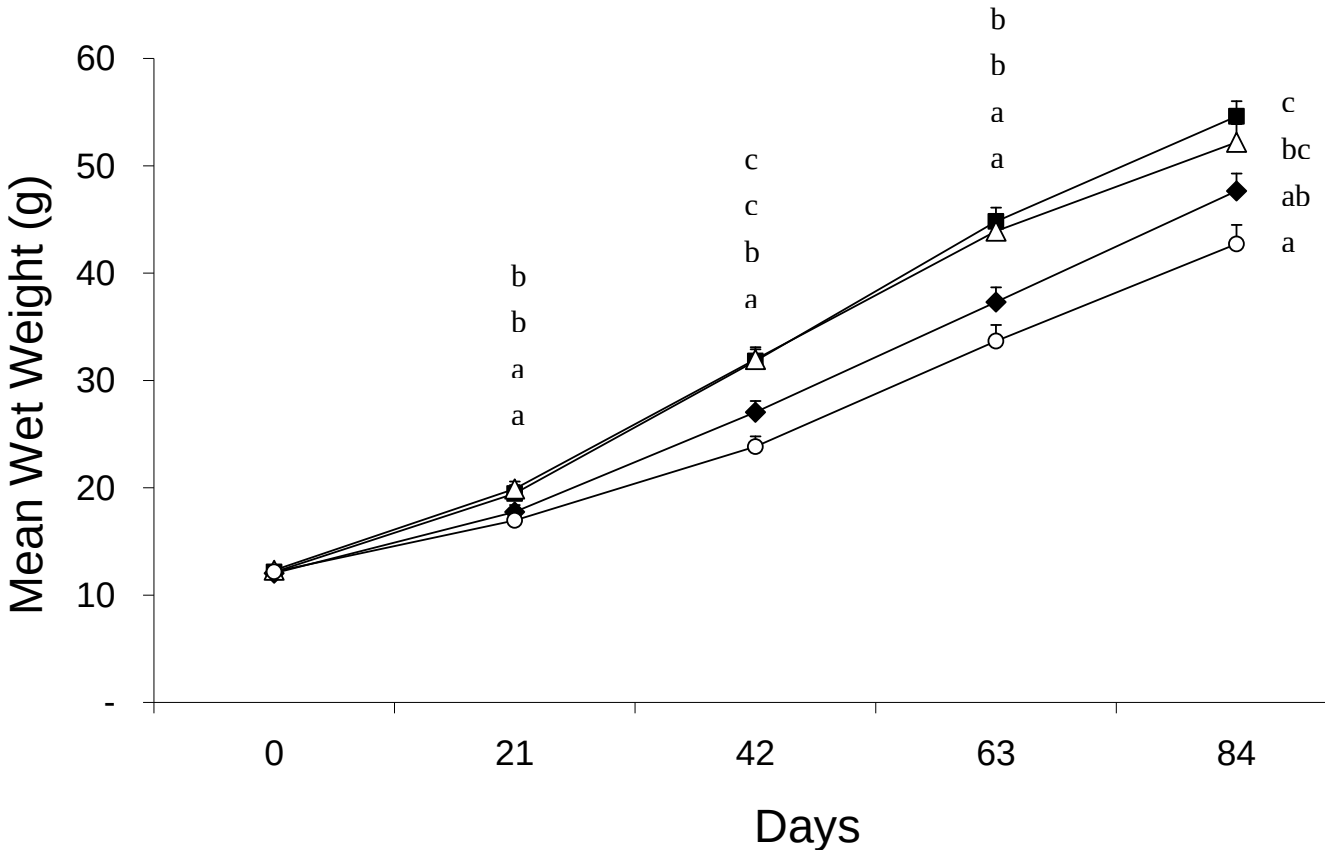

Figure 15.2 Growth (mean WW ± SE) of striped trumpeter post-larvae at four different temperatures (■ = 14^{o}C, Δ = 16^{o}C, ○ = 18^{o}C, ♦ = 12 ^{o}C) over 84 d. Significant differences in wet weight are represented using different letters at each 21 d measurement ($P < 0.05$).

Table 15.1 Total length (TL) and coefficient of variation of wet weight (CV) (mean ± SE) of striped trumpeter at four different temperatures.

Mean TL (mm)	12 °C	14 °C	16 °C	18 °C
0-days	112.8 ± 0.3[a]	114.0 ± 0.2[a]	115.0 ± 0.03[a]	114.4 ± 0.2[a]
21-days	124.2 ± 0.3[a]	125.5 ± 0.4[b]	125.8 ± 0.18[b]	122.2 ± 0.4[a]
42-days	137.4 ± 0.3[b]	141.8 ± 0.5[c]	142.4 ± 0.26[c]	132.3 ± 0.5[a]
63-days	148.1 ± 0.2[a]	155.5 ± 0.7[b]	154.8 ± 0.41[b]	142.6 ± 0.7[a]
84-days	159.2 ± 0.2[ab]	164.4 ± 0.7[c]	163.2 ± 0.71[bc]	152.3 ± 0.7[a]

CV of WW (%)	12 °C	14 °C	16 °C	18 °C
0-days	24.8 ± 1.9	25.7 ± 1.0	23.3 ± 0.8	25.9 ± 1.2
21-days	29.2 ± 2.2	32.0 ± 3.0	29.7 ± 2.1	29.5 ± 2.7
42-days	30.0 ± 1.9	27.5 ± 1.0	28.3 ± 1.7	32.2 ± 3.8
63-days	28.1 ± 2.0[ab]	22.7 ± 0.3[a]	27.2 ± 0.3[ab]	34.0 ± 3.6[b]
84-days	26.1 ± 1.8[ab]	20.3 ± 0.2[a]	25.6 ± 1.1[ab]	32.3 ± 3.4[b]

Within periodic measurements, treatments not sharing a common letter are significantly different ($P < 0.05$).

The CV of WW among treatments was not significantly different at 0 d, 21 d or 42 d. The WW of fish at 18 °C had significantly higher CV compared to the 14 °C treatment at 63 d (df = 3, F = 5.2, P = 0.02) and at 84 d (df = 3, F = 6.5, P = 0.007), (Table 15.1).

Fish at 14 °C and 16 °C showed a significantly higher SGR at 21 d compared to fish at 12 °C and at 18 °C (df = 3, F = 8.4, P = 0.003). At 42 d, fish at 14 °C and at 16 °C showed significantly higher SGR compared to fish at 12 °C and at 18 °C (df = 3, F = 20.6, P < 0.001). There were no significant differences in SGR found among treatments neither at 63 d nor at 84 d (Table 15.2). From the beginning of the experiment to 42 d, majority of the population across all treatments did not metamorphose and were classified as post-larvae. During the post-larvae period SGR was highest at 14 °C and 16 °C, followed by 12 °C lowest at 18 °C (df = 3, F = 20.4, P < 0.001). From 43 d to 84 d, metamorphosis into juveniles was recorded. During this metamorphosis period, no significant differences in SGR were found. For the whole growth period, SGR at 14 °C and at 16 °C was significantly higher compared to 18 °C (df = 3, F = 10.1, P = 0.001). Fish at

18 ^{o}C showed the lowest overall SGR (Figure 15.3). Maximum SGR was predicted to be at 14.4 ^{o}C (Table 15.3).

Table 15.2 Specific growth rate (SGR) and thermal growth coefficient (TGC) of striped trumpeter (mean ± SE) at four different temperatures over time.

SGR (% increase day^{-1})	12 ^{o}C	14 ^{o}C	16 ^{o}C	18 ^{o}C
0-21 days	1.9 ± 0.1^{ab}	2.2 ± 0.1^{b}	2.1 ± 0.1^{b}	1.7 ± 0.1^{a}
22-42 days	1.9 ± 0.1^{a}	2.3 ± 0.1^{b}	2.3 ± 0.1^{b}	1.6 ± 0.1^{a}
43-63 days	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
64-84 days	1.2 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.1 ± 0.2
0-42 days	1.9 ± 0.1^{b}	2.3 ± 0.1^{c}	2.2 ± 0.0^{c}	1.6 ± 0.1^{a}
43-84 days	1.3 ± 0.0	1.3 ± 0.1	1.2 ± 0.1	1.4 ± 0.1
0-84 d	1.6 ± 0.0^{ab}	1.8 ± 0.0^{b}	1.7 ± 0.0^{b}	1.5 ± 0.0^{a}
TGC (% increase day^{-1})	12 ^{o}C	14 ^{o}C	16 ^{o}C	18 ^{o}C
0-21 days	12.1 ± 0.2^{b}	13.3 ± 1.0^{b}	11.0 ± 0.5^{b}	7.0 ± 0.3^{a}
22-42 days	19.0 ± 1.0^{b}	22.4 ± 0.6^{b}	19.4 ± 0.7^{b}	9.7 ± 0.7^{a}
43-63 days	21.6 ± 0.6^{b}	23.6 ± 1.3^{b}	18.7 ± 1.6^{ab}	13.9 ± 1.8^{a}
64-84 days	22.2 ± 1.0^{b}	17.8 ± 1.4^{ab}	13.0 ± 1.1^{a}	11.8 ± 2.3^{a}
0-42 days	15.5 ± 0.6^{b}	17.8 ± 0.7^{b}	15.2 ± 0.3^{b}	8.4 ± 0.5^{a}
43-84 days	22.0 ± 0.7^{b}	20.7 ± 1.2^{b}	15.8 ± 1.3^{a}	12.9 ± 0.8^{a}
0-84 d	18.7 ±0.1^{c}	19.3 ± 0.8^{c}	15.5 ± 0.6^{b}	10.6 ± 0.6^{a}

Within periodic measurements, treatments not sharing a common superscript are significantly different ($P < 0.05$).

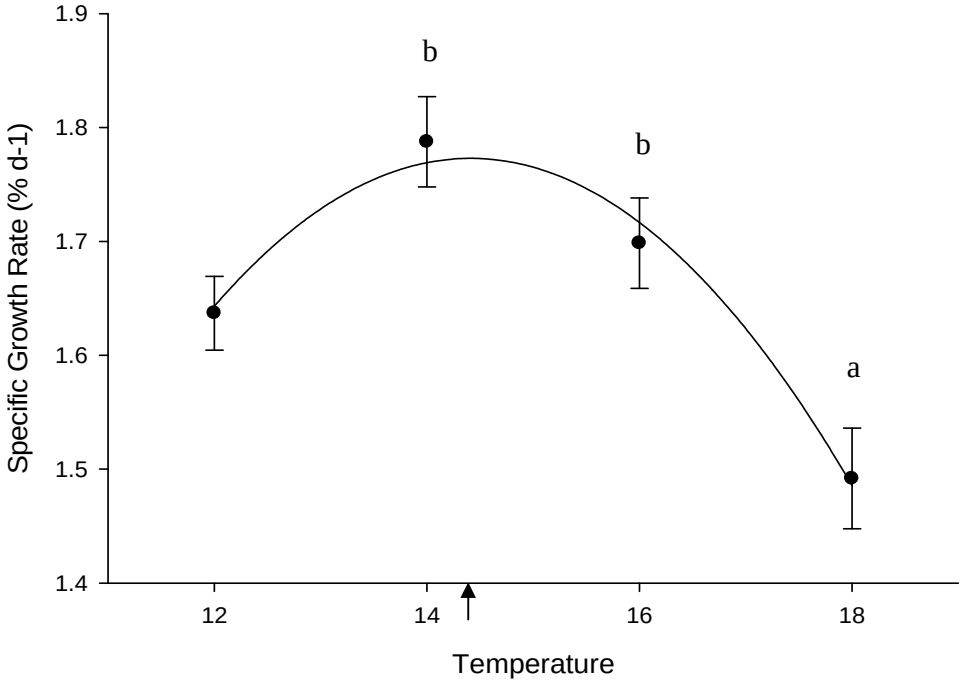

Figure 15.3 Relationship between specific growth rate (% d^{-1}) and temperature described with a quadratic polynomial curve. Significant differences are represented using different letters ($P < 0.05$). Arrow indicates predicted maximum.

Table 15.3 Relationships between feed intake (FI), growth (SGR), productive protein value (PPV) and productive energy value (PEV) and temperature (T, ° C)

	$y = a+bT+cT^2$					
	a	b	c	R^2	$F_{2,15}$	*P*
SGR (% d^{-1})	-2.86	0.64	-0.02	.70	15.5	.0004
TGC	-37.03	8.71	-0.34	.91	59.6	<.0001
FI (g d^{-1})	-56.07	9.24	-0.30	.83	29.3	<.0001
PPV (%)	-5.54	4.33	-0.17	.80	23.6	<.0001
PEV (%)	-45.04	10.17	-0.37	.69	13.2	.0009

Temperature related growth expressed as TGC of fish at 18 ^oC was significantly lower at 21 d compared to the other treatments which had similar TGC (df = 3, F = 23.5, P < 0.001). At 42 d,

fish at 18 °C again had significantly lower TGC compared to the other treatments which were similar (df = 3, F = 56.2, P < 0.001). At 63 d, TGC of fish at 12 °C and at 14 °C were significantly higher compared to the TGC at 18 °C (df = 3, F = 8.2, P = 0.004). At 84 d, TGC of fish at 12 °C was significantly higher compared to those of fish at 16 °C and 18 °C (df = 3, F = 6.8, P = 0.007). Growth during pre-metamorphosis (0 to 42 d) was significantly lower at 18 °C compared to the other treatments which were similar (df = 3, F = 58.0, P < 0.001). During the period of metamorphosis (42 to 84 d), fish at 12 °C and at 14 °C had similar TGC which were significantly higher compared to fish at 16 °C and at 18 °C (df = 3, F = 14.8, P < 0.001) (Table 15.2). Overall TGC was significantly higher at 12 °C and at 14 °C compared to 16 °C. Fish at 18 °C showed the lowest overall TGC (df = 3, F = 44.1, P < 0.001), (Figure 15.4). Maximum TGC was predicted to be at 12.9 °C (Figure 15.4).

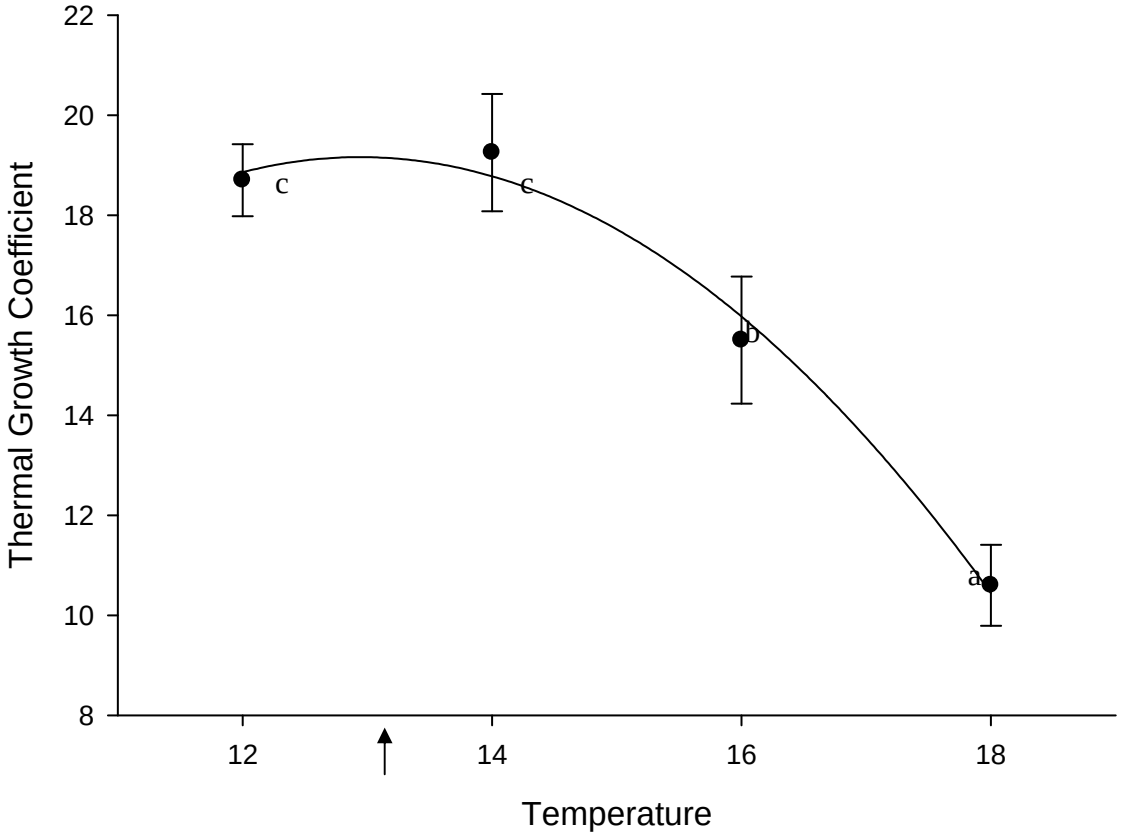

Figure 15.4 Relationship between temperature (°C) related growth expressed as thermal growth coefficient and temperature described with a polynomial curve. Significant differences are represented using different letters ($P < 0.05$). Arrow indicates predicted maximum.

Feed intake (g d-1) was significantly higher at 14 ^{o}C and 16 ^{o}C. Fish at 12 ^{o}C ate the least amount of food (df = 3, F = 20.1, P < 0.001), (Figure 15.5). Maximum feed intake was predicted at 15.4 ^{o}C (Figure 15.5).

At 12 ^{o}C, 14 ^{o}C and 16 ^{o}C FCE were similar and were significantly higher than at 18 ^{o}C at 21 d (df = 3, F = 16.1, P < 0.001) and at 42 d (df = 3, F = 30.6, P < 0.001). There was no significant difference in FCE among treatments at 63 d. At 84 d, the FCE of fish at 12 ^{o}C was significantly higher compared to 16 ^{o}C (df = 3, F = 5.1, P = 0.02). During the post-larval growth phase (0 to 42 d), FCE was lowest at 18 ^{o}C and similar at 12 ^{o}C, 14 ^{o}C and 16 ^{o}C (df = 3, F = 57.4, P < 0.001). During the period of metamorphosis (43 d to 84 d), fish at 12 ^{o}C had significantly higher FCE compared to fish at 16 ^{o}C (df = 3, F = 5.2, P = 0.01) (Table 15.4). Overall FCE was significantly higher at 12 ^{o}C compared to the FCE at 16 ^{o}C and at 18 ^{o}C. Fish at 18 ^{o}C had the lowest overall FCE (df = 3, F = 18.6, P < 0.05), (Table 15.4); (Figure 15.6).

Table 15.4 Feed conversion efficiency (%; mean ± SE) of striped trumpeter at four different temperatures.

FCE (%)	12 ^{o}C	14 ^{o}C	16 ^{o}C	18 ^{o}C
0-21 days	74.2 ± 1.9^{b}	72.2 ± 2.7^{b}	65.6 ± 2.4^{b}	46.7 ± 2.1^{a}
22-42 days	76.7 ± 2.7^{b}	69.9 ± 1.4^{b}	71.8 ± 2.7^{b}	45.5 ± 2.4^{a}
43-63 days	59.0 ± 2.1^{a}	60.9 ± 2.0^{a}	54.0 ± 4.1^{a}	53.3 ± 4.8^{a}
64-84 days	44.5 ± 2.0^{b}	35.4 ± 2.6^{ab}	29.5 ± 1.9^{a}	34.9 ± 3.9^{ab}
0-42 days	75.0 ± 1.5^{b}	71.0 ± 1.6^{b}	69.3 ± 1.3^{b}	45.8 ± 2.3^{a}
43-84 days	50.7 ± 1.8^{b}	46.5 ± 1.9^{ab}	40.3 ± 2.6^{a}	42.8 ± 1.5^{ab}
0-84 days	58.8 ± 0.5^{c}	55.3 ± 0.7^{bc}	50.8 ± 0.8^{b}	43.8 ± 0.8^{a}

Within periodic measurements, treatments not sharing a common superscript are significantly different ($P < 0.05$).

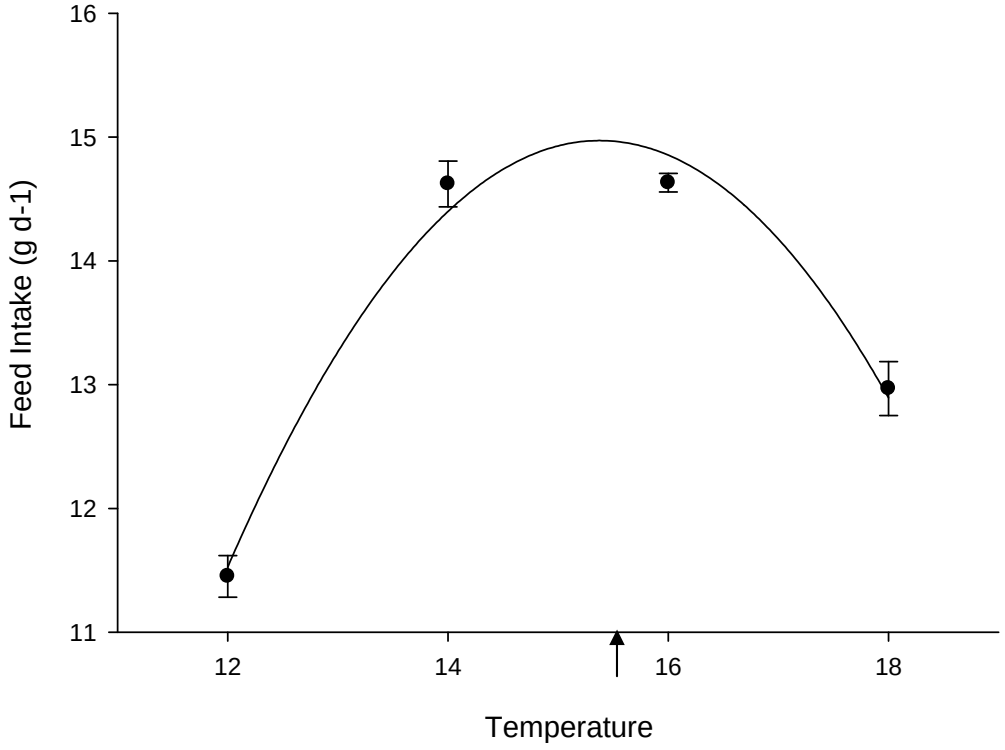

Figure 15.5 Relationship between feed intake (g d^{-1}) and temperature (^{o} described with a quadratic polynomial curve. Significant differences are represented using different letters ($P <$ 0.05). Arrow indicates predicted maximum.

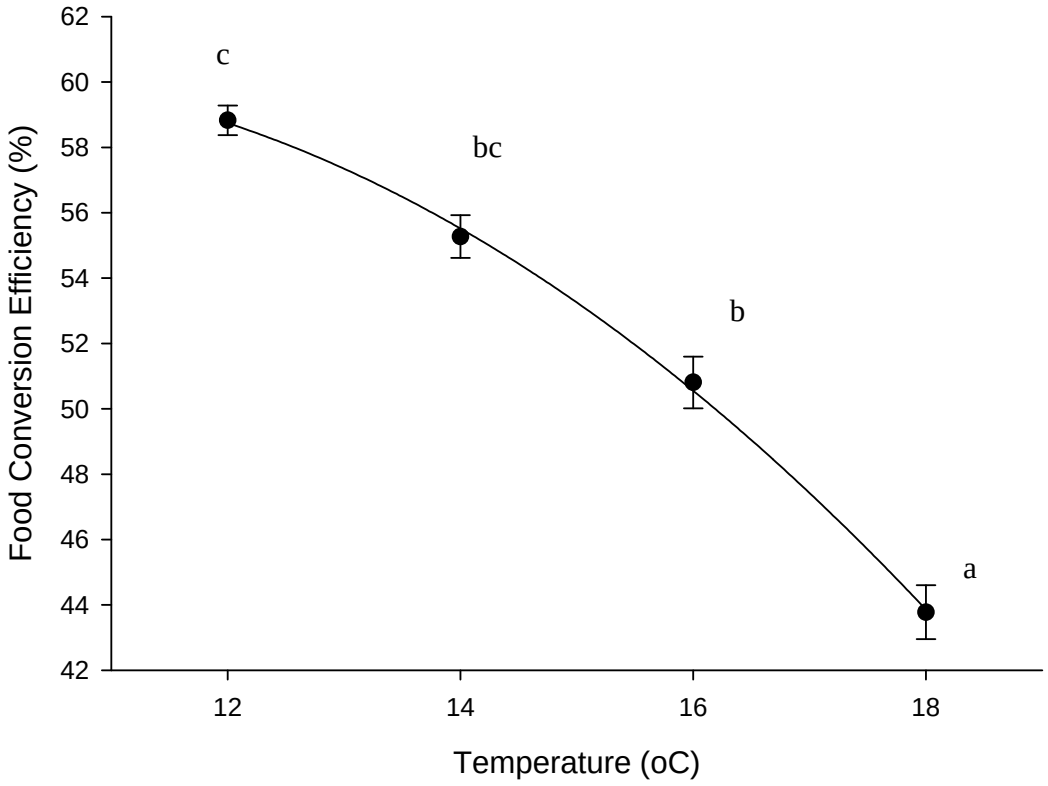

Figure 15.6 Relationship between food conversion efficiency (%) and temperature described with a polynomial curve. Significant differences are represented using different letters (P < 0.05).

15.4.3 *Chemical composition*

The crude protein content of fish at 16 ^{o}C was significantly higher compared to fish at 12 ^{o}C (df = 3, F = 5.1, P < 0.05). The total lipid content of fish reared at 14^{o}C was significantly higher than fish at 12 ^{o}C and at 18 ^{o}C (df = 3, F = 5.1, P< 0.05). Fish at 18^{o}C had significantly higher ash content compared to the other three treatments which were similar (df = 3, F=8.2, P < 0.05). The moisture content of fish at 12^{o}C was significantly higher than those at 14^{o}C and 16^{o}C (df =3 , F = 7.7, P < 0.05) (Table 15.5). A significant linear relationship between k and carcass total lipid content was found (df = 63, F = 32.1, P < 0.05), (Figure 15.7).

Table 15.5 Whole body chemical composition of striped trumpeter (mean ± SE) at four temperatures after 84 d.

Whole body composition	12 °C	14 °C	16 °C	18 °C
Moisture (% WW)	71.2 ± 0.4[b]	69.0 ± 0.20[a]	69.1 ± 0.3[a]	70.4 ± 0.6[ab]
Crude protein (% WW)	16.7 ± 0.2[a]	17.1 ± 0.14[ab]	17.7 ± 0.1[b]	17.2 ± 1.0[ab]
Total lipid (% WW)	9.0 ± 0.4[a]	11.4 ± 0.24[b]	10.5 ± 0.4[ab]	9.1 ± 0.6[a]
Ash (% WW)	2.8 ± 0.1[a]	2.8 ± 0.05[a]	2.9 ± 0.0[a]	3.1 ± 0.1[b]

Within periodic measurements, treatments not sharing a common superscript are significantly different ($P < 0.05$).

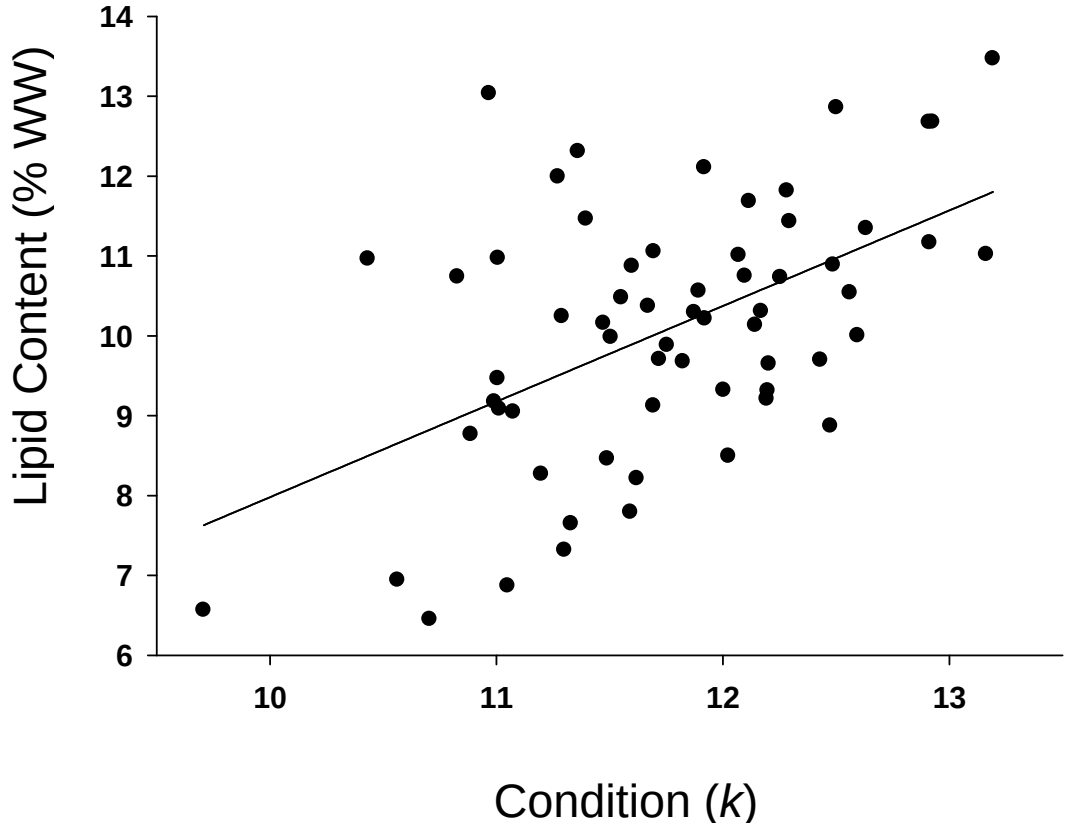

Figure 15.7 The linear relationship between condition (*k*) and lipid content Lipid content (% WW) = 1.2 X Condition (*k*) - 0.4 ($r^2 = 0.28$, F = 22.3, $P < 0.001$)

15.4.4 *Nutrient retention efficiency*

At 18 ^{o}C fish showed significantly lower PPV values compared to fish at 12 ^{o}C , 14 ^{o}C and at 16 ^{o}C which had similar PPV (df = 3, F = 15.6, $P < 0.05$) (Figure 15.8). Maximum PPV was predicted to be at 12.7 ^{o}C (Figure 15.8) using a polynomial curve (Table 15.3). At 18 ^{o}C fish showed significantly lower PEV compared to fish at 12 ^{o}C , at 14 ^{o}C and at 16 ^{o}C which had similar PEV (df = 3, F = 7.7, $P < 0.05$) (Figure 15.9). Maximum PEV was predicted to be at 13.8 ^{o}C (Figure 15.9) using a polynomial curve (Table 15.3).

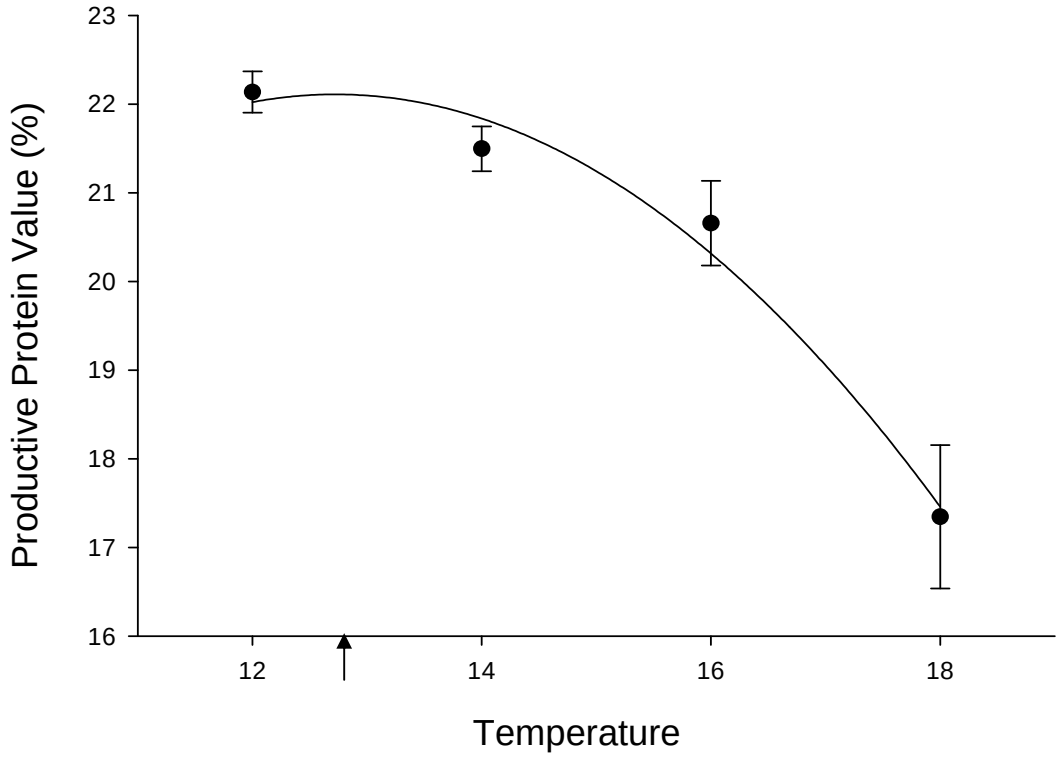

Figure 15.8 Relationship between productive protein value (%) and temperature (^{o}C) described with a polynomial curve. Significant differences are represented using different letters ($P < 0.05$).

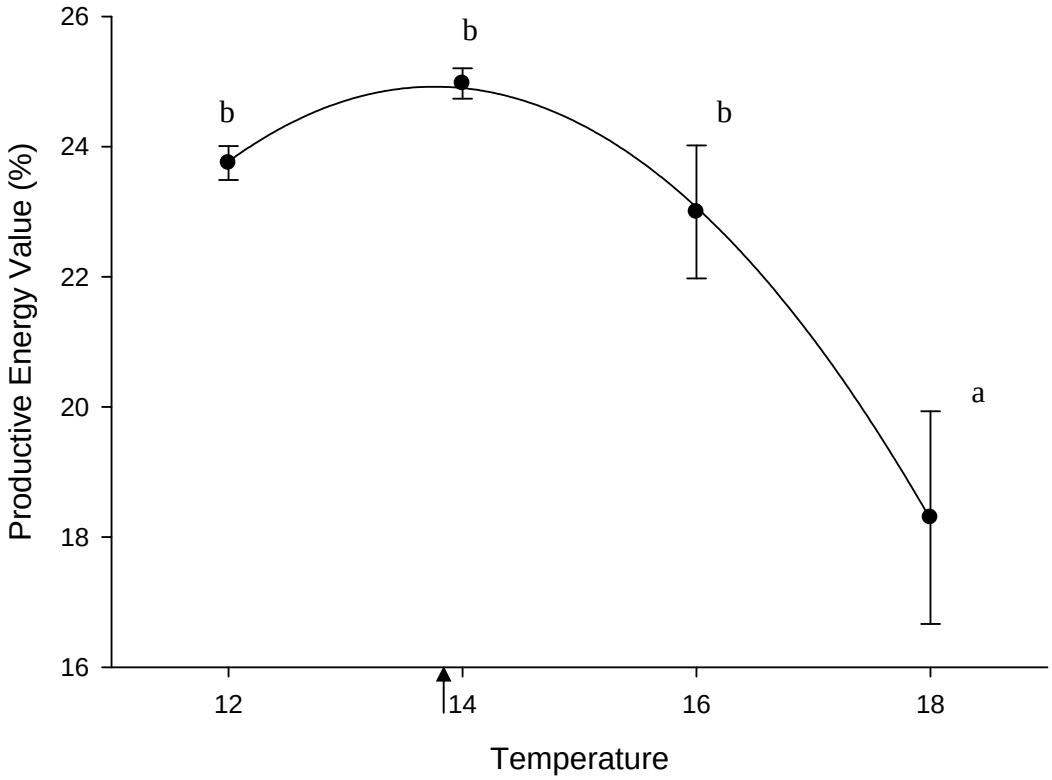

Figure 15.9 Relationship between productive energy value (%) and temperature (^{o}C) described with a polynomial curve. Significant differences are represented using different letters (P < 0.05)

15.4.5 *Metamorphosis*

Metamorphosis into juveniles was first observed in two fish at 42 d at 12 ^{o}C (Figure 15.10). At 63 d, the 14 ^{o}C treatment (47.08 ± 13.45 %) had a significantly higher incidence of metamorphosed individuals compared to the 18 ^{o}C treatment (11.04 ± 3.94 %) (df = 3, F = 4.4, P < 0.05). At 84 d, fish in the 12 ^{o}C (100 %) and 14 ^{o}C (98.44 ± 1.56 %) treatments had higher incidence of metamorphosed individuals which was significantly higher than the 16 ^{o}C treatment (66.2 ± 3.0 %). The 18 ^{o}C treatment (33.1 ± 6.2 %) had the lowest proportion of metamorphosed individuals (df = 3, F = 92.6, P < 0.05) (Figure 15.10). The logistic curve of all observations of WW against metamorphosis score for each temperature showed that fish metamorphosed at a smaller size in the 12 ^{o}C treatment and metamorphosed at progressively larger sizes with higher temperatures (Figure 15.11). Aggressive behaviour was observed in the larger fish which had completed metamorphosis into juveniles. Aggressive behaviour was characterised by larger fish chasing the smaller fish and was pronounced immediately before the first feed of the day. As more feed was dispensed throughout the day, aggression towards the smaller fish decreased.

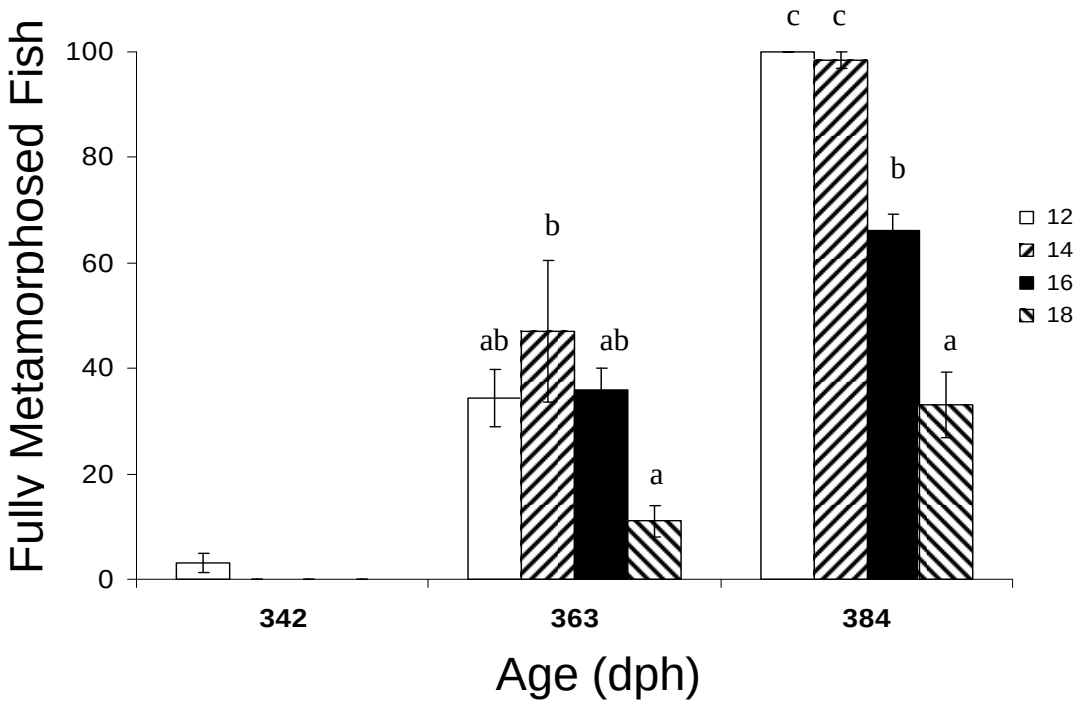

Figure 15.10 Percentage of fully striped fish in each treatment. Within measurements, treatments not sharing the same letter are significantly different ($P < 0.05$). Bars represent standard error.

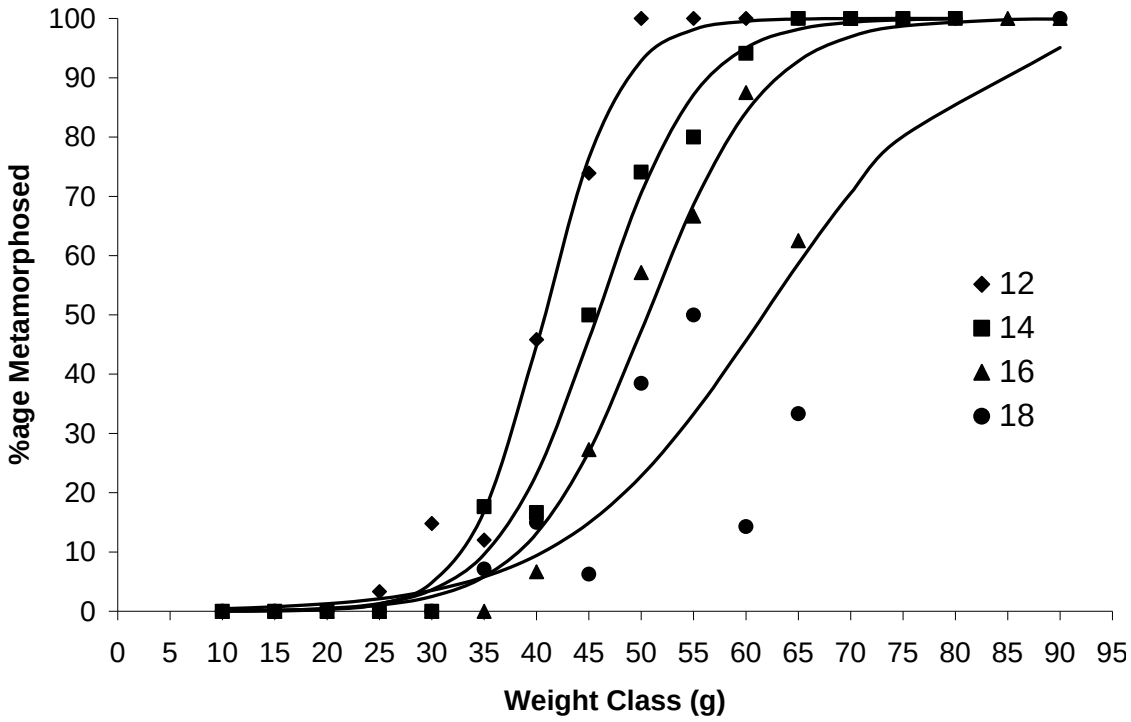

Figure 15.11 The incidence of completely metamorphosed individuals at each weight class (all observed individual wet weights) with best fit logistic curves. 12 ^{o}C = (exp(-11.3 + 0.3x) / 1 + exp(-11.3 + 0.3x))*100; 14 ^{o}C = (exp(-9.5 + 0.2x) / 1 + exp(-9.5 + 0.2x))*100; 16 ^{o}C = (exp(-9.0 + 0.2x) / 1 + exp(-9.0 + 0.2x))*100; 18 ^{o}C= (exp(-6.4 + 0.2x) / 1 + exp(-6.4 + 0.2x))*100; where x = weight class (g)

15.5 Discussion

This is the first study to identify the optimum temperature for rearing late-stage striped trumpeter post-larvae (< 15 g) to metamorphosis into juveniles (~50 g). The effect of temperature on growth of striped trumpeter was clearly shown in this study. Growth was highest at 14 ^{o}C and 16 ^{o}C with a predicted maximum between 12.9 to 14.4 ^{o}C, depending on the growth indices used. This agrees with other studies where optimum temperature for growth was found to be between the temperatures where feed intake is highest, 15.4 ^{o}C for our study, and where food conversion efficiency is highest, <12 ^{o}C (Kestemont and Barras, 2001). Striped trumpeter are adapted to a specific oceanic surface water niche as post-larvae and the narrow range of temperatures where growth is maximised is similar to other temperate species such as pollack (*Pollachius pollachius*) (12-15 ^{o}C), (Le-Ruyet et al., 2006) and Atlantic cod (*Gadus morhua*) (14.5 ^{o}C), (Kling et al., 2007). We conclude that the optimum temperature for growth is a reflection of the unique life history of fish species and their physiological adaptations to their environment.

The effect of temperature on growth is related to its influence on feed intake and metabolic activity (Jobling, 1994). Higher metabolic costs associated with elevated temperatures and the physical limitations to assimilate enough energy to meet this increase explain the

decrease in growth and nutrient retention efficiency (Katersky and Carter 2007; Handeland et al., 2008). This was clearly shown at 18 ^{o}C where fish had the lowest and therefore least efficient FCE, PPV and PEV values. As a consequence of low nutrient retention, ash content of fish reared in 18 ^{o}C was also highest, whereas fish in 14 ^{o}C had the highest total lipid content and fish in 16 ^{o}C reported the highest total lipid content. We suggest that temperature effects on composition of the mass gained was an indirect consequence of higher metabolic costs associated with the higher temperatures, which lead to depressed growth rates and smaller fish at temperatures beyond the optimum.

The FCE and nutrient retention values in our study are relatively low compared to other commercially cultured species but typical of data for species new to aquaculture and where nutrient requirements are not well known such as studies on Atlantic cod (Kling et al., 2007) and pollack (Le-Ruyet et al., 2006). It is possible that the commercial diet used in the study was suboptimal in meeting the nutrient requirements of post-larval striped trumpeter and lead to low retention rates. The fish used in the study also possessed varying degrees of jaw malformations, typical of the hatchery reared striped trumpeter at the time, and this possibly had further negative impacts on feeding capacity (Battaglene and Cobcroft, 2007; Cobcroft and Battaglene, 2009). However, growth rates on the best treatment were high and fish grew by a factor of nearly five times.

The unique life history of the striped trumpeter characterised by its prolonged post-larval phase also influences nutrient retention as smaller fish have a greater metabolic demand (Jobling 1994; Shearer et al., 1994). Metamorphosis from larvae into juveniles occurs through a progression of saltatory steps (Balon, 1999). Juveniles are fish that have taken on the appearance of adults but do not possess mature gonads and the metamorphosis into juveniles is often associated with a change in habitat (Balon, 1999). It is assumed that functional morphology is determined by the fish's environment (Long, 1995). In our study, the further metabolic demand imposed by temperatures higher than the optimum meant that fish at 18 ^{o}C, and to a lesser degree those at 16 ^{o}C, spent more ingested nutrients on maintenance rather than towards nutrient accretion in tissues. The decelerated rate of building nutrient reserves at the higher temperatures had an adverse effect on the progress of metamorphosis into juveniles. In our study, a greater proportion of the population, at smaller sizes, were fully metamorphosed at 12 ^{o}C and at 14 ^{o}C. At the higher temperatures even large individuals failed to metamorphose. These findings should ideally be compared to the development of striped trumpeter post-larvae in the wild as it has been found that metamorphosis into juveniles occurs at different developmental thresholds in some fish e.g., sofie (*Chondrostoma toxostoma*) (Gozlan et al., 1999). However, almost no data are available on wild striped trumpeter post-larvae (Furlani and Ruwald, 1999; Tracy and Lyle, 2005).

In a review by Shearer et al., (1994) using salmonids, fish size, diet and life-history stage were identified as factors that primarily influenced fish composition. The influence of temperature on metabolic functions, nutrient retention and ultimately on chemical composition has cascading effects on the progression of life-history changes in other species such as Atlantic salmon and brown trout (*Salmo trutta*) (Jonsson and Jonsson, 2005). In our study, the predicted optimum temperatures for PPV (12.7 ^{o}C) and PEV (13.8 ^{o}C) agrees with our finding that more individuals metamorphose into juveniles at 12 ^{o}C and 14 ^{o}C. For the entire population, we found that fish with higher k values had higher lipid content but fish at the high temperatures with high k values did not metamorphose successfully. Therefore, composition is not the only cue for metamorphosis. Temperature clearly has a strong influence on the onset and completion of metamorphosis into juveniles, whether directly as

an environmental cue or indirectly by influencing metabolic activity and nutrient retention. Lower water temperatures could also act as a signal to migrating late-stage post-larvae indicating an appropriate environment to progress in their development into juveniles. Understanding nutrient accretion and energetic demands therefore has benefits for aquaculture where environmental conditions can be controlled by the culturist to yield superior growth.

Striped trumpeter has not been subjected to a commercial fishery quota in Tasmania, possibly as a consequence of inadequate catches to sustain a commercial fishery. It is a species with commercial potential and managing the population could increase abundance. Tracey and Lyle (2005) were able to model the growth and population dynamics of striped trumpeter stocks in Tasmania. Another stock management model, developed by Tracey (2007), incorporated reproductive biology and recruitment of the striped trumpeter. Both of these models would benefit by incorporating the data collected in the current study on the effects of temperature on growth. This would further refine understanding the effects of temperature on growth, reproduction and exploitation limits of striped trumpeter stocks. Studies into environmental conditions for the culture of striped trumpeter are important because of the inability of confined animals to select the optimum conditions for growth. The findings will find immediate application in the development of aquaculture of the striped trumpeter. Studies are currently underway to assess if post-larvae can be reared in sea cages and the best time to introduce them to cage culture can now be assessed within known temperature optima. It is highly desirable to reduce the period between post-larvae and metamorphosis into juveniles due to the extended duration of the paper fish stage compared to other fish species, the delicate nature of post-larvae and the possibility of significant mortalities caused by stress during this period (Tracey et al., 2006; Battaglene and Cobcroft, 2007).

15.6 Acknowledgements

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16 THE EFFECTS OF RATION AND DIETARY LIPID ON GROWTH OF POST-LARVAL STRIPED TRUMPETER (*LATRIS LINEATA*)

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16.1 Abstract

Striped trumpeter (*Latris lineata*) is a new candidate species for adoption by the Tasmanian aquaculture industry. In preparation for future trials in sea cages, an experiment was conducted to investigate feeding strategies and the effects of dietary lipid inclusion level. Post-larval striped trumpeter (8.1 ± 0.1 g fish-1) were reared using a combination of 33 %, 67 % or 100 % satiation rations and low (18 %) or high lipid (24 %) diets at a constant temperature of 15 ^{o}C. The diets were formulated to only vary nutritionally in the fish oil component. The 33 % and 67 % rations were determined by adjusting proportionally to the feed intake of fish fed to satiation (100 % ration) at the start of each week. Fish were reared for 63 d and at the end of the experiment, three fish representing post-larvae (incomplete metamorphosis) and three fish representing juveniles (complete metamorphosis) were taken from each replicate tank (n = 4) and measured for whole body chemical composition. The 100 % (22.7 ± 1.0 g) and 67 % ration (21.1 ± 1.1 g) produced similar weight gain; the 33 % ration (15.4 ± 0.7 g) produced significantly smaller fish at the end of the experiment. Food conversion efficiency was highest at the 33 % ration (40.9 ± 3.2 %) compared to the 67 % (37.6 ± 3.0 %) and 100 % (30.1 ± 2.7 %) rations which were similar. The 67 % (22.6 ± 0.0) and 100 % (22.7 ± 0.0) ration showed significantly higher proportions of fully metamorphosed individuals compared to the 33 % ration (8.6 ± 0.0). Dietary lipid did not have a significant effect on growth nor on food conversion efficiency. Data from starvation trials on similar sized fish reared at similar temperatures were incorporated to estimate optimum rations. The optimum ration for late stage post-larvae was found to be 4 % biomass d-1. A dietary lipid content of 24 % of dry matter produced post-larvae with significantly higher carcass lipid content (5.8 ± 0.3 % of wet weight). Post-larvae were shown to have significantly higher carcass ash content and significantly lower carcass lipid content compared to juveniles. Metamorphosis into juveniles required fish to reach a minimum weight of 23 g and a carcass lipid content of at least 4 %. Metamorphosis of the majority of post-larvae (> 50 %) was predicted to occur at weights above 20 g and carcass lipid content of 7 %. All post-larvae were predicted to have metamorphosed by 40 g. This is the first experiment to investigate the effects of ration and different dietary lipid levels on post-larval striped trumpeter metamorphosis and growth. The results emphasise the need for proper feed management to increase growth and feed efficiencies to shorten the post-larval rearing period.

16.2 Introduction

To remain competitive in the rapidly developing global seafood industry, the Tasmanian aquaculture industry is studying the striped trumpeter (*Latris lineata*) as an alternative

species for aquaculture to Atlantic salmon (*Salmo salar*) (Searle and Zacharin 1994; Battaglene et al., 2008). The striped trumpeter is endemic to Tasmania and can potentially be cultured in other temperate regions. It produces firm white flesh that has high levels of polyunsaturated fatty acids (Nichols et al., 1994; Nichols et al., 2005). Research on the striped trumpeter has advanced larviculture and broodstock management to the extent that production of post-larvae is now routine (Battaglene and Cobcroft, 2007). However, the production of commercial quantities of juveniles is complicated by the prolonged neustonic, oceanic, post-larvae or 'paperfish' phase that lasts up to nine months (Furlani and Ruwald, 1999; Tracey et al., 2006; Battaglene and Cobcroft, 2007). Striped trumpeter post-larvae are susceptible to stress induced mortality and rearing under optimum conditions to increase growth is seen as a viable option to shorten the duration of the post-larval stage. Temperature has a significant effect on growth and the optimum temperature for rearing post-larvae has been determined experimentally as 14 ^{o}C (see Chapter 15).

Feeding is another important factor that has a significant influence on growth rates and performance of cultured fish species (Brett and Groves, 1979; Bureau et al., 2006; Ahmed 2007). Inadequate feed input leads to slower growth rates, delayed development and increases size variation and aggression (Carter et al., 1992; McCarthy et al., 1992; Berrill et al., 2006). Overfeeding does not produce higher growth and leads to water quality deterioration, feed wastage and organic pollution (Tsevis et al., 1992; Mihelakakis et al., 2002; Yokoyama et al., 2009). Optimising feeding regimes and diet composition results in more efficient growth, decreased environmental impacts and increases profitability by minimising feed cost; this can account for up to 50% of variable costs in farm production (De Silva and Anderson, 1995; Lovell, 2002).

The objective of this study was to determine the optimum ration levels for post-larvae to produce the fastest growth at optimum temperatures. Two levels of dietary lipid inclusion (18% and 24%) were also tested for their effects on growth and metamorphosis. Lipid is the primary substrate for energy storage in fishes and is directly correlated to the condition and development of the fish (Weatherley and Gill, 1987; Shearer 1994). Striped trumpeter larvae have a high requirement for essential fatty acids, specifically arachidonic acid (20:4 n-6) and docosahexaenoic acid (22:6 n-3) (Bransden et al., 2005a,b). It is expected that faster growth will shorten the post-larvae stage and increase survival, ultimately lowering the production costs for striped trumpeter juveniles.

This study reared striped trumpeter post-larvae over a period of 63 d and the data were combined with studies on starvation from Choa (2010) to provide a preliminary estimate of the optimum ration for maximum growth and to investigate the effect of two dietary lipid inclusion levels on growth and performance of striped trumpeter post-larvae. Further, whole body chemical composition data for post-larvae and juveniles at the end of 63 d of growth were used to determine the thresholds for metamorphosis (Klemetsen et al., 2003; Berrill et al., 2004; Jonsson and Jonsson, 2005).

16.3 Materials and methods

16.3.1 *Experimental diets*

Two diets which varied nutritionally only in dietary lipid content were formulated to contain low dietary lipid (LDL) or high dietary lipid (HDL) (Table 16.1). Dry ingredients were

mixed with a Hobart mixer until homogenous (Fish meal, Skretting, Australia; Pre-gelatinised starch, Penford Australia Limited; Bentonite, Sigma Aldrich; α-Cellulose, Sigma Aldrich; Stay-C, Argent Laboratories; Inositol, Sigma Aldrich; Yttrium oxide, Sigma Aldrich; Carboxymethylcellulose, Sigma Aldrich; Choline chloride, Sigma Aldrich; Vitamin mix, Rabar, Queensland, Australia; Mineral mix, ingredients listed in Table 16.1; Potassium phosphate, Sigma Aldrich). Fish oil (Skretting, Australia) was then added to the diets and mixed until homogenous. Diets were cold pellet pressed to 1 mm diameter using a California Pellet Mill (Cl-2 laboratory pellet mill, California Pellet Mill Co., USA). Pellets were sieved and dried at 36 ^{o}C and stored at 4 ^{o}C.

Table 16.1 Ingredient and proximate composition of the experimental diets

Ingredient composition (g kg^{-1})	Low dietary lipid	High dietary lipid
Fish meal	690.00	690.00
Fish oil	90.00	140.00
Pre-gelatinised starch	100.00	100.00
Bentonite	44.50	14.50
α-Cellulose	40.00	20.00
Carboxymethylcellulose	10.00	10.00
Vitamin mix	3.00	3.00
Mineral mix[a]	5.00	5.00
Potassium phosphate (KH_2PO_4)	10.00	10.00
Stay-C	3.00	3.00
Choline chloride	2.00	2.00
Inositol	0.50	0.50
Yttrium oxide	2.00	2.00
Chemical composition (g kg^{-1} dry matter)		
Dry matter (g kg^{-1})	893.20	890.41
Crude protein	442.50	434.69
Total lipid	187.49	237.25
Ash	179.11	149.88
Gross energy (MJ g^{-1})	19.47	21.21

[a] Mineral mixture (mg kg^{-1} mixture): α-Cellulose 612.3; Fe 544.7 ($FeSO_4.7H_2O$); Zn 197.9 ($ZnSO_4.7H_2O$); Mn 92.3 ($MnSO_4.7H_2O$); Cu 35.4 ($CuSO_4$ anhydrous); Co 14.3 ($CoSO_4.6H_2O$); I 2.2 (KI); Se 1.0 (Na_2SeO_3); all mineral premix ingredients were obtained from Sigma Aldrich.

16.3.2 *Source of animals and experimental system*

Larvae were hatched from eggs originating from captive broodstock and were subsequently reared using established hatchery protocols (Battaglene and Cobcroft, 2007). The experimental system at the Marine Research Laboratories, Tasmanian Aquaculture and Fisheries Institute consisted of twenty-four 150 L round flat-bottomed tanks with marbled sides and black bottoms and external stand pipes. Over each tank was an automatic feeding unit (Curnow et al. 2006). Flow rates into each tank were maintained at 162 L h-1. Lighting was provided by fluorescent globes, light intensity measured from the water surface was of 2.8 ± 0.1 µmol. Photoperiod was maintained at 16h light : 8h dark. Incoming seawater was filtered through one micron bag filters and passed through heat chillers. Temperature was maintained at 14.5 ± 0.4 ^{o}C. Water quality was measured at 10:00 am daily using a YSI 660 Multi-probe (YSI Incorporated, USA) for temperature, pH and salinity and an Oxyguard™ Handy Polaris probe (Oxyguard International, Denmark) for oxygen. Water quality parameters were: pH, 8.1 ± 0.1, salinity, 33.9 ± 0.2 ppt, oxygen saturation, 97.9 ± 2.59 %. Faeces and uneaten food were removed daily from the tanks using a siphon and 70 % water replaced every afternoon to maintain high water quality.

16.3.3 *Experimental design*

At 287 days post-hatch (dph), 20 striped trumpeter post-larvae (8.1 ± 0.1g, mean ± SE, n = 480) were randomly stocked into the experimental tanks. Fish that had severe jaw malformations which hindered their capacity to feed were not used. Twenty five fish were euthanased using Aqui-S™ (Aqui-S, New Zealand) at a dose of 10 200-1 v v-1 Aqui-S™ in seawater for assessment of initial whole-body chemical composition. Fish in tanks were acclimated for a period of 24 d and fed with a 50:50 mix of the LDL and HDL diets at a fixed ration of 10 g d-1. Feeders were programmed to dispense feed equally every hour throughout the 16 h light period. Mortalities during acclimation were replaced with fish from the mass rearing tank.

At the beginning of the experiment, fish were anaesthetised using Aqui-S™ (Aqui-S, New Zealand) at a dose of 1 200-1 v v-1 seawater and individually measured for wet weight (WW) to 0.1 g and total length (TL) to 1 mm. Each tank was randomly assigned a ration of 33, 67 or 100% of satiation and either the LDL or HDL diet in a fully orthogonal design. At the beginning of each week, the 100% ration tanks were hand fed their respective diets to satiation every hour throughout the light period. Satiation was determined to be reached when approximately five pellets were left on the tank bottom. Feed intake (FI) was calculated by subtracting the uneaten pellets from the amount of food fed. The 67% and 33% rations were calculated weekly from the feed intake of the 100% ration groups. Fish were anaesthetised and weighed (to 0.1 g) and measured (to 1 mm) individually every 21 d. Fish were scored for development using the characteristic appearance of lateral stripes scored on a scale of zero to three, with zero indicating the absence of stripes and three indicating complete development into juveniles (see Chapter 15). At the end of the experiment, three post-larvae and three metamorphosed post-larvae were taken randomly from each tank, euthanased and frozen. Whole fish were autoclaved (Williams et al., 1995) and freeze dried to a constant weight prior to whole-body chemical composition analysis.

16.3.4 *Chemical Analysis*

Samples of diets were freeze dried to constant weight and ground to a homogenous powder. Standard methods were used to determine dry weight (freeze-drying to a constant weight); crude protein (Thermo Finnigan 1112 Series elemental analyser, N X 6.25); total lipid

(Bligh and Dyer, 1959); ash by combustion at 550 °C for 6 h (AOAC, 1995). Energy content of the diets was determined by bomb calorimetry (AOAC, 1995). Three replicate measurements for crude protein, total lipid and ash were performed for each fish. All composition data are presented as a mean percentage of the WW of each individual fish sampled from each treatment (Shearer et al., 1994).

16.3.5 *Calculations*

Condition factor (k) was calculated using the formula k = (WW/TL3) x 100 where WW is wet weight and TL is total length (Weatherley and Gill, 1987). Specific growth rate (SGR, % day-1) was calculated from mean WW using the formula: SGR= 100 x (ln final mean WW – ln initial mean WW) / D; where is D the number of days. Coefficient of variation for WW per tank (CV, %) was calculated as: 100 x SD / mean WW. Feeding rate was expressed a percentage of biomass (FR, % BM d-1) and was calculated using the formula: FR = 100 x (total feed input / D) / ((initial biomass + final biomass) / 2); where D is the number of days. Food conversion efficiency (FCE) was calculated by dividing the total gain in biomass by the total food consumed for each growth period. The total food consumed was adjusted to account for food consumed by mortalities on a pro rata basis. Energy content of whole fish was calculated by using the conversion factors of Brafield (1985) for protein (23.6 KJ g-1) and lipids (36.2 KJ g-1). Productive protein value (PPV, %) was calculated using the formula: PPV = 100 x (Fish protein gain (g) / Total protein consumed (g)). Productive lipid value (PLV, %) was calculated using the formula: PLV = 100 x (Fish lipid gain (g) / Total lipid consumed (g)). Productive energy value (PEV, %) was calculated using the formula: PEV = 100 x (Fish energy gain (kJ) / Total energy consumed (kJ)).

16.3.6 *Statistical analysis*

All results are reported as mean ± SE. Data were tested for normality and homogeneity using Levene's test of equality. Percent data were arcsine square-root-transformed prior to analysis. Univariate ANOVA with ration and dietary lipid as the two fixed factors was used to detect differences in survival, mean WW, mean TL, condition factor, coefficient of variation of WW, SGR, metamorphosis success, FCE, PPV, PLV, and PEV. Univariate ANOVA with ration, dietary lipid content and life history stage (post-larvae or juveniles) as the fixed factors was used to analyse differences in whole carcass chemical composition. Samples were pooled according to ration when dietary lipid was not found to have a significant effect ($P > 0.25$). When significant interactions were found, a univariate ANOVA was used to detect differences between the groups. A Tukey's post-hoc multiple comparison test was used to separate means. Statistical significance was accepted at $P<0.05$. Statistical analyses were performed using SPSS version 15.0 statistical software.

Data from starvation trials in Choa (2010) were used in estimating optimum ration using quadratic polynomial curves. Logistic curves were fitted to the proportion of metamorphosed individuals using wet weight and carcass lipid content as categories. SigmaPlot version 11.0 was used to formulate best fit curves.

16.4 Results

16.4.1 *Survival*

There were no significant differences in survival attributable to ration (df = 2, F = 1.14, P = 0.34, n = 8), dietary lipid (df = 1, F = 0.33, P = 0.58, n = 12), nor was there any interaction

between ration and dietary lipid (df = 2, F = 0.08, P = 0.92, n = 4). Survival was high and the overall mean survival rate was 95.6 ± 2.3 %.

16.4.2 *Growth and development*

There were no significant differences in the mean WW between treatments at the beginning of the experiment (df = 2, F = 1.37, P = 0.28, n = 8). There was no significant difference in growth and development with dietary lipid content at 21 d (df = 1, F = 1.6, P = 0.22, n = 12); 42 d (df = 1, F = 0.5, P = 0.50, n = 12) and 63 d (df = 1, F = 0.1, P = 0.93, n = 12). There was no significant interaction between ration and dietary lipid content at 21 d (df = 2, F = 0.7, P = 0.50, n = 4); 42 d (df = 1, F = 1.1, P = 0.37, n = 4) and 63 d (df = 1, F = 1.2, P = 0.33, n = 4). Ration was found to have a significant effect on mean WW, mean TL, condition, SGR and success of metamorphosis. Fish fed with 33 % ration were significantly smaller than fish fed with 67 % and 100 % rations, which had reported similar mean WW, at 21 d (df = 2, F = 14.0, P < 0.001) at 42 d (df = 2, F = 18.2, P < 0.001) and at 63 d (df = 2, F = 16.3, P < 0.001) (Figure 16.1). There were no significant differences in the coefficient of variation of WW of individual fish in any sampling period.

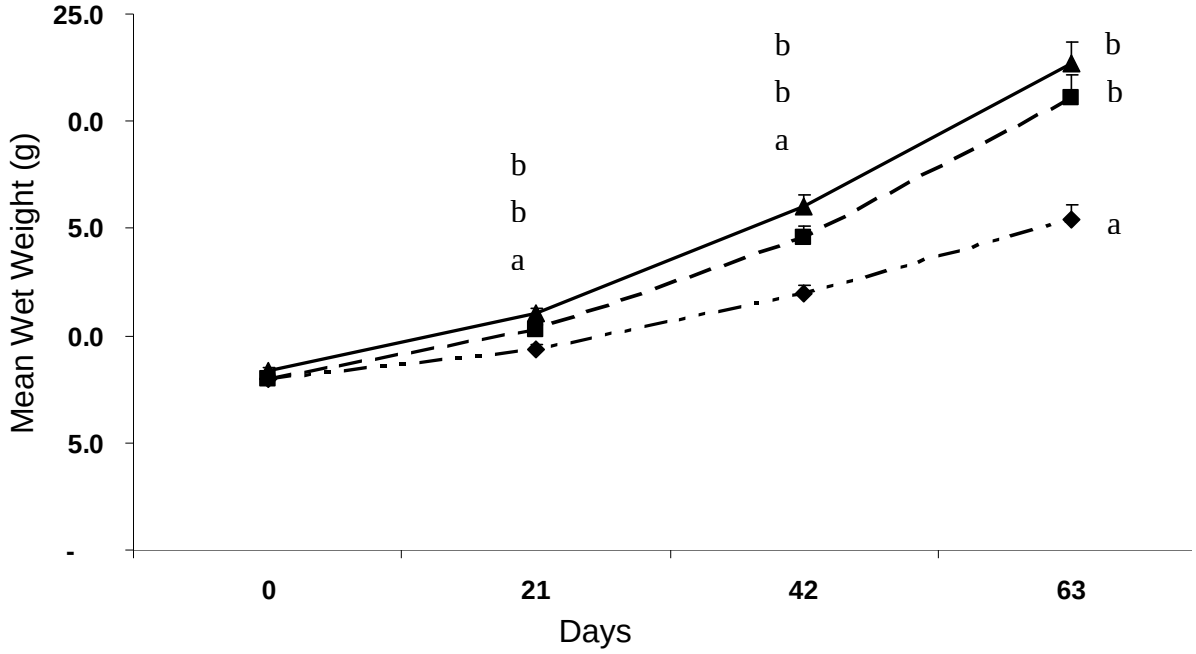

Figure 16.1 Growth (mean WW ± SE) of striped trumpeter post-larvae fed three different levels of ration (♦ = 33 %, ■ = 67 %, ▲= 100 %) over 63 d. Significant differences in wet weight are represented using different letters at each 21 d measurement ($P < 0.05$).

Mean TL of fish showed similar trends to mean WW with ration producing significant differences in mean TL but not dietary lipid. At 21 d, fish fed the 100 % ration were significantly longer than fish fed with 33 % ration (df = 2, F = 7.1, P = 0.005). Fish fed with 33 % ration were significantly shorter than fish fed with 67 % and 100 % rations, which had

similar mean TL, at 42 d (df = 2, F = 14.0, P < 0.001) and at 63 d (df = 2, F = 14.1, P < 0.001) (Table 16.2).

At 21 d, mean condition of fish fed with 33 % ration was significantly lower than fish fed with 67 % ration, fish fed with 100 % ration had the highest condition (df = 2, F = 20.7, P < 0.001). Fish fed with 33 % ration showed the lowest mean condition compared to the 67 % and 100 % ration fed fish, which had similar mean condition , at 42 d (df = 2, F = 7.7, P = 0.003) and at 63 d (df = 2, F = 7.4, P = 0.005) (Table 16.2).

Table 16.2 Mean total length and condition *K* (mean ± SE) of striped trumpeter post-larvae fed with different rations. Within sampling periods, treatments not sharing the same letter are significantly different ($P < 0.05$).

Mean TL (mm)	33 % Ration	67 % Ration	100 % Ration
21 d	112.1 ± 2.7^{a}	114.0 ± 3.0^{ab}	116.2 ± 2.8^{b}
42 d	118.3 ± 3.1^{a}	123.3 ± 3.5^{b}	126.6 ± 3.4^{b}
63 d	125.1 ± 3.6^{a}	133.5 ± 4.3^{b}	137.1 ± 4.1^{b}
Condition (*K*)			
21 d	0.6 ± 0.0^{a}	0.7 ± 0.0^{b}	0.7 ± 0.0^{c}
42 d	0.7 ± 0.0^{a}	0.7 ± 0.0^{b}	0.7 ± 0.0^{b}
63 d	0.7 ± 0.0^{a}	0.8 ± 0.0^{b}	0.8 ± 0.0^{b}

The SGR of fish fed with 33 % ration was significantly lower than fish fed with 67 % and 100 % rations, which were similar, after 21 d (df = 2, F = 18.2, P < 0.001), 41 d (df = 2, F = 17.3, P < 0.001) and 63 d (df = 2. F = 7.1, P = 0.005). When SGR was calculated for the whole growth period, fish fed 33 % ration showed significantly lower SGR compared to 67 % and 100 % ration fed groups which showed similar SGR (df = 2, F = 17.6, P < 0.001) (Table 16.3).

Table 16.3 Mean specific growth rate (SGR; mean ± SE) of striped trumpeter post-larvae fed with different rations. Within sampling periods, treatments not sharing the same superscript are significantly different ($P < 0.05$).

Mean SGR (% d^{-1})	33 % Ration	67 % Ration	100 % Ration
0-21 d	0.7 ± 0.1^{a}	1.2 ± 0.1^{b}	1.4 ± 0.1^{b}
22-42 d	1.2 ± 0.1^{a}	1.7 ± 0.1^{b}	1.8 ± 0.1^{b}
43-63 d	1.2 ± 0.1^{a}	1.7 ± 0.1^{b}	1.6 ± 0.1^{b}
0-63 d	1.0 ± 0.1^{a}	1.5 ± 0.1^{b}	1.6 ± 0.1^{b}

Groups fed with 67 % (22.6 ± 0.0) and 100 % (22.7 ± 0.0) ration showed significantly higher proportions of fully metamorphosed individuals compared to the 33 % ration (8.6 ± 0.0) groups (df = 2, F = 6.0, P = 0.01).

16.4.3 *Feed intake and food conversion efficiency*

Measurements of feed intake are confounded by the behaviour of the fish which sometimes chewed the pellet and regurgitated it out through the mouth and gill openings. Pellets which were regurgitated appeared as fine particles on the tank bottom and were missed during counting of leftover pellets. Feed intake measurements are overestimated because of this phenomenon. By controlling the feed input, total feed fed to the 33, 67 and 100 % ration groups was significantly different at 21 d (df = 2, F = 284.26, P < 0.001), at 42 d (df = 2, F = 159.84, P < 0.001), at 63 d (df = 2, F = 43.56, P < 0.001) and for the entire experimental period (df = 2, F = 68.46, P < 0.001). The LDL and HDL diets were fed at the same rate according to the ration treatment and were not significantly different. Fish fed 100 % ration consumed more food during the first 42 d of the experiment regardless of diet fed (df = 2, F = 12.8, P < 0.001). No significant differences were found in the feeding rates for the 67 % ration (df = 2, F = 2.8, P = 0.09). A significantly higher amount of food was provided to the 33 % ration treatment from 0 to 21 d compared to 22 to 63 d (df = 2, F = 5.9, P = 0.01). Mean feeding rates for each 21 d period and the entire experimental period are summarised in Table 16.4.

Table 16.4 Mean feeding rates per ration treatment (% BM d-1; mean ± SE). Treatment rations were significantly different and are noted with different letters. For each ration, feeding periods not sharing the same superscript are significantly different ($P < 0.05$).

Mean Feeding Rate	33 % Ration[a]	67 % Ration[b]	100 % Ration[c]
0-21 d	2.2 ± 0.1[a]	4.0 ± 0.1	5.4 ± 0.1[b]
22-42 d	2.5 ± 0.1[b]	4.4 ± 0.1	5.2 ± 0.1[b]
43-63 d	2.6 ± 0.1[b]	3.9 ± 0.2	4.5 ± 0.2[a]
0-63 d	2.4 ± 0.1	3.8 ± 0.1	4.7 ± 0.2

Fish fed with 33 % ration showed significantly higher FCE compared to fish fed with 100 % ration for the period from the beginning of the experiment to 21 d ($df = 2$, $F = 6.0$, $P = 0.010$) and from 21 d to 42 d ($df = 2$, $F = 4.8$, $P = 0.022$) . There were no significant differences in FCE between 33 %, 67 % and 100 % ration fed groups for the period from 42 d to 63 d. There were no significant differences over the entire growth period in FCE between the three rations (Table 16.5).

Table 16.5 Mean food conversion efficiency (FCE; mean ± SE) of striped trumpeter post-larvae fed with different rations. Within sampling periods, treatments not sharing the same superscript are significantly different ($P < 0.05$).

Mean FCE (%)	33 % Ration	67 % Ration	100 % Ration
0-21 d	32.4 ± 2.8[b]	29.6 ± 2.5[ab]	22.2 ± 1.3[a]
22-42 d	44.2 ± 2.7[b]	38.2 ± 2.7[ab]	31.9 ± 3.2[a]
43-63 d	42.9 ± 4.8	41.4 ± 4.4	33.5 ± 3.5
0-63 d	40.9 ± 3.2	37.6 ± 3.0	30.1 ± 2.7

16.4.4 *Chemical composition*

Ideally three post-larvae (not metamorphosed) and three juveniles (metamorphosed) were sampled from each tank. However, some tanks did not show a high degree of metamorphosis, therefore fish closest to juveniles in appearance were sampled resulting in an unbalanced number of post-larvae (n = 88) and juveniles (n = 52).

Moisture

The moisture content (% WW; mean ± SE) of 100 % ration fed tanks (72.6 ± 0.3 %) was significantly lower than 33 % ration groups (74.4 ± 0.4 %) (df = 2, F = 6.27, P = 0.003). Moisture content of juveniles (72.5 ± 0.3 %) was significantly lower than the moisture content of post-larvae (74.1 ± 0.2 %) (df = 1, F = 16.02, P<0.001). A significant interaction between ration and diet was found; groups fed a 100 % ration with HDL diet showed significantly lower moisture content (70.8 ± 0.6 %) compared to groups fed 33 % rations with LDL (75.0 ± 0.7 %) and HDL diets (74.4 ± 0.4 %) (df = 2, F = 4.27, P = 0.016).

Ash

The ash content (% WW; mean ± SE) of 100 % ration groups (3.5 ± 0.0 %) was significantly lower than the ash content of the 33 % ration groups (3.8 ± 0.1 %) (df = 2, F = 5.79, P = 0.004). A significant interaction between ration and life history stage was found; juveniles (3.6 ± 0.1 %) and post-larvae (3.5 ± 0.1 %) from 100 % rations groups and juveniles (3.6 ± 0.1 %) from 33 % ration groups had significantly lower ash content compared to post-larvae (3.9 ± 0.1 %) from 33 % ration groups (df = 2, F = 4.77, P = 0.010). A significant interaction between ration level, dietary lipid level and life history stage was also found; post-larvae from 100 % ration groups fed with the LDL diet (3.4 ± 0.1 %) and juveniles fed a 33 % ration with the LDL diet (3.4 ± 0.1 %) had significantly lower ash content compared to post-larvae fed a 33 % ration with the LDL (3.9 ± 0.1 %) and HDL diets (3.9 ± 0.1 %) (df = 2, F = 3.77, P = 0.026).

Crude protein

No significant differences in crude protein content were found attributable to the different ration and dietary lipid level treatments and life history stages. There were also no significant interactions found between the fixed factors that influenced crude protein content. Overall mean crude protein content was 16.9 ± 0.0 %.

Total lipid

Total lipid content of fish from 33 % ration groups (3.8 ± 0.3 %) was significantly lower compared to fish from the 67 % (5.5 ± 0.4 %) and 100 % ration groups (6.0 ± 0.4 %) (df = 2, F = 12.56, P < 0.001). Fish fed with the LDL diet (4.5 ± 0.3 %) had significantly lower total lipid content compared to fish fed with the HDL diet (5.8 ± 0.3 %) (df = 1, F = 12.90, P < 0.001). Lipid content of post-larvae (4.0 ± 0.2 %) were significantly lower than total lipid content in juveniles (7.0 ± 0.3 %) (df = 1, F = 61.78, P < 0.001).

16.4.5 *Nutrient retention efficiency*

Fish fed with 33 % ration (18.3 ± 1.3 %) had significantly higher PPV compared to the 67 % (15.6 ± 0.9 %) and 100 % ration groups (13.3 ± 1.0 %) (df = 2, F = 6.4, P = 0.008). Ration did not influence PEV and PLV. Overall mean PEV was 15.1 ± 0.7 % and overall mean PLV was 14.4 ± 0.8 %. Dietary lipid content and the interaction between ration and dietary lipid did not influence PPV, PEV and PLV.

16.4.6 *Estimation of optimum ration*

Data for loss of weight and depletion of body nutrient reserves during starvation was determined from a separate series of experiments where fish of different size classes were starved in individual tanks for a period of 10 d (see Choa [2010]. Data obtained from 16 g and 32 g fish starved at 14 ^{o}C and 16 ^{o}C (n = 4) were determined to be closest to the sizes of fish and rearing conditions of this study. Optimum rations for growth expressed in gains of

biomass, mass of protein, mass of lipid and energy content were calculated using fitted quadratic polynomial curves. The equations of the curves are summarized in Table 16.6. Predicted maximum rations for SGR, SGR Protein, SGR Lipid and SGR Energy were 4.2 %, 4.1 %, 4.2 % and 4.2 %, respectively (Figure 16.2 and Figure 16.3).

A calculated maintenance ration of 0.9 % BM d-1 was found using the SGR curve. This was combined with the FCE data for individual tanks over the 63 d experimental period to provide a predictive model for estimating optimal rations. Maximum FCE was predicted to be achieved with a ration of 3.1 % BM d-1 (r2 = 0.550, F2,22 = 13.45, P = 0.0002) (Table 16.4).

Table 16.6 Quadratic polynomial equations describing best-fit lines for SGR, SGR Protein, SGR Lipid and SGR Energy at different rations; where R represents ration (% BM d-1).

	$y = a+bR+cR^2$					
	a	b	c	R^2	$F_{2,25}$	*P*
SGR (% d^{-1})	-1.007	1.241	-0.148	0.941	197.602	<0.0001
SGR Protein (% d^{-1})	-1.175	1.340	-0.165	0.924	152.013	<0.0001
SGR Lipid (% d^{-1})	-3.240	3.203	0.385	0.923	149.293	<0.0001
SGR Energy (% d^{-1})	-1.346	1.586	-0.190	0.952	246.925	<0.0001

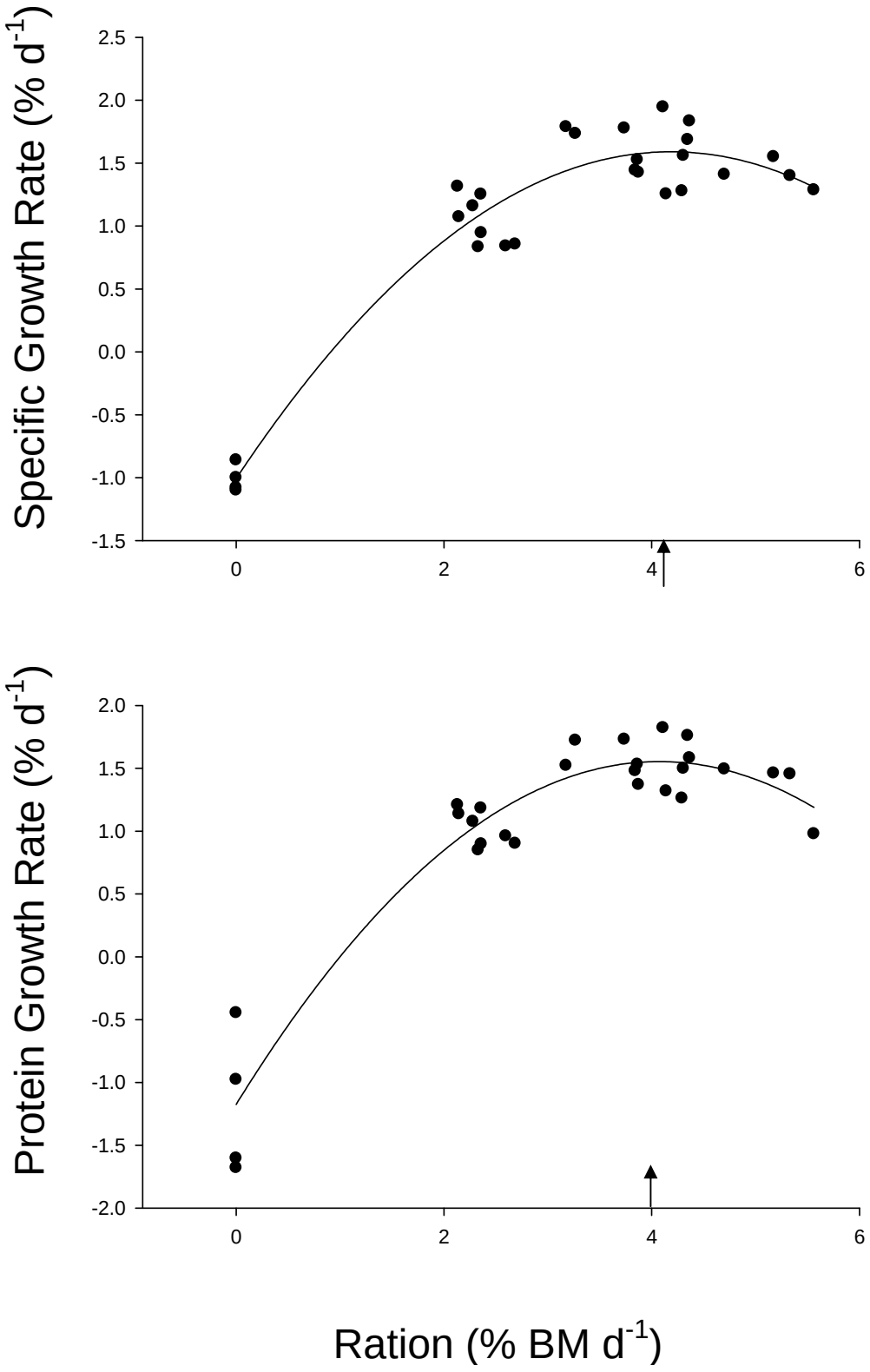

Figure 16.2 Relationship between specific growth rate (% d-1) and specific protein growth rate (% d-1) and ration (% BM d-1) described with a quadratic polynomial curve. Arrows indicate predicted maximum.

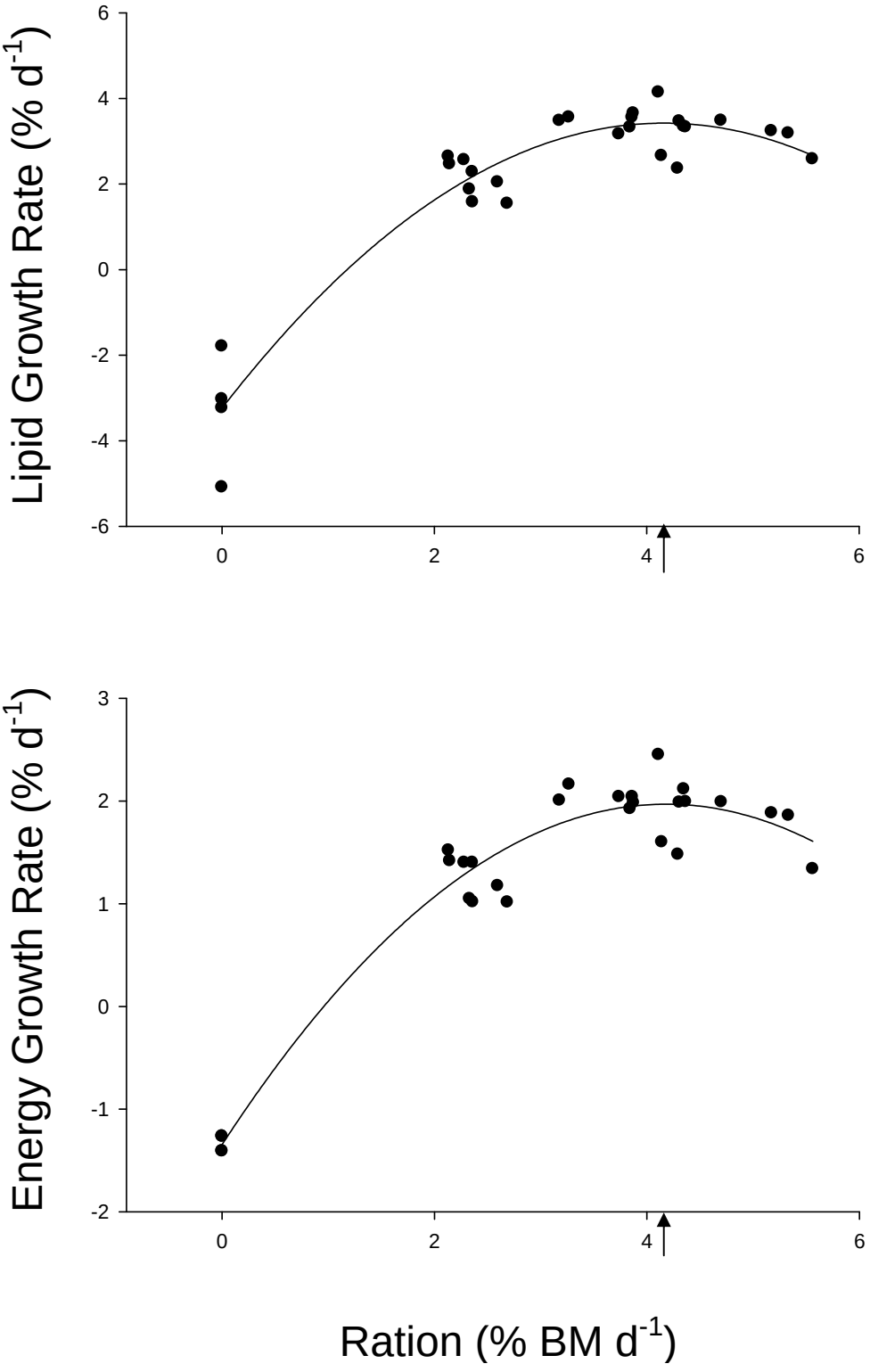

Figure 16.3 Relationship between specific lipid growth rate (% d-1) and specific energy growth rate (% d-1) and ration (% BM d-1) described with a quadratic polynomial curve. Arrows indicate predicted maximum.

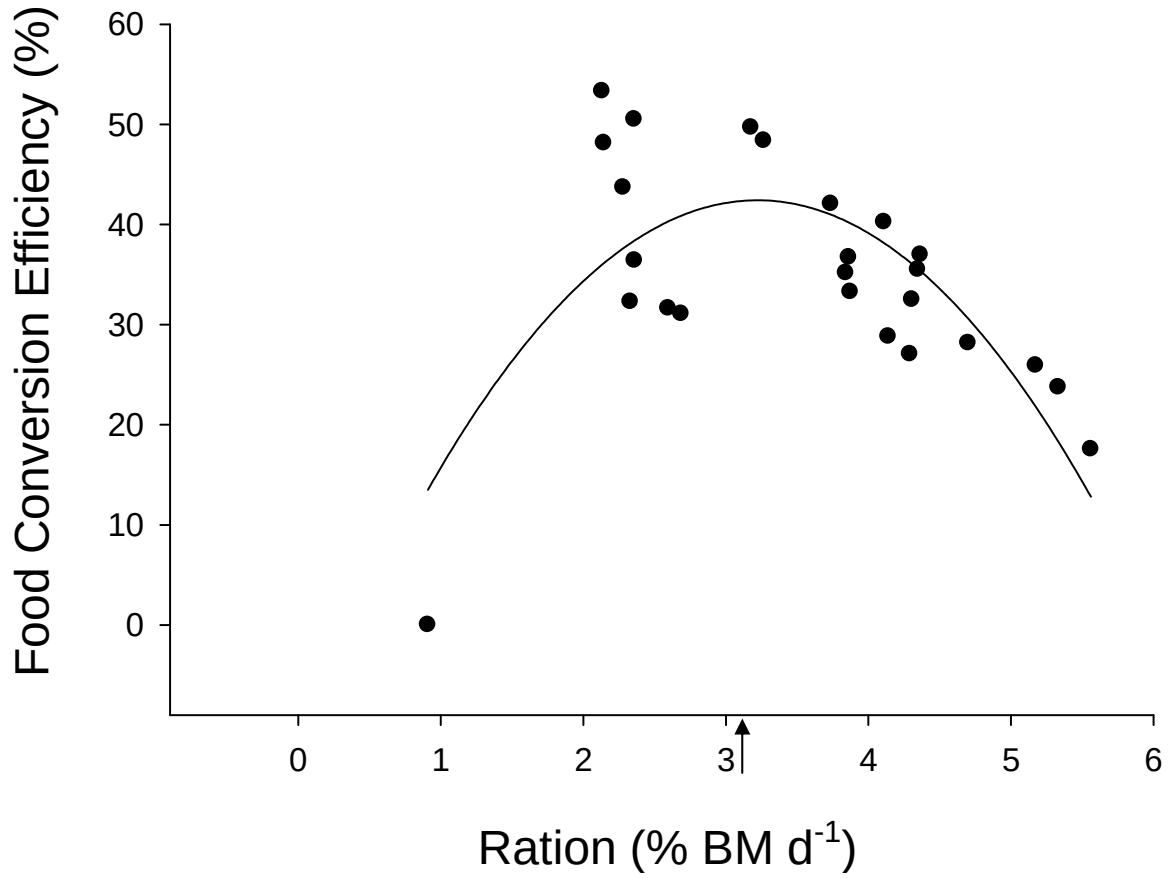

Figure 16.4 Relationship between food conversion efficiency (%) and ration (% BM d-1) described with a quadratic polynomial curve with the equation y = -13.74 + 34.86 X R-5.41 X R2; where R is ration (% BM d-1). Arrows indicate predicted maximum.

16.4.7 *Estimation of thresholds for metamorphosis into juveniles*

It was noted during weekly measurements that the progression of metamorphosis of individuals of similar sizes varied. Examples of fish of the same size but of different life history stages are presented (Figure 16.5). Data from the 67 % and 100 % ration treatments were pooled based on previous ANOVA analysis for both wet weight and carcass lipid content. The proportion of metamorphosed fish from the 33 % ration treatment were compared to the proportion of metamorphosed fish from the pooled 67 % and 100 % ration treatments according to wet weight (Figure 16.6). Fish from the 67 % and 100 % ration treatments showed a higher proportion of metamorphosed individuals from 20 to 35 g. At 40 g all three ration treatments showed complete metamorphosis. Data for sizes beyond 40 g for the 33 % ration treatment were not available and it was assumed that fish would show complete metamorphosis beyond this size.

Figure 16.5 Photograph of specimens of similar sizes showing different life history stages; the fish on top shows signs of metamorphosing into the juvenile form while the bottom fish is still a post-larvae in appearance.

The proportion of metamorphosed fish from the 33 % and the pooled 67 % and 100 % ration treatments were compared according to carcass lipid content (Figure 16.7). The carcass lipid content of fish from the 33 % ration treatment reached a maximum of 7 % and did not reach the point at which complete metamorphosis could be expected. Complete metamorphosis of fish from the 67 % and 100 % ration treatment occurred at 11 % carcass lipid content. Equations for the logistic curves are summarised in Table 16.7.

Table 16.7 Equations for fitted logistic curves describing best-fit for metamorphosed individuals according to weight class (g) and carcass lipid content (%).

	$y = a / 1+(x / x^0)^b$					
	a	b	x^0	R^2	F	*P*
Weight class						
33 % ration	114.3	-5.2	30.9	0.95	80.8	<0.001
67 % and 100 % ration	95.8	-7.4	24.7	0.96	75.2	<0.001
Carcass lipid content						
33 % ration	90.7	-3.5	5.1	0.89	15.8	0.012
67 % and 100 % ration	129.6	-3.0	8.0	0.86	31.4	0.0002

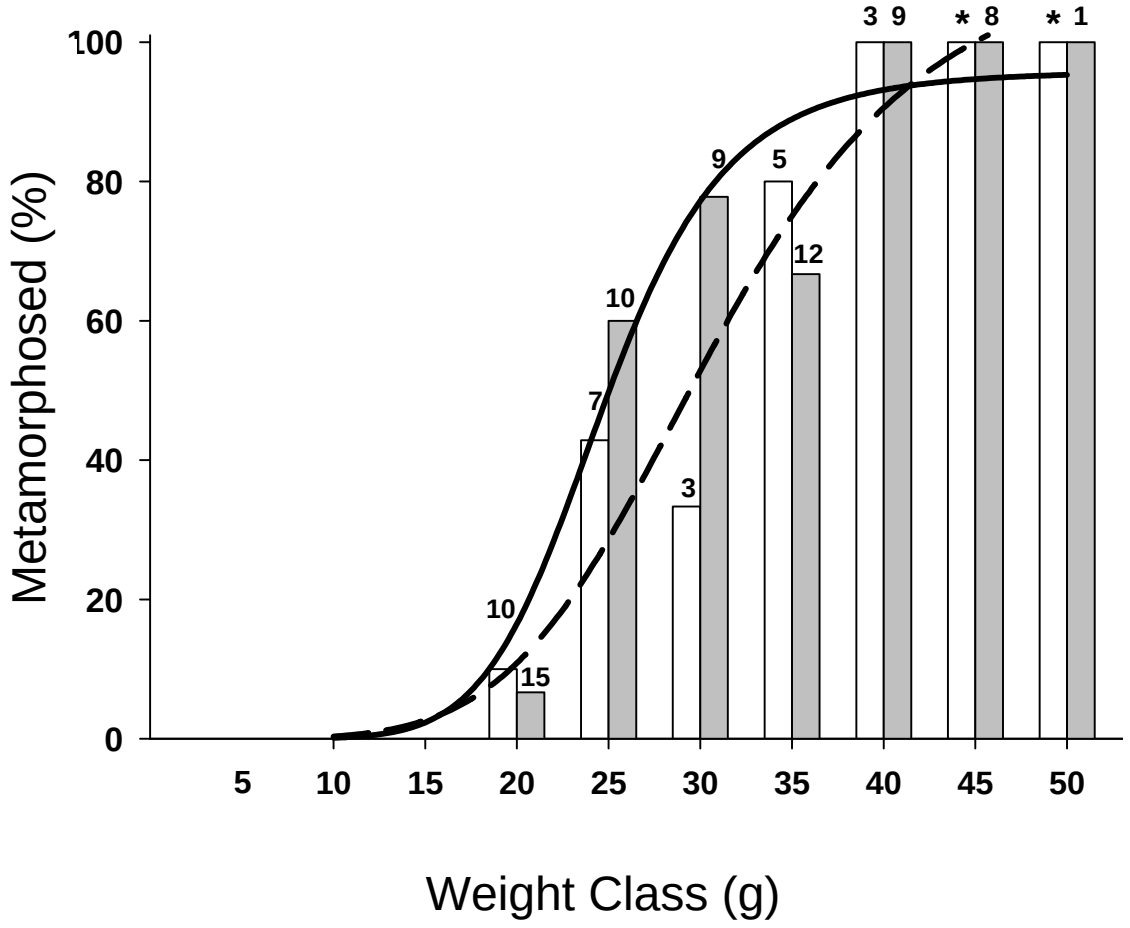

Figure 16.6 Proportion of metamorphosed individuals per weight class from the 33 % ration treatment (□) and the pooled 67 % and 100 % treatments (■). The number of fish in each weight class is indicated above the respective bars. The * represent assumed values of 100 % metamorphosed individuals at 45 and 50 g categories for the 33 % ration. Logistic curves for the 33 % ration treatment (hatched line) and the pooled 67 % and 100 % (solid line) treatments.

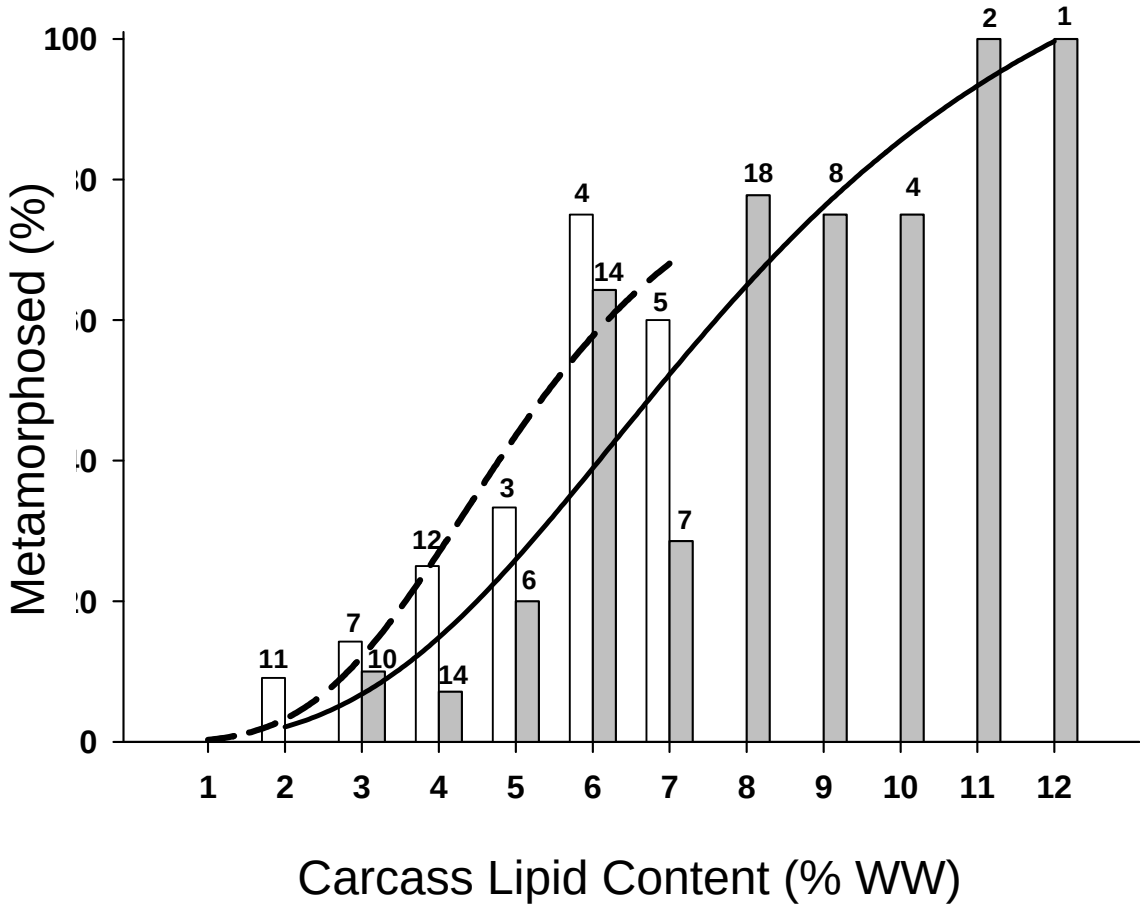

Figure 16.7 Proportion of metamorphosed individuals grouped according to carcass lipid content from the 33 % ration treatment (□) and the pooled 67 % and 100 % treatments (■). The number of fish in each categorical class is indicated above the respective bars. Logistic curves for the 33 % ration treatment (hatched line) and the pooled 67 % and 100 % (solid line) treatments.

The whole population was sampled at 63 d and individual wet weights plotted against carcass lipid content. Majority (> 95 %) of the post-larvae population weighed between 6 g and 20 g. Metamorphosed individuals were first observed at 19 g. Majority (> 95 %) of the metamorphosed individuals weighed from 23 g upwards and had a carcass lipid content of 4 % upwards (Figure 16.8).

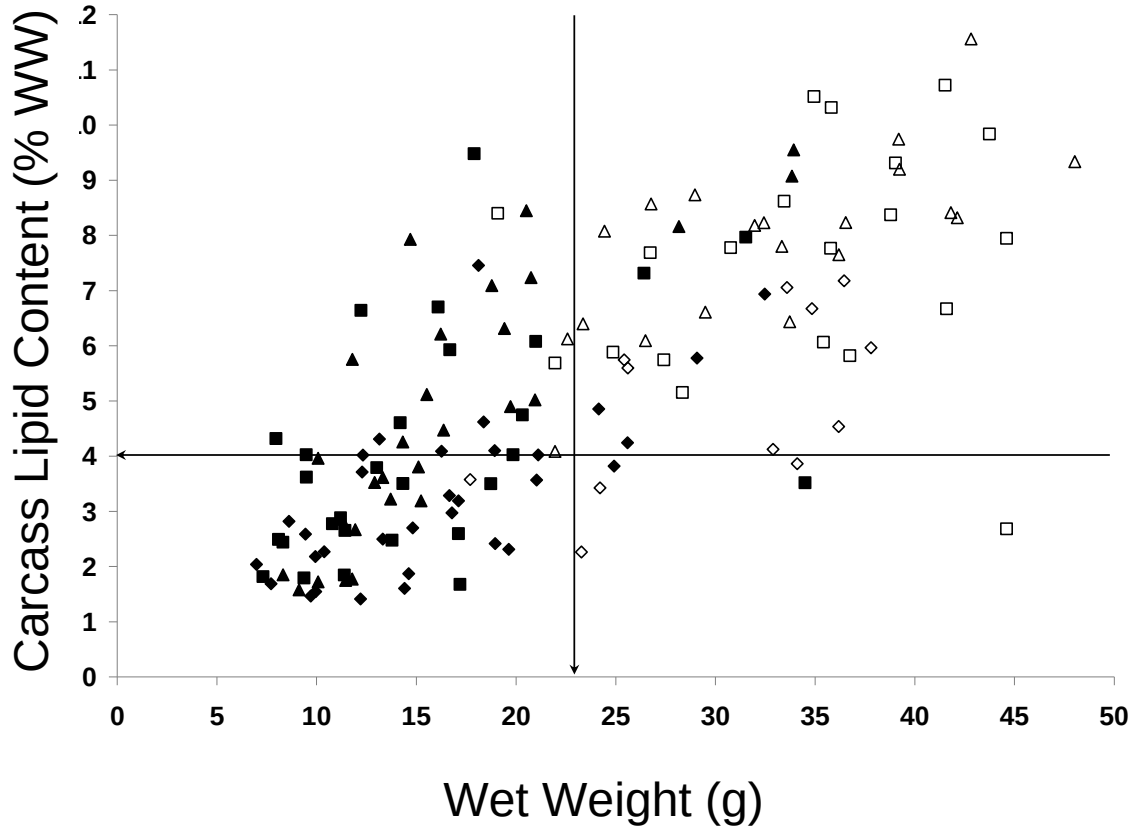

Figure 16.8 Relationship between wet weight and carcass lipid content of post-larvae and juvenile striped trumpeter. Solid data markers represent post-larvae and hollow data markers represent juveniles from the 33 % ration (◇), the 67 % ration (□) and 100 % ration (△) treatments. Arrows indicate the point where majority (> 95 %, n = 140) of the metamorphosed population is found.

16.5 Discussion

16.5.1 *Growth and identifying the optimum ration*

The current study expands our understanding of optimal culture conditions for striped trumpeter post-larvae by investigating the effects of feeding, specifically ration and dietary lipid content, on growth and other indicators of performance. Prior to this study, there were no empirical studies on feeding rates for post-larval striped trumpeter. This study quantified the relationship between feeding rate and growth rate for post-larval striped trumpeter. Many other studies have shown that as ration increases, wet weight growth increases up to an optimum point and then decreases at rations beyond the optimum (Brett and Groves, 1979; Weatherley and Gill, 1987; Jobling, 1994). Feeding 33 % of satiation, which translated to 2.4 % BM-1 actual feed intake, resulted in the lowest growth. Growth of fish fed at 67 % of satiation and to satiation, which translated to 3.8 % and 4.7 % BM d-1, produced equally high growth rates. Therefore, increasing feed intake from 3.8 to 4.7 % BM d-1 did not result in an incremental increase in wet weight growth. These relationships were also found for

other measures of growth and performance, namely, total length, condition (K) and SGR. These findings indicated that the optimum ration lies between 3.8 and 4.7 % BM d-1, respectively.

The chemical composition of wet weight growth was also affected by ration. Feeding below the optimum range resulted in lower total lipid and higher ash and moisture content. Protein content of the carcass did not differ between treatments. These findings agree with those of Shearer (1994) and Bureau et al., (2006) which showed an inverse relationship between body lipid and moisture and that protein content in the carcass remained stable as weight increases. Further, protein content was not affected by growth rate, diet or environment. The higher ash content found in the group fed below the optimum ration reflected the lower condition of fish from those treatments.

Fish fed with the HDL diet (24 %) showed higher total lipid which indicated a higher accumulation of energy reserves (Jobling, 2001). This increase in reserves did not result in higher wet weight growth compared to fish fed with the LDL diet but has implications for health and husbandry if fish with higher energetic reserves are less susceptible to stress induced mortality. In future work, it will also be useful to identify the optimum dietary lipid inclusion rates and the potential replacement of fish oil with alternative oils in the face of increasing pressure on worldwide fish oil supplies (Miller et al., 2008).

To identify the optimum ration, we combined the collected growth data with results from separate starvation trials on similar sized fish held for 10 days at 14 ^{o}C and 16 ^{o}C (see Choa 2010). Polynomial curves fitted to SGR, SGR-Protein, SGR-Lipid, SGR-Energy and FCE all indicate that the optimum ration is 4.0 % BM d-1. Feeding above this level led to overfeeding and lower feed conversion efficiency. Other studies have also found this effect of overfeeding on growth and conversion efficiency (Tsevis et al., 1992; Mihelakakis et al., 2002; Fiogbe and Kestemont, 2003; Wang et al., 2007).

The observed growth efficiency of striped trumpeter post-larvae was lower compared to results from other research studies on cultured species. The highest FCE achieved in this study was 41 %. Studies on other species have found higher FCE such as those on European sea bass (*Dicentrarchus labrax*) with an FCE of 90 % (Eroldogan et al., 2004) and an FCE of 75 % (Peres and Oliva-Teles, 2005); a study on barramundi (*Lates calcarifer*) reported an FCE of 146 % (Katersky and Crater, 2005); and a study on Atlantic salmon (*Salmo salar*) parr was able to achieve an FCE of 144 % (Bendiksen et al., 2003).

Growth efficiency of striped trumpeter post-larvae was lower compared to other cultured species. The highest FCE achieved was 41 %. Likewise the highest PPV (18 %) and PEV (15 %) values of striped trumpeter post-larvae were much lower compared to species such as barramundi which had a PPV of 50 % and a PEV of 45 % (Katersky and Carter 2005) but were similar for greenback flounder which showed a PPV of 17 % and a PEV of 26 % (Carter and Bransden, 2001). Poor digestibility of the diet can lead to reduced efficiency because of poor absorption of nutrients (Bureau et al., 2002). The quality of ingredients can also affect growth efficiency because of an amino acid deficiency which leads to reduced muscle deposition (Carter and Houlihan, 2001). A high inclusion of protein or lipid in fish diets can also reduce growth efficiency caused by catabolism of protein and reductionin growth rates. The use of fish meal and fish oil based diets in this study avoided any concerns regarding digestibility of the diet. The amino acid profile of fish meal is also ideal for growth (NRC, 1993). It is hypothesised that the inaccurate measure of feed intake led to an

overestimation of satiation and protein and energy intake. This is supported by the high SGR of 1.6 % found in this study.

The measurement of feed intake is a continuously evolving field and has been discussed extensively by various authors (McCarthy et al., 1993; Jobling et al., 2001b). The precision of measuring feed intake and nutrient uptake in the current experiment was limited due to the following factors. First, counting uneaten pellets is in some instances inaccurate due to striped trumpeter consuming and rejecting, possibly regurgitating, some pellets which then rapidly disintegrated. Feed ingestion rates reported here could therefore be overestimated, particularly on the satiation ration. Second, striped trumpeter post-larvae did not produce large amount of faeces and stripping the fish would lead to mortalities so traditional measurements of digestibility were not possible.

In comparison with standard feeding rates for striped trumpeter post-larvae culture reared at the Marine Research Laboratories, Tasmania the optimum ration of 4.0 BM-1 was higher. During production, post-larvae and juveniles are fed at a daily ration of 2.5 % to 3.0 % BM d-1 with a commercial extruded diet (R. Goldsmid, Marine Research Laboratories, University of Tasmania, personal communication). However, the optimum ration found was similar to a recommended ratio of 3.5 % bdw d-1 for European sea bass, another temperate marine aquaculture species (Eroldogan et al., 2004). A direct comparison of rations and growth and conversion efficiency cannot be made because of other factors which influence conversion efficiency rates such as feed formulation, rearing environment and fish size to name a few (Bureau et al., 2006).

The quality of the experimental diet used was different from the commercial extruded diet used at MRL. The experimental diet was not extruded and was not as water stable as an extruded diet. The experimental diet also incorporated bulking agents such as bentonite and cellulose. It was observed that fish would masticate and then forcefully regurgitate the experimental pellets and would sometime subsequently ingest them again or reject them. The experimental diets yielded similar SGR and intake rates to post-larvae fed with a commercial extruded diet (see Chapter 15) but reduced feed conversion efficiency.

Feed intake of fish is affected by sensory qualities of the feed, pellet size and the quality of the ingredients (Jobling et al., 2001a). The importance of the physical properties of the feed to match the feeding behaviour of the cultured species should be taken into consideration, for example in Deng et al., (2003) sturgeon were unable to feed on pellets until they were forced to the bottom. Floating pellets may perform better than the sinking pellets used in the current study by prolonging the window for capture. Striped trumpeter post-larvae generally fed at the water surface but were also observed to feed on pellets on the tank bottom. Producing a feed with superior physical and nutritional properties will minimise feed wastage and negative environmental impacts and achieve higher economic gains (Alanara et al., 2001; Read and Fernandes, 2003).

Jaw malformations are typical of fish cultured at the Marine Research Laboratories and have been linked to walling behaviour (Cobcroft et al., 2001; Cobcroft and Battaglene, 2009). Jaw malformations of varying severity were observed in all of the experimental animals and could have affected the feeding ability of some animals; efforts were made to select only the individuals with functioning mouths. It is also possible that post-larvae were damaged during the course of the experiment because of their flight response to external disturbances and greater interaction with tank surfaces in smaller tanks.To reduce walling tanks were lined with marble backgrounds (Cobcroft and Battaglene, 2009). The feeding activity of

experimental animals was not affected by the jaw malformations and the small size of the pellets (1 mm) made them easy to ingest. By the post-larvae stage, striped trumpeter jaws are fully developed and malformations did not increase in severity during the experiment.
In a review by Madrid et al., (2001), rhythms in fish feeding behaviour were found to respond to changing abiotic factors such as light, temperature and oxygen concentration; and also to endogenous responses. In the present study fish were fed continuously throughout the light period managed by an automatic feeding system. Feeding time and frequency were found to affect nutrient utilisation in cuneate drum (*Nibea miichthioides*) and European sea bass (Bolliet et al., 2001; Wang et al., 2007). However, in a study on Australian snapper (*Pagrus auratus*) by Booth et al., (2008) it was found that the time of feeding did not affect feed utilisation nor did it affect gastric evacuation rates. These studies report opposing findings and it is worth investigating the effects of feeding time, frequency and meal size on striped trumpeter performance on a production scale.

Identifying the optimum ration is useful not only for research but also for its implications on commercial culture. Feeding below the optimum ration has been shown to increase competition and aggressive behaviour in aquacultured finfish species such as sea bream (*Sparus aurata*) (Andrew et al., 2004), Atlantic cod (*Gadus morhua*) (Hatlen et al., 2006), coho salmon (*Oncorhynchus kisutch*) (Ryer and Olla, 1996) and Atlantic salmon (*Salmo salar*) (Noble, et al., 2008). In contrast, flatfish species such as greenback flounder (Rhombosolea taparina), yellowtail flounder (*Limanda ferrugine*) and turbot (*Scophthalmus maximus*) do not exhibit aggressive behaviour under restricted rations but still show evidence of inter-individual variation in feed consumption and growth (Carter et al., 1996; Dwyer et al., 2002; Irwin et al., 2002). Striped trumpeter post-larvae did not exhibit physical damage as a result of aggression and the similar coefficient of variation for the three treatment rations indicate an absence of aggressive behaviour even under restricted rations which is desirable for high density culture. Overfeeding, on the other hand, results in increased organic loading. This can cause a decrease in water quality particularly increased ammonia concentrations which can suppress appetite in fish (Ortega et al., 2005). This is particularly important to avoid for striped trumpeter because striped trumpeter are sensitive to deterioration of water quality (Battaglene and Cobcroft, 2007).

16.5.2 *Metamorphosis and development*

The protracted post-larval stage of striped trumpeter metamorphosing into juveniles at nine months is highly unique. During this time post-larvae are more susceptible to stress induced mortality than juveniles possibly because of their lower energetic reserves. Adequate energetic reserves are needed to cope with stressors. Studies on rainbow trout and roach (*Rutilus rutilus*), in the wild show that larger individuals with higher lipid reserves survive better under winter starvation conditions (Kirjasniemi and Valtonen, 1997; Biro et al., 2004). Striped trumpeter post-larvae did consume more food compared to juveniles; which agrees with other studies that show a higher feed intake for smaller fish (Jobling, 1994; Fiogbe and Kestemont, 2003). However, this increased intake is offset by the active swimming behaviour of striped trumpeter post-larvae which is a greater energetic burden for small fish (Boisclair and Tang, 2005). This resulted in higher ash and lower total lipid content in post-larvae compared to juveniles and provides an insight into the delicate nature of the post-larvae.

Striped trumpeter juveniles were found to weigh a minimum of 23 g and have at least 4 % lipid in their carcass. Wet weight is a more accurate predictor of metamorphosis than total lipid content because the most marked changes in carcass content are lipid and moisture and

is directly correlated with size (Shearer, 1994). Observations of post-larvae and juveniles of similar sizes indicate that a combination of size and nutritional fitness is needed to be fulfilled before metamorphosis is completed. Using a logistic curve model of wet weight and carcass lipid the point at which 50 % of the population would metamorphose into juveniles was determined as 20 g and 7 % carcass lipid content, all of the post-larvae are predicted to be fully metamorphosed into juveniles when they reach 40 g wet weight and greater than 10 % carcass lipid. In another study by Tracey (2007), 50 % of hatchery reared striped trumpeter post-larvae were predicted to be fully metamorphosed at 150 mm, findings in this experiment are in agreement with the mean length of metamorphosed individuals found to be 150.8 mm. Large post-larvae, which met the minimum size requirement for metamorphosis, were not able to complete metamorphosis due to their inability to meet a nutritional threshold (Shearer, 1994; Thorpe et al., 1998; Berill et al., 2004).

It should be noted that environmental conditions also have an influence on metamorphosis. Fish under culture conditions experience less diel variability which ensures their consistent growth and reduces stress (Gozlan et al., 1999). Temperature was previously found to have an influence on metamorphosis rates of striped trumpeter post-larvae with fish reared at 12 ^{o}C and 14 ^{o}C showing higher metamorphosis rates and metamorphosing at smaller sizes compared to fish reared at 16 ^{o}C and 18 ^{o}C (see Appendix 4). Photoperiod has also been found to have an effect on parr-smolt transformation in Atlantic salmon (Berrill et al., 2003). The effect of photoperiod regimes on striped trumpeter growth is an opportunity for future research.

16.5.3 *Conclusion*

The success of the striped trumpeter research program has created an impetus to research the rearing of striped trumpeter post-larvae. The striped trumpeter post-larvae is a challenging animal to work with and it is highly desirable to increase its growth rates to hasten metamorphosis into juveniles. Optimising feeding rates will increase efficiency, increase fish performance and promote sustainable aquaculture practices. This study recommends a 4.0 % BM d-1 ration of at least 18 % dietary lipid to support high growth rates. Metamorphosis into juveniles should be expected to occur once fish reach a weight of 20 g. Verification of these findings in a commercial setting is also advised to ensure applicability in cage culture.

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17 JAW MALFORMATION IN STRIPED TRUMPETER *LATRIS LINEATA* LARVAE LINKED TO WALLING BEHAVIOUR AND TANK COLOUR

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17.1 Abstract

Jaw malformations are a recurrent obstacle in the hatchery production of high quality juveniles of many marine finfish species. Whilst nutrition and temperature are often cited as the most likely causes, this study investigated manipulation of the physical culture environment and larval behaviour to reduce jaw malformations. The onset of jaw malformation after metamorphosis in striped trumpeter *Latris lineata* follows changes in larval behaviour from an even distribution throughout the water column to close association with the tank walls, often with vigorous swimming into the walls known as 'walling' behaviour. Larvae were reared through metamorphosis, 16 to 44 days post-hatching (dph), in twenty four 300 L hemispherical tanks with six different wall colours, black, blue, green, marble (a black, grey and white mottled pattern), red and white. Walling behaviour, jaw malformation and swim bladder hyperinflation were assessed. The highest proportion of severely malformed jaws at 44 dph occurred in red tanks, followed by green, white, blue, black and marble. More fish walled in coloured tanks (25-44%) than in black and marble tanks (9.6 and 3.4%, respectively). The proportion of fish with jaw malformations at 44 dph was positively correlated with fish walling behaviour. Both black and marble tanks had more than 50% of fish with normal jaws at 44 dph, and close to 80% with no or very minor malformations. Growth and survival to 44 dph were highest in the black (15.7 ± 1.3 mm fork length, 7.9 ± 0.9 mg dry weight, 71 ± 6%) and marble (15.6 ± 1.2 mm, 7.6 ± 0.5 mg, 58 ± 17%) tanks, compared with the lowest values in red tanks (14.2 ± 1.1 mm, 6.4 ± 0.4 mg, 11 ± 6%). Swim bladder hyperinflation, an apparent stress response, was greatest in red tanks and surface mortality was positively correlated with the proportion of fish with hyperinflated swim bladders. Potential mechanisms for the influence of walling behaviour on jaw malformation are mechanical damage and poor nutrition, via reduced feed intake and increased energy expenditure. The study highlights the often overlooked importance of hard-surface interactions in the growth and survival of cultured marine fish and demonstrates a cheap and effective technique for assessing tank background colour as a means of reducing malformations in cultured fish.

Keywords: marine fish larvae, tank colour, jaw malformation, walling behaviour, swim bladder, hyperinflation, deformity.

17.2 Introduction

Skeletal malformations are a frequent problem in cultured marine fish, with potentially detrimental effects on growth, survival and marketability of aquaculture product (Divanach, et al., 1996; Koumoundouros, et al., 2002; Cahu, et al., 2003a; Sweetman, 2004; Verhaegen,

et al., 2007). Jaw malformations with a variety of morphologies occur in several fish species and the onset of their manifestation occurs at different stages during development, from yolksac larvae through to juveniles (ayu, *Plecoglossus altivelis* (Kanazawa, et al., 1981); Atlantic halibut, *Hippoglossus hippoglossus* (Morrison and MacDonald, 1995; Lein, et al., 1997); sea bass, *Dicentrarchus labrax* (Divanach, et al., 1996; Villeneuve, et al., 2005); Japanese flounder, *Paralichthys olivaceus*, (Suzuki, et al., 2000); Atlantic salmon, *Salmo salar* (Sadler, et al., 2001); striped trumpeter, *Latris lineata* (Cobcroft, et al., 2001a); yellowtail kingfish, *Seriola lalandi* (Cobcroft, et al., 2004); common carp, *Cyprinus carpio* (Kocour, et al., 2006); seven-band grouper, *Epinephelus septemfasciatus* (Nagano, et al., 2007). Jaw development is sensitive to a range of factors that give rise to malformations, including nutrition (Kanazawa, et al., 1981; Suzuki, et al., 2000; Villeneuve, et al., 2005), temperature (Lein, et al., 1997) and pollutants (Rosenthal and Alderdice, 1976), although the cause of many jaw malformations remains unknown. Fundamental system design factors are often overlooked when investigating the cause of malformations, particularly when they can have an origin in complex interactions between biotic and abiotic factors (e.g. nutrition and temperature).

Striped trumpeter, *Latris lineata*, is a candidate species for marine aquaculture in Tasmania that presently has a high incidence of jaw malformation in all batches of juveniles produced (Cobcroft, et al., 2001a; Battaglene and Cobcroft, 2007). The apparent onset of jaw malformation occurs after metamorphosis and follows changes in larval behaviour from an even distribution throughout the water column to close association with the tank walls. Striped trumpeter have a long pelagic larval phase, with flexion occurring from 22 to 28 dph (days post-hatching) and metamorphosis to a post-larval 'paperfish' at 36-44 dph, prior to settlement as a striped juvenile around nine months old (Battaglene and Cobcroft, 2007). Larvae and post-larvae often display vigorous swimming into the walls known as 'walling' behaviour (Cobcroft, et al., 2001a; Shaw, et al., in review). This behaviour has been observed in other species and has been explained as phototactic behaviour of the larvae, where the water surface and the tank wall are highly reflective and attract larvae (Bristow and Summerfelt, 1994; Naas, et al., 1996). Whilst nutritional factors cannot be eliminated, the apparent force exerted by striped trumpeter larvae during walling implicates mechanical damage as a potentially significant contributor to jaw malformation in this species. In addition, time spent walling is likely to reduce the time that larvae are feeding and cause increased nutrient utilisation, due to increased swimming activity, with consequent detrimental effects on growth and survival. The application of turbid culture conditions reduced walling in striped trumpeter larvae through the rotifer-feeding period via addition of microalgae to the culture water (greenwater) (Shaw, et al., in review) and in larval walleye with inert particles added to the water (Bristow and Summerfelt, 1994). In addition, tank wall colour has ameliorating effects on larval distribution, with black tanks recommended to reduce accumulation of larvae near the walls (Naas, et al., 1996). However, walling persists in older striped trumpeter post-larvae to 100 dph even in greenwater and black tanks. Striped trumpeter larvae are particularly sensitive to changes in their visual environment and small scale, short duration experiments demonstrate that background colour plays an important role in feed intake (Cobcroft, et al., 2001b; Shaw, 2006).

We examined the effect of an apparently simple abiotic factor, tank colour, which is a visual cue that elicits behavioural responses in striped trumpeter, on larvae and post-larvae during the *Artemia* feeding period. Previous studies investigating the effect of tank colour in larval fish culture have largely examined black, white and grey, green or tan combinations (Hinshaw, 1985; Ostrowski, 1989; Duray, et al., 1996; Martin-Robichaud and Peterson, 1998; Downing and Litvak, 1999; Tamazouzt, et al., 2000; Bransden, et al., 2005b; Pena, et

al., 2005; Jentoft, et al., 2006). Experimentation with a large range of colours is rare, although eight colour treatments were examined in juvenile seahorses as an environmental stimulus to change skin colour and to optimise feed intake (Martinez-Cardenas and Purser, 2007). Black, blue, green or white tanks are the most common in finfish hatcheries (Tucker, 1998; Kolkovski, et al., 2004; Olsen, et al., 2004). One notable exception is the use of orange or yellow tanks for tropical culture of grouper and milkfish in southeast Asia (Sim, et al., 2005). The objectives of this study were to determine: (i) the effect of six different tank wall colours on walling behaviour, and (ii) the relationship between walling behaviour and jaw malformation, growth and survival.

17.3 Materials and Methods

17.3.1 *Larval culture*

Egg incubation and early larval culture protocols followed standard procedures for striped trumpeter production at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories (Morehead and Hart, 2003; Bransden, et al., 2005c; Battaglene and Cobcroft, 2007). Briefly, gametes were collected from wild-caught, acclimated broodstock. Eggs were stripped from one female and fertilised with milt from three males. Fertilised eggs were disinfected with ozone at 1 mg O_3 l^{-1} for 1 min (Battaglene and Morehead, 2006) prior to stocking into 270-l upwelling incubation tanks supplied with seawater at 160 $l.h^{-1}$. All seawater used for egg incubation and larval rearing was 1µm filtered and ozonated to >700mV ORP for 10 min, followed by UV treatment (254 nm) and carbon filtration prior to delivery to tank systems at 300-350mV ORP and a salinity range of 34.0-34.6. Eggs were incubated at a density of 430 l^{-1} with a 14 h:10 h L:D photoperiod, at 14.2 ± 0.1°C (values are mean ± SD throughout) and hatched 5 days post-fertilisation. Following hatching, water temperature was gradually increased from 14.2°C to 15.8°C by 2 dph. At 2 dph, yolksac larvae were stocked at a density of 7.5 larvae l^{-1} into two 3000-l cylindroconical black fibreglass tanks, with a 'marble'-coloured adhesive panel on the walls from the surface to ~45 cm depth. Photoperiod was changed to 16 h:8 h L:D and light intensity was 11 $\mu mol.s^{-1}.m^{-2}$ at the water surface provided by a single 50 W, 12 V halogen light above each tank. All light intensities were measured with a Li-Cor LI-250 light meter with an LI-192SA sensor, calibrated for use in air or water as appropriate. Larvae were held in static clear seawater, with low aeration until 5 dph. From 6 dph, 300 l of algae *Nannochloropsis oculata* was added daily at 08:30, providing a greenwater environment during the light-phase with a turbidity of ~1.3 NTU. Larvae were fed daily at 09:00 from 6 dph with rotifers *Brachionus plicatilis* Austria at 10 ml^{-1}, enriched with Algamac 2000 (Aquafauna Biomarine, USA; 0.2 g per million rotifers for 12 h). From 6 dph, flow of clean seawater at 760-800 $l.h^{-1}$ flushed out uneaten food and provided water exchange during the dark-phase (23:00 to 08:30).

17.3.2 *Experiment*

Larvae were cultured from 16 dph to 44 dph in tanks with one of six different wall colours (described by CMYK colour separation model, Cyan: Magenta: Yellow: Black): black (0:0:0:98) , blue (100:72:0:32), green (100:91:42:0), marble (a black, grey and white mottled pattern), red (0:94:100:0), and white (0:0:0:0). The base colour of the 24, 300-l hemispherical fibreglass tanks in the experimental system was a matt black gel-coat. Alkor adhesive strips (Alkor-Venilia, Germany) were applied to a depth of 40-42 cm around the walls of the tanks, equivalent to 38 cm water depth, for all colours except black which was the control matt black (no Alkor). There were four replicate tanks of each colour, randomly

distributed. Light intensity was measured in several positions around each tank, 1 cm above and 1 cm below the water surface, to describe the light environment.

At 16 dph, 500 larvae were stocked into each of the 300-l tanks (1.7 larvae l^{-1}), 250 larvae sourced from each of the 3000-l production tanks. To reduce transfer shock, larvae were stocked into greenwater (*N. oculata* 3.5 x 10^5 cells ml^{-1}), which was slowly cleared with a constant incoming flow of seawater at 111 ± 1 l h^{-1} (n = 24). From 17 dph, total flow was increased to 214 ± 4 l h^{-1} with recirculation to facilitate the clearance of uneaten live food. Tanks had a central outlet fitted with a 390µm screen and a submerged side inlet creating a circular flow. The photoperiod on 16 and 17 dph was 22 h:2 h L:D (07:00 to 09:00 dark phase) and was continuous light thereafter (24 h L) to overcome problems with nocturnal downward movement of larvae (Bransden, et al., 2005a). Light intensity was 9.97 ± 1.05 µmol.s^{-1}.m^{-2} at the water surface provided by a single 50W, 12V halogen light located centrally above each tank. Water quality was monitored daily and temperature was 15.9 ± 0.1 °C (range 15.4 – 16.1), pH 8.16 ± 0.04 (7.94 – 8.24), dissolved oxygen 101 ± 4% (87 – 110%), and salinity 34.1 ± 2.1 (34.0 – 34.6). Larvae were fed with enriched rotifers on 16 and 17 dph at 5 ml^{-1}, prior to stocking and at 09:00, respectively. On 16 and 17 dph, *Artemia* nauplii enriched with Algamac 3050 (Aquafauna Biomarine, USA; 0.2 g per 100000 *Artemia* for 16 to 20 h) were added at 17:00 and 21:00 at 0.25 ml^{-1}. From 18 dph, enriched *Artemia* were fed four times daily at 0.25 ml^{-1} at 09:00, 13:00, 17:00 and 21:00. The tanks were spot siphoned daily to remove uneaten feed and faeces and mortalities were counted.

17.3.3 *Morphological sampling*

Larvae were randomly sampled from each of the two 3000-l production tanks at 16 dph (n = 20 per tank; initial measures), and during the experiment at 23, 30, 37 (n = 20 per tank each day) and 44 dph (n = 50 per tank). Larvae were anaesthetised in 0.06% 2-phenoxyethanol, and larval length measured using an eyepiece graticule fitted to an Olympus SZ stereomicroscope. Larvae were scored for swim bladder inflation (absent, normal, hyperinflated) and presence or absence of bacterial disease (white patches on fins and/or skin). The appearance of the jaws was rated on a scale of 0 to 3 according to the jaw malformation index in Table 17.1 (modified from Cobcroft, et al., 2004). The index of 0.5 indicated a very minor variation from normal structure that was unlikely to impair larval performance and that would not be considered a malformation from a commercial perspective. Larvae were defined as malformed when the jaw index was 1, 2 or 3. The dry weight of 20 larvae from each tank was determined on the same sampling days.

Table 17.1 Jaw malformation index used to score the condition of striped trumpeter larvae and post-larvae. Examples from 44 day-old post-larvae. All same scale.

Jaw index	Examples of appearance	Description
0		Normal position and shape of jaw elements.
0.5		Very minor variation from normal, e.g. slightly short lower jaw (this example), slightly snub nose (shortened distance from rostral tip to eye). Considered 'normal' for commercial production.
1		Minor variation from normal structure, e.g. with some resistance to closing mouth (this example), or with shortened lower jaw with normal movement.
2		Intermediate where some elements are abnormal in shape or position although limited movement occurs to open and close the mouth.
3		Severe malformation where jaw elements have abnormal shape or are in abnormal positions and do not move to close the mouth.

17.3.4 *Larval distribution and walling behaviour*

Larval distribution and behaviour were described daily in all tanks within 1 h of the 09:00 live food addition and the number of larvae touching the tank walls (from surface to 38 cm depth) was counted. In order to quantify horizontal distribution, digital photographs were taken approximately 3 h after food addition over a white Secchi disc placed at three positions within the tanks (edge, mid and centre) at 12 cm depth (three replicate photographs at each position) on 21, 24, 28, 31, 35, 38 and 42 dph. The number of larvae visible over the Secchi disc was counted for each digital photograph. Distribution was assessed at different times during the day on 28, 35 and 42 dph to assess changes in relation to food addition as a surrogate for prey density (08:00, no prey; 09:30, prey recently added; 12:30, prior to 13:00 prey addition). Vertical distribution of larvae was determined by the number of larvae counted over the Secchi disc (total of edge, mid and centre) as a proportion of the remaining population.

17.3.5 *Statistical Analysis*

Separate one way ANOVAs were used to test the effects of tank colour on larval performance indicators, survival, distribution, and the light intensity in the tanks. Data were transformed to achieve homogeneity of variance and normal distribution of residuals by arcsin $\sqrt{p}$, where p is the proportion, and by $\log_{10}$ for dry weight. Relative light intensity from different directions, as a proportion of the centre downwelling light intensity, and the ratio of downwelling:upwelling light intensity were compared at the tank centre and edge positions by separate one way ANOVAs. Where significant treatment effects were detected, a Tukey test was used to determine differences between means. One replicate blue tank incurred very high mortality due to a bacterial infection (see below), and was not included in ANOVAs and mean comparisons on 44 dph. Unbalanced ANOVAs were considered robust to the loss of one replicate in the 6 colour treatment × 4 replicate design (Sokal and Rohlf, 1995). Survival to 44 dph was low in red tanks, so this treatment was excluded from ANOVAs testing larval performance indicators at 44 dph. The effects of time and tank wall colour on horizontal distribution of larvae were assessed by comparing the frequency distribution among treatments with a Chi-Square test of independence. Observed frequencies were identified as significantly different to the expected value when the standardised residual was <-1.96 or >1.96. Separate linear regressions were used to describe the relationships between the proportion of fish walling (mean of whole tank observations to the day of jaw assessment) and the proportion of fish with jaw malformation at different ages, and between swim bladder hyperinflation and cumulative surface mortality. Statistical analyses were performed with JMP 5.1 (SAS Institute Inc.) and SPSS 14.0 (SPSS Inc.). Results are reported as mean ± SD and significance accepted as $P < 0.05$.

17.4 Results

17.4.1 *Jaw malformation*

Overall, the incidence and severity of jaw malformation increased with age and development from 23 to 44 dph (Figure 17.1). Tank colour significantly affected overall incidence of jaw malformation (jaw index 1, 2, and 3) on day 44 ($F_{4, 14} = 40.35$, $P < 0.001$). White (61 ± 7%) and green (57 ± 7%) tanks had a significantly greater proportion of malformed fish than black (21 ± 4%) and marble (18 ± 4%) tanks, with an intermediate incidence in blue (44 ± 9%) tanks on day 44. Fish in red tanks had the most jaw malformations (64 ± 9%), including the most severe malformations (14 ± 4%), but were not included in the statistical analysis due to low survival (Figure 17.1D). No fish exhibited severe jaw malformations in the marble tanks at 37 and 44 dph, with <4% on 30 dph, and there were <3% in black tanks at 37 and 44 dph. Both black and marble tanks had >50% of fish with normal jaws at 44 dph, and approximately 80% had no or very minor variation (jaw index 0 and 0.5).

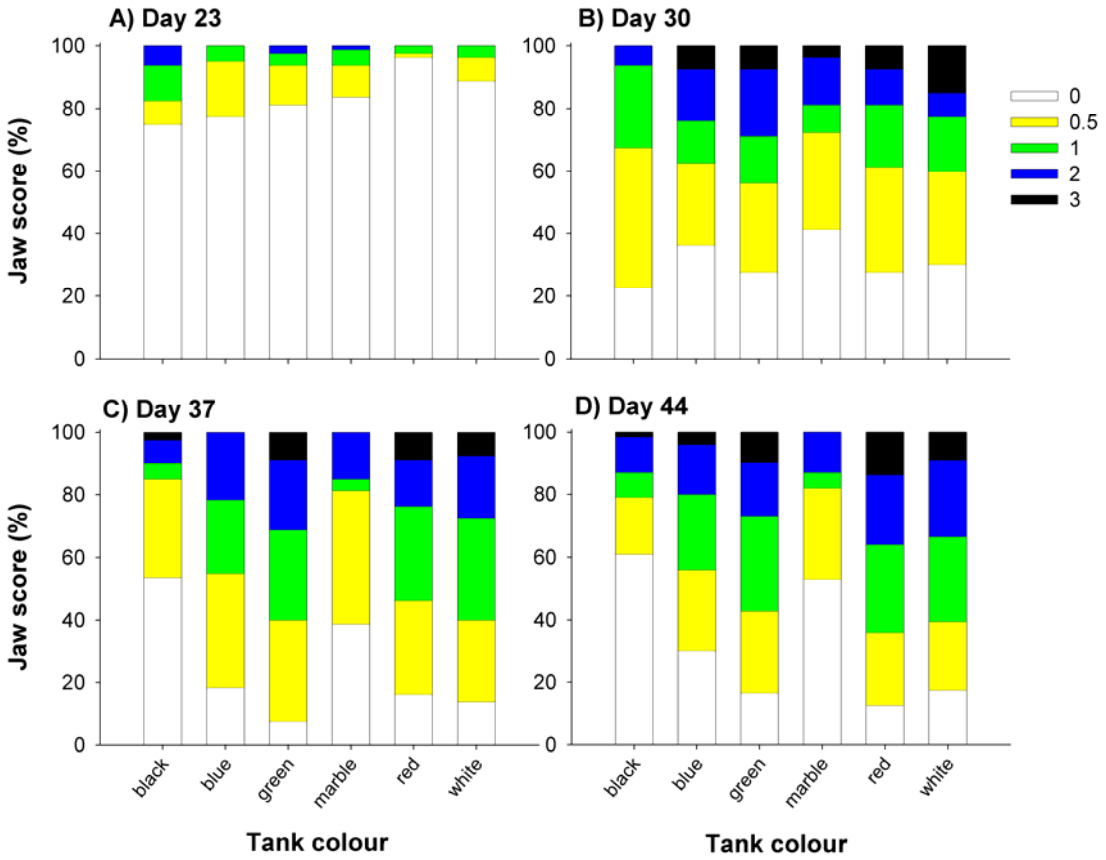

Figure 17.1 Incidence, severity and progression of jaw malformation in striped trumpeter larvae and post-larvae reared in tanks with different wall colour with age. Four replicate tanks per colour, n = 20 fish per tank on days 23, 30 & 37, with n = 50 on day 44, except n = 3 tanks on days 37 & 44 for blue, and n < 50 fish for all four red tanks (n = 11, 26, 38 & 42) and one green tank (n = 44) on day 44.

17.4.2 *Walling behaviour*

Larval distribution and behaviour were significantly affected by tank colour. Overall, there was an increase in the proportion of fish walling from the start of the trial to around 33 dph in most tanks, but walling remained lower and relatively constant in black and marble tanks (Figure 17.2). The mean daily proportion of larvae walling was lowest in marble (3.4 ± 0.2%) and black (9.6 ± 2.0%) tanks, significantly higher in blue (27.0 ± 4.2%) and white (31.5 ± 3.3%), followed by green (37.3 ± 1.5%), and significantly highest in red (44.3 ± 3.9%) tanks ($F_{5, 17}$ = 130.64, P < 0.001).

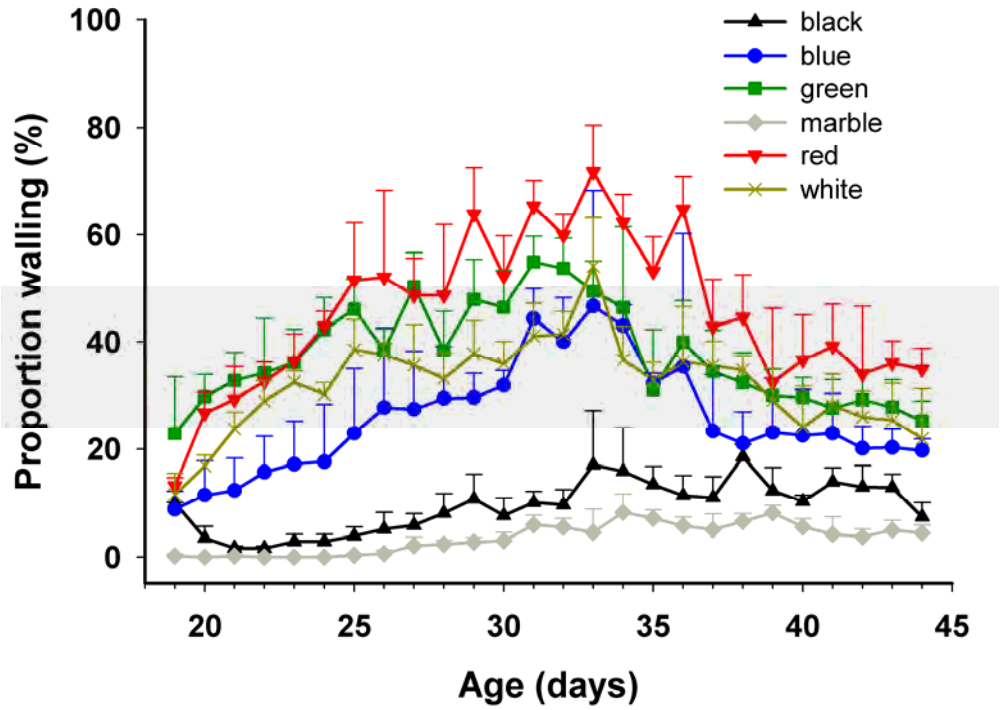

Figure 17.2 Proportion of striped trumpeter at different ages exhibiting walling behaviour in tanks with different wall colours. Values are mean + SD, n = 4 tanks each day, except for blue tanks which are a mean of three tanks from 30 dph.

The patterns observed in the proportion of fish walling from the whole tank observations in the morning and in the frequency distribution determined by Secchi disc counts were similar. Horizontal distribution of larvae, assessed with a Secchi disc, was not significantly affected by time around feed addition (data not shown), so counts at different times were pooled within day for further analysis. Tank wall colour had a significant effect on horizontal distribution (Figure 17.3). From days 21 to 35, the observed frequencies of larvae walling in white, green and red tanks were significantly higher than expected, except for red tanks at 21 dph when the number at the edge position, but not walling, was higher. In these tanks walling remained high on 38 and 42 dph, although the number of larvae counted declined with reducing larval density due to mortality and sampling, and there was a shift in the distribution of larvae in white tanks away from the walls, toward the tank centre on 42 dph. In contrast, there was a significantly lower number than expected walling in black and marble tanks to 42 dph, except for marble tanks at 42 dph when the number was not significantly different from expected (Figure 17.3). Reduced walling coincided with a higher number of fish than expected in the centre in black tanks, and close to the wall in the marble tanks. The distribution of fish in blue tanks was similar to black until 24 dph, followed by a higher number walling than expected on 28 and 31 dph.

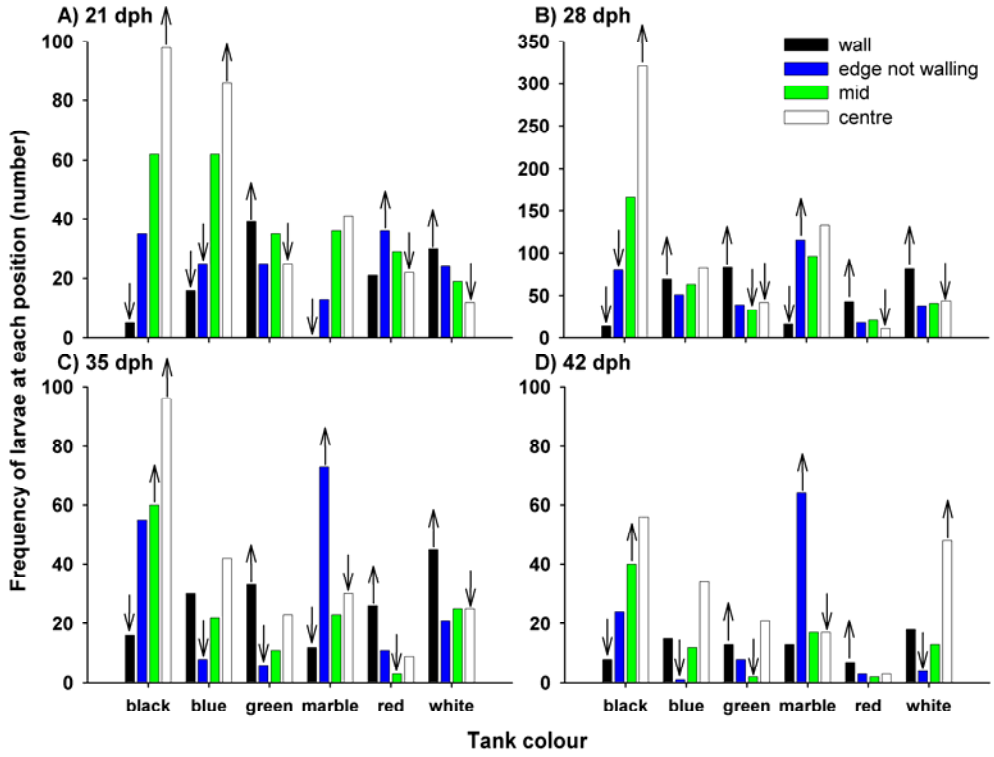

Figure 17.3 Horizontal frequency distribution of larvae walling, at the edge, middle and centre positions in tanks of different wall colours and at different ages. Bars are total number of larvae counted, pooled from n = 4 tanks each day, except for blue tanks which are a mean of three tanks from 35 dph, and n = 9 replicate counts at each position in each tank, except for 21 dph where n = 3 replicate counts. Arrows indicate observed frequencies that were significantly higher (arrow up) or lower (arrow down) than expected.

Vertical distribution of larvae was affected by tank colour (F_5 = 23.85, P = 0.000) and age (F_6 = 38.31, P = 0.000), with a significant interaction between the two factors (F_{30} = 3.62, P = 0.000). In black, blue, green and red tanks the proportion of larvae above the Secchi disc decreased to approximately half of initial levels with age (Figure 17.4). In marble tanks, there was an increase between 21 and 28 dph to 3.4%, indicating a shift of the population higher in the water column, followed by a decrease to the initial level (<2%). A similar, but not significant, increase from 21 dph was observed in black (28 dph) tanks. The proportion of larvae above the Secchi disc was similar in white tanks across all ages (1.8%).

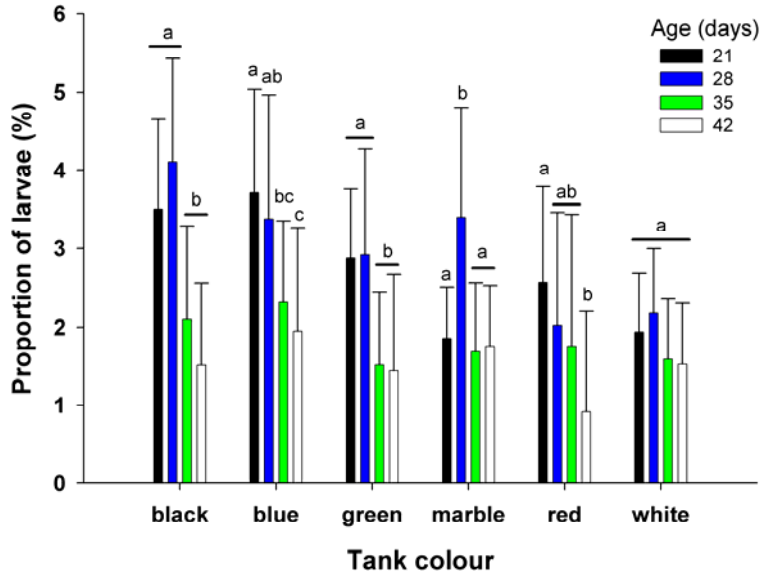

Figure 17.4 Striped trumpeter larvae counted above the Secchi disc as a proportion of the remaining population. Bars are mean + SD, n = 4 tanks each day, except for blue tanks which are a mean of three tanks from 35 dph, and n = 9 replicate counts per tank, except for 21 dph where n = 3 replicate counts. Different letters indicate significant differences between means within tank colour ($P < 0.05$). Horizontal bars denote means with the same letter(s).

17.4.3 *Relationship between jaw malformation and walling behaviour*

There was no significant relationship between the proportion of fish walling and jaw malformations at 23 dph (Figure 17.5). There were progressively more fish exhibiting jaw malformations with age in tanks with greater walling behaviour (day 30 and 37 data not shown). The proportion of fish with jaw malformations at 44 dph was positively correlated with the average proportion of fish walling ($y = 12.97 + 1.224.x$, $r^2 = 0.871$, $P < 0.001$) (Figure 17.5).

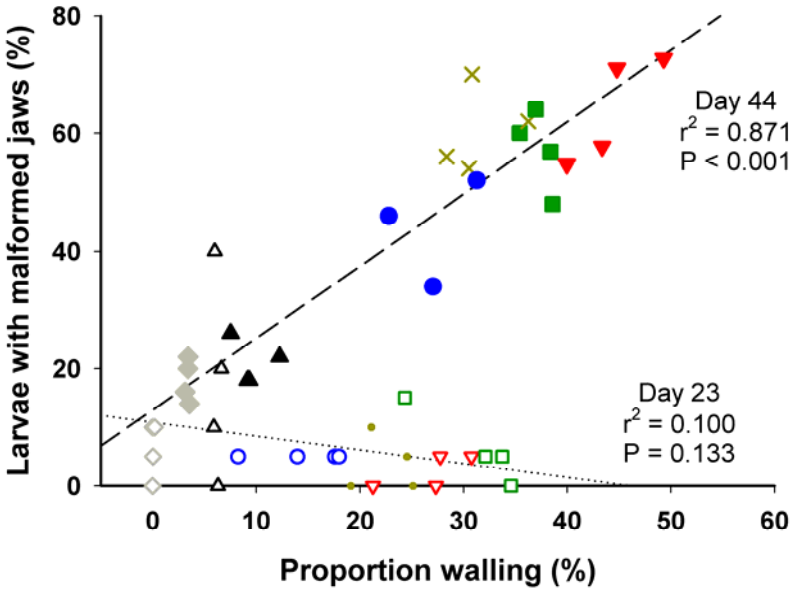

Figure 17.5 Relationship between the average proportion of larvae walling and jaw malformation (jaw index 1, 2 and 3) of striped trumpeter larvae on day 23 (open symbols) and 44 (closed symbols) post-hatching. Values are for each tank, and tank colour is represented by symbol either black (triangle up), blue (circle), green (square), marble (diamond), red (triangle down), white (cross). n = 20 fish per tank on day 23, and on day 44 n = 50 except for all red and one green tank where n < 50.

17.4.4 *Survival and bacterial lesions*

Survival to 44 dph was significantly affected by tank wall colour ($F_{5, 17}$ = 19.46, P <0.001), and was highest in black (71%), followed by marble, white, blue, green, and red (11%) tanks (Figure 17.6). One blue tank had very high mortality from 25 dph due to a Flavobacteria-type infection and was not included in the statistical analysis of survival. The last surviving fish in that tank were sampled for morphometric assessment on 29 dph, when 85% showed gross signs of dermal necrosis. Overall, the proportion of fish affected by bacteria was variable among tanks of all colours (range 0 - 30%, mean 9 ± 8%, n = 69) and between sampling days (
Table 17.2).

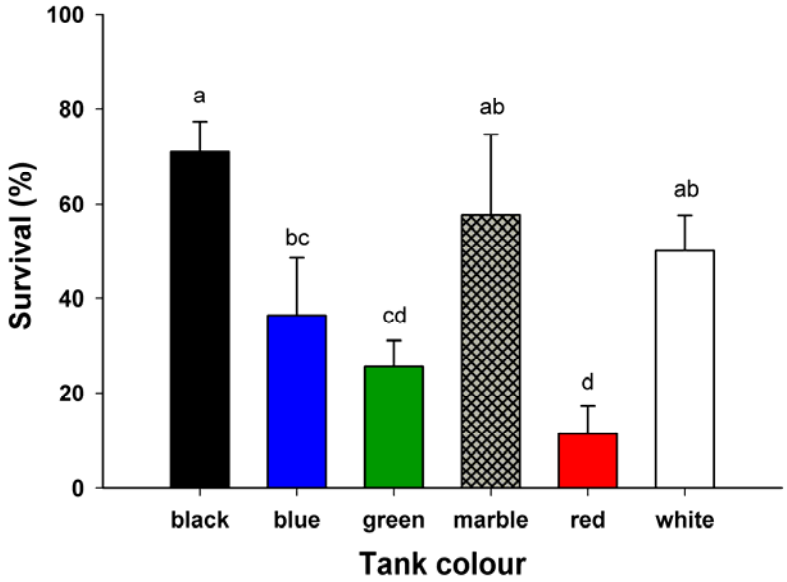

Figure 17.6 Total survival of striped trumpeter larvae from 16 to 44 dph reared in tanks with different wall colours. Values are mean + SD, n = 4, except for blue tanks, n = 3. Different letters indicate significant differences between means ($P < 0.05$).

Table 17.2 Performance of striped trumpeter larvae at 23, 30, 37, and 44 dph reared in tanks with different wall colours.†

Treatment	**Unit**	**Black**	**Blue**	**Green**	**Marble**	**Red**	**White**		
Age Parameter								P	Sig.
23 dph									
Bacteria	%	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0		
Swim bladder hyperinflation	%	0 ± 0 a	6 ± 8 a	13 ± 10 a	10 ± 12 a	48 ± 20 b	1 ± 3 a	0.000	***
30 dph									
Bacteria	%	6 ± 5 ab	17 ± 3 ab £	16 ± 9 ab	4 ± 5 a	21 ± 8 b	4 ± 8 a	0.006	**
Swim bladder hyperinflation	%	0 ± 0 a	0 ± 0 a	3 ± 3 a	0 ± 0 a	29 ± 19 b	1 ± 3 a	0.000	***
37 dph			n = 3						
Bacteria	%	3 ± 3 a	3 ± 3 a	9 ± 6 ab	8 ± 3 ab	18 ± 9 b	13 ± 3 ab	0.014	*
Swim bladder hyperinflation	%	1 ± 3 a	2 ± 3 a	3 ± 3 ab	0 ± 0 a	13 ± 10 b	0 ± 0 a	0.000	***
44 dph ‡			n = 3						
Bacteria	%	4 ± 3	9 ± 8	13 ± 7	3 ± 2	9 ± 6	12 ± 4	0.022 #	*
Swim bladder hyperinflation	%	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0		

† Values are mean ± SD, n = 4 tanks per treatment, except for blue, where n = 3 on 37 and 44 dph.
£ Value is mean ± SD, n = 3 tanks, to exclude the one tank with very high incidence of bacterial lesions. Including this tank, the incidence of bacteria was 34 ± 34%.
‡ Red tanks excluded from statistical analyses.
# Significant ANOVA, but no significant differences between treatment means with Tukey test.

17.4.5 *Growth*

Tank colour had a significant effect on larval length and dry weight at 44 dph ($F_{4, 938}$ = 39.82, P = 0.000, and $F_{4, 33}$ = 8.31, P = 0.000, respectively). Larvae cultured in black and marble coloured tanks were significantly longer than fish in blue and green tanks, which were in turn significantly longer and heavier than fish in white tanks (P < 0.05) (Figure 17.7a). Dry weight of larvae followed a similar pattern (Figure 17.7b). Fish in the red tanks were shorter and had the smallest dry weight compared with those reared in all other tank colours, although there were too few surviving on 44 dph for statistical comparison. Larvae from black, marble and blue tanks completed flexion earlier than those from the other tank colours.

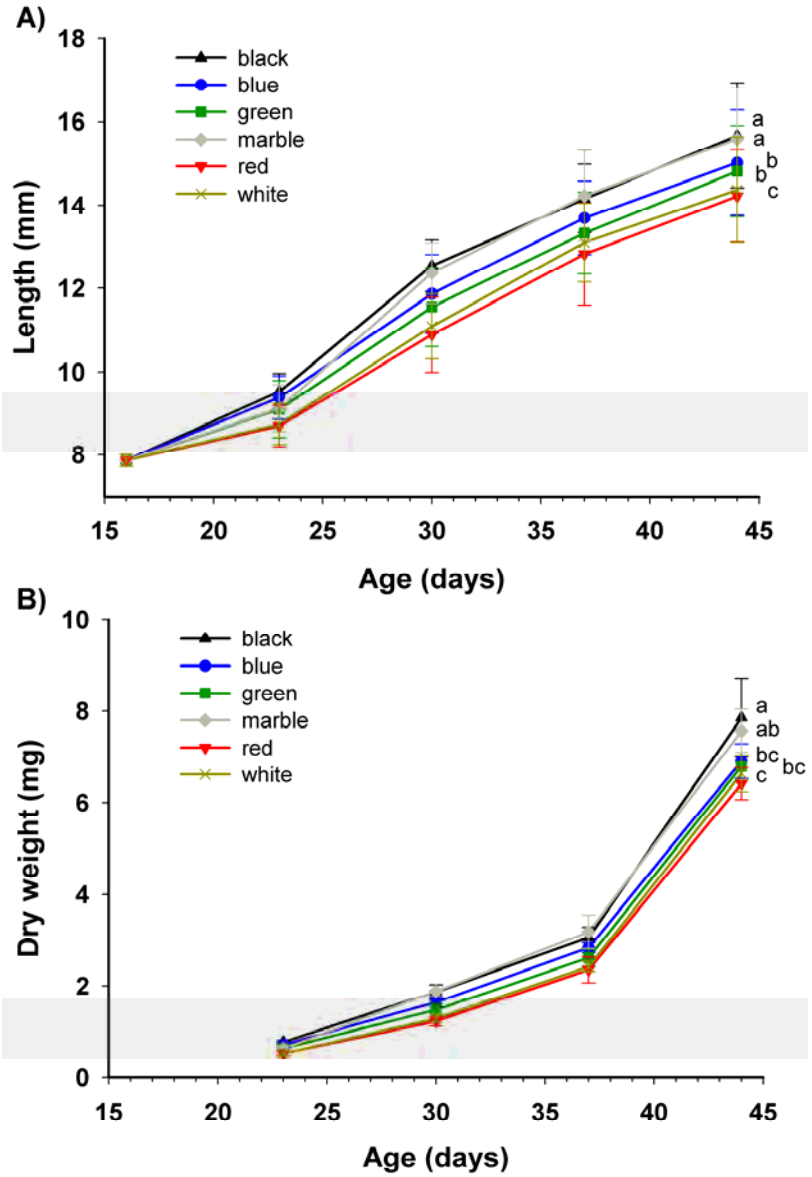

Figure 17.7 Changes in A) length and B) dry weight of striped trumpeter larvae grown in tanks with different wall colours with age. Day 23 is standard length, days 30 and 37 are total length and day 44 is fork length. Values are mean ± SD, n = 4. Different letters indicate significant differences between means at 44 dph (P < 0.05).

17.4.6 *Swim bladder hyperinflation*

At stocking on 16 dph, 95% of larvae had inflated swim bladders and the overall proportion remained unchanged at 23 and 30 dph across treatments. In contrast, transfer to coloured tanks affected swim bladder hyperinflation. Almost 50% of larvae transferred to red tanks exhibited swim bladder hyperinflation at 23 dph, decreasing to 13% on 37 dph (Table 17.2). Hyperinflation occurred in significantly lower proportions and with reduced severity in other treatments. There was a significant positive relationship between fish dying at the water surface (surface mortality) and swim bladder hyperinflation ($y = 54.331 + 2.694.x$, $r^2 = 0.548$, $P < 0.001$). Peak surface mortality occurred between 19 and 26 dph and was highest at 22 dph (18.0 ± 17.1), with very few surface deaths after 30 dph. Surface mortality, as a proportion of total mortality, was significantly greater in red tanks than in marble and black tanks, with intermediate levels in green, white and blue ($F_{5, 18} = 6.16$, $P = 0.002$) (Figure 17.8).

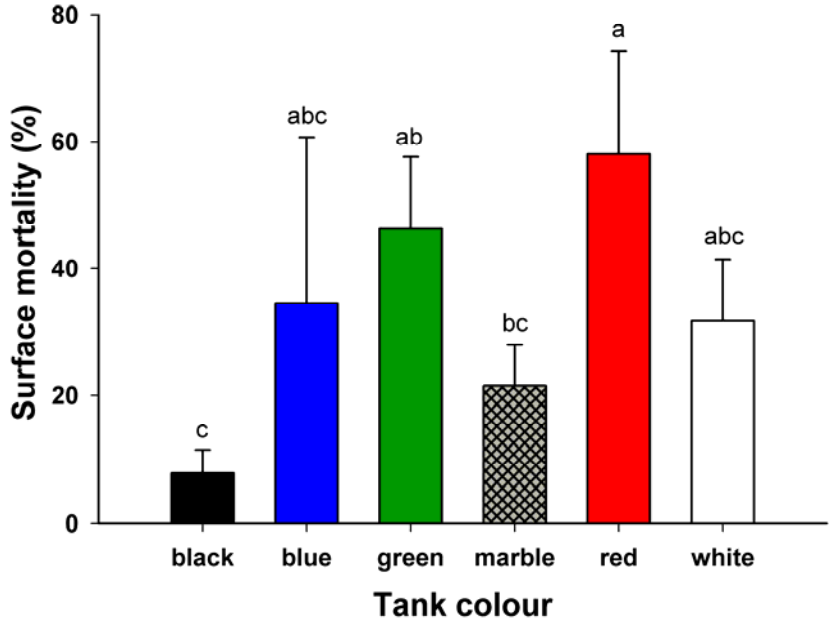

Figure 17.8 Surface mortality of striped trumpeter larvae as a proportion of total mortality to 44 dph in tanks with different wall colours. Values are mean + SD, n = 4 tanks. Different letters indicate significant differences between means ($P < 0.05$).

17.4.7 *Light environment*

Light intensity at different positions within the tanks was affected by tank wall colour (Figure 17.9a-f). There was no significant difference in downwelling light in air at the tank centre with tank colour ($F_{5, 18} = 0.99$, $P = 0.450$) (Figure 17.9a). However, reflected light from the walls and bottom of the tank, were significantly affected by tank wall colour (Figure 17.9b-f). The highest reflected light occurred in white-walled tanks, followed by marble and red, with lowest reflected light in green, blue and black tanks. Downwelling light was also lower at the edge than in the centre of the tanks. The ratio of downwelling to upwelling light ranged from 6:1 in white tanks to 40:1 in black tanks in the tank centre (Figure 17.9g) and 3:1 in white to 107:1 in black at the edge (Figure 17.9h).

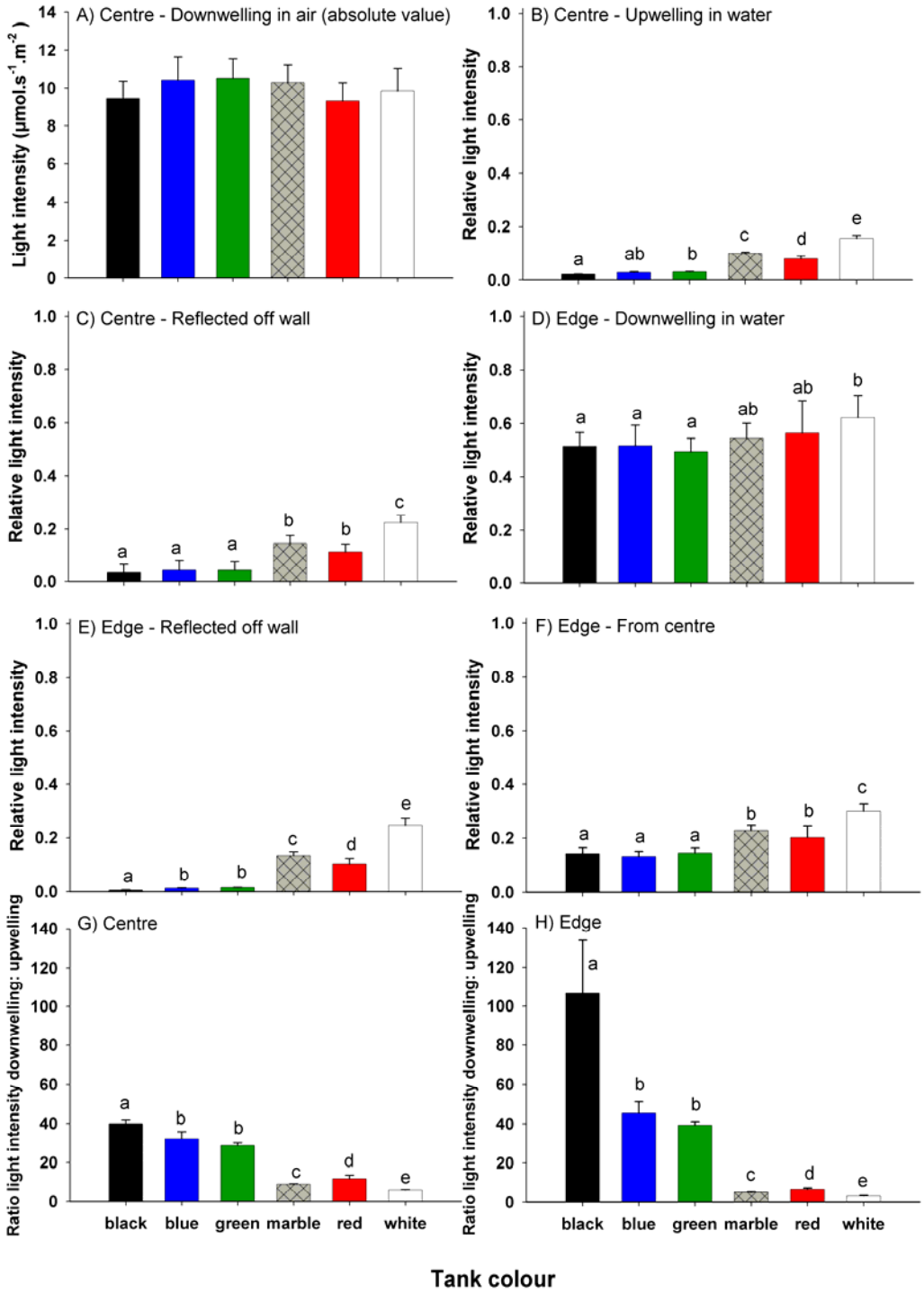

Figure 17.9 Light intensity at the centre and edge of each tank colour treatment. A) is absolute light intensity in air, B)-F) are relative intensities measured in water as a proportion of the downwelling light in air at the tank centre, and G)-H) are ratios of downwelling to upwelling light intensity 1 cm below the water surface. In the centre of the tank, A) downwelling in air, B) upwelling in water, and C) reflected off the wall. At the edge of the tank, D) downwelling, E) reflected off the wall, and F) from centre of tank. Ratios at G) the centre and H) the edge of the tanks. Values are mean + SD, n = 4 tanks and 1-4 measurements per tank depending upon meter position and orientation.

17.5 Discussion

Tank wall colour had a significant effect on growth, survival, swim bladder hyperinflation, behaviour and jaw malformation of striped trumpeter larvae. Fish reared in black and marble tanks exhibited a lower proportion of walling behaviour, they grew longer, had higher survival and a lower incidence of malformations than fish in other tank colours. The incidence and severity of malformations remained relatively constant from 30 dph in the black and marble tanks with around 80% of fish with no or very minor jaw malformations, but became progressively worse in tanks with greater amounts of walling behaviour, especially in red, green and white tanks.

There are four possible mechanisms for the positive correlation between walling behaviour, which was induced by tank colour, and jaw malformation in larval striped trumpeter. First, mechanical abrasion around the mouth and head from striking the wall may have damaged the soft tissues or skeletal jaw elements, alignment and subsequent development of the structures. Gross observations of post-larvae indicated evidence for physical damage around the jaws caused by walling, ranging from erosion of tissues and chronic lesions to lateral breakage of the lower jaw. Second, larval nutrition was compromised by walling behaviour, as the time that fish spent foraging was reduced and feed intake was lowered. Third, energy consumption may have been higher in fish exhibiting vigorous walling behaviour in comparison with fish in the water column. Poor larval nutrition, as a result of an imbalance between nutrient intake and requirements, has a detrimental effect on developmental processes, including skeletal development in a range of marine fish (Kanazawa, et al., 1981; Gapasin, et al., 1998; Suzuki, et al., 2000; Cahu, et al., 2003a; Cahu, et al., 2003b; Villeneuve, et al., 2006). Finally, soft tissue damage caused by abrasion in combination with contact with tank surfaces may have resulted in infection by pathogenic agents (e.g. bacteria and fungi) that disrupt normal jaw development, similar to that found in Atlantic halibut yolksac larvae (Morrison and MacDonald, 1995). Whilst a bacterial infection was apparent in some tanks during the experiment there were no apparent relationships between incidence of lesions and tank colour or jaw malformation. The absence of other significant skeletal malformations, besides that of the jaw elements, is strong evidence that physical contact with the walls was the primary cause of malformations in striped trumpeter, although compromised nutritional status and infections may exacerbate the extent and severity of jaw malformations.

The theory that walling behaviour is caused by the phototactic response of larval fish to aggregate near the high intensity reflected light from the tank walls (Bristow and Summerfelt, 1994; Naas, et al., 1996) does not explain all of the differences in walling behaviour observed in larval striped trumpeter. While reflected light conditions were similar in white and marble tanks, the proportion of larvae walling was significantly higher in white tanks. In addition, walling was minimal in black tanks compared with blue and green tanks that had similar light intensity reflection as measured by the quantum light sensor, although the different tank colours demonstrated that the reflected spectral composition was different. The marble pattern had a beneficial effect by reducing walling, potentially because the mixture of light and dark pixels in the pattern created a visual plane of focus for the larvae and a reference point for a solid surface to be avoided. However, older larvae demonstrated an attraction to the marble-patterned wall (indicated by larval distribution) without coming in contact with the surface. This behaviour could be interpreted as a refuge response, with larvae seeking a surface against which to hide as opposed to open water (Bradner and McRobert, 2001), or as a move into an area with higher visual contrast between prey and background to increase feeding (Browman and Marcotte, 1987; Utne-Palm, 1999). In contrast, the beneficial effect of black tanks was likely due to the ratio of downwelling to

upwelling light, which was higher than other tank colours especially at the wall (107:1). This condition simulates the natural light environment of the ocean, where the majority of light is downwelling, with only a small amount of upwelling light (Lythgoe, 1988). Naas, et al. (1996) recommended black-walled tanks in order to mimic natural conditions to achieve a high ratio of downwelling to upwelling light in a culture tank environment, and whilst black provided for best larval performance in some studies (Hinshaw, 1985; Ostrowski, 1989; Martin-Robichaud and Peterson, 1998; Jentoft, et al., 2006), white or tan tanks were optimal in others (Duray, et al., 1996; Downing and Litvak, 1999; Tamazouzt, et al., 2000).

Manipulation of larval behaviour and distribution by tank colour, as shown in this study, was successful in reducing walling behaviour. Larvae in the wild do not presumably come into contact with hard surfaces and their inability to leave a tank wall when contacted is possibly an innate flight response. Many oceanic larvae are excellent swimmers and they have an acute response to food shortages being conditioned to travel large distances in search of patches of zooplankton or suitable settlement habitat (Hunter and Thomas, 1974; Leis, et al., 2006; Gerlach, et al., 2007). This innate capacity for sustained swimming by pelagic larvae provides challenges in aquaculture. The appearance and onset of jaw malformation, around flexion to metamorphosis, in striped trumpeter (Cobcroft, et al., 2001a) is very similar to that which occurs in cultured yellowtail kingfish (Cobcroft, et al., 2004). Both species have oceanic, pelagic life histories, with rapid larval development for temperate species (especially kingfish, Moran, 2007; Moran, et al., 2007). It can be difficult to maintain prey/food densities and distribution that encourage fish away from hard surface interactions, especially when satiated fish reduce feeding behaviour and increase swimming. The interaction with hard surfaces can also result from a flight response initiated by changes in shadows or noise (e.g. due to human activity around tanks) or by reduced water quality (e.g. when dissolved oxygen drops after feeding).

Swim bladder hyperinflation has been recorded in the larval rearing of a number of species, including grey mullet, *Mugil cephalus* (Nash, et al., 1977), barramundi, *Lates calcarifer* (Bagarinao and Kungvankij, 1986), sea bass (Johnson and Katavic, 1984; Katavic, 1986), and Atlantic cod, *Gadus morhua* (Shields, et al., 2003). It is usually linked to a multitude of stress inducing factors including inappropriate temperatures, light conditions, handling, change in feeding regime and poor water quality (Nash, et al., 1977; Johnson and Katavic, 1984; Bagarinao and Kungvankij, 1986; Shields, et al., 2003). The mortality of striped trumpeter larvae at the tank surface, associated with hyperinflation, is consistent with mass mortality of fish caused by swim bladder stress syndrome (Nash, et al., 1977; Bagarinao and Kungvankij, 1986; Katavic, 1986; Shields, et al., 2003). Hyperinflation in striped trumpeter was observed in larvae undergoing flexion and occurred during the transition from nocturnal upward movement at night up to 23 dph to a downward migration when larvae are around 9.5 mm in length from 25 to 30 dph (Bransden, et al., 2005a; Trotter, et al., 2005b). Peak surface mortality followed handling and transfer of larvae, the transition from greenwater to clearwater culture, weaning from rotifers to *Artemia* and increasing light period to 24 h L. All of these factors may have contributed to hyperinflation and associated mortality. The mechanisms involved in hyperinflation are not well understood but may be related to the effect that a variety of stressors have on the complex processes of gas solubility in blood and acidic metabolites associated with deposition and resorption of gas from the swim bladder (Pelster, 1998). Nash, et al. (1977) suggested excessive gulping of air from the surface was the cause in *Mugil cephalus* larvae. Striped trumpeter were beyond the swim bladder inflation window and without a functional pneumatic duct, so excessive gulping was not the cause of hyperinflation (Trotter, et al., 2005a). Likewise, seahorse *Hippocampus abdominalis* juveniles that were prevented from ingesting air at the water surface, also

developed hyperinflation (Woods, 2000). It appears that a combination of secretion of too much gas into the swim bladder and/or an inability for excess gas to diffuse out can result in hyperinflation.

17.6 Conclusion

By inducing different degrees of walling behaviour in striped trumpeter, through manipulation of the visual environment with different tank wall colours, our study has clearly demonstrated the close link between walling and jaw malformation. Growth, survival, swim bladder hyperinflation, and distribution of striped trumpeter larvae were also significantly affected by tank colour. Our technique of using coloured Alkor adhesive strips overcomes the difficulty of painting tanks. The product is quick, cost effective and provides great versatility of choice of colour or patterns, leaving no permanent mark on the tanks when removed. Both a high ratio of downwelling to upwelling light (black) and a patterned wall (marble) reduced walling behaviour and produced fish with the lowest malformations. It remains to be confirmed whether a combination of these light conditions may further reduce larval walling. Now that a link has been demonstrated between walling behaviour and jaw malformation in striped trumpeter, research will be focused on rearing conditions (e.g. lighting regimes, tank wall colours, feeding protocols, larval density) and system design (e.g. large tanks, water movement) to reduce this behaviour. The findings are particularly relevant to other pelagic species like yellowtail kingfish which have similar jaw malformations and pelagic larval development.

17.7 Acknowledgments

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18 JAW MALFORMATION IN STRIPED TRUMPETER LARVAE IS CORRELATED WITH CULTURE CONDITIONS, WHITE TANKS AND CLEARWATER, WALLING BEHAVIOUR AND LIVE FEED ENRICHMENT

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18.1 Abstract

A high incidence of jaw malformation has hindered the production of quality striped trumpeter *Latris lineata* juveniles and has been correlated with walling behaviour in *Artemia*-fed larvae. In this study, striped trumpeter were reared from first feeding in different coloured tanks (black or white), culture conditions (clear or greenwater) and fed different diets (enriched or non-enriched rotifers and *Artemia*), to examine the effects on behaviour and jaw malformation to 29 days post-hatching (dph). The highest incidence and severity of jaw malformations occurred in clearwater and enriched diet treatments, being significantly more common in white (70 ± 15%) than in black tanks (26 ± 15%). In black tanks, jaw malformation was significantly more common in larvae fed enriched diets (18 ± 14%) than in those fed non-enriched diets (8 ± 8%) and in clearwater (19 ± 14%) versus greenwater (7 ± 6%). At the end of the rotifer feeding phase, larvae were significantly larger in black (8.44 ± 0.42 mm, 0.79 ± 0.09 mg) than white tanks (7.51 ± 0.52 mm, 0.55 ± 0.06 mg), and longer in greenwater (8.74 ± 0.23 mm) than in clearwater (8.44 ± 0.42 mm). In the *Artemia* feeding phase, larval growth was slower and mortality was higher in non-enriched treatments compared with those fed enriched diet. Larval length and survival were higher in black (9.4 ± 0.9 mm, 26 ± 13%) than in white tanks (8.0 ± 1.0 mm, 10 ± 10%) and higher in enriched (9.3 ± 1.0 mm, 24 ± 16%) than in non-enriched (8.1 ± 1.0 mm, 12 ± 10%) diet treatments, whilst survival was higher in greenwater (46 ± 18%) versus clearwater (x± ??%). Swimbladder inflation was significantly higher in fish reared in black (50 ± 20%) than in white (25 ± 11%) tanks and in greenwater (95 ± 5%) versus clearwater (25 ± 11%). The average proportion of larvae walling over the duration of the experiment was higher in white than black tanks, and higher in enriched than non-enriched treatments, with lowest walling in greenwater. There was no consistent relationship between feed intake and jaw malformation. However, there was a significant positive correlation between walling and the incidence of jaw malformation at 29 dph in larvae fed enriched diets, but not in larvae fed non-enriched diets. The use of greenwater, black tanks and enriched live feeds are required for good growth, development and survival of striped trumpeter. The study emphasises the importance of reducing walling in the culture of oceanic larvae and may have direct application in the rearing of other marine fish with similar malformations such as yellowtail kingfish.

18.2 Introduction

The production of marine finfish through aquaculture is dependent upon the reliable supply of high-quality hatchery produced juveniles. An important determinant of juvenile quality is the incidence and severity of skeletal malformations. Both cranial and vertebral malformations can detrimentally affect performance of fish during on-growing, increase production costs, reduce the value of harvested product, and affect animal welfare in culture (Divanach, et al., 1996; Georgakopoulou, et al., 2007; Verhaegen, et al., 2007). In marine fish, many skeletal malformations arise early in larval development and become apparent around metamorphosis (Cobcroft, et al., 2001a; Cobcroft, et al., 2004; Lewis and Lall, 2006; Verhaegen, et al., 2007). Malformations occurring in larval culture have been associated with unbalanced nutrition (Cahu, et al., 2003a), poor swim bladder inflation (Chatain, 1994), inappropriate abiotic rearing conditions (temperature, salinity and dissolved oxygen) (Santerre, 1976; Lein, et al., 1997; Sawada, et al., 2006; Sfakianakis, et al., 2006), and association of larvae with tank walls causing tissue damage (Morrison and MacDonald, 1995; Cobcroft and Battaglene, 2009).

Improvements in larval quality have been achieved by investigating the effects of abiotic culture conditions and nutritional requirements. Appropriate rearing temperature reduced cranial malformations in Atlantic halibut *Hippoglossus hippoglossus* (Lein, et al., 1997) and in sea bass *Dicentrarchus labrax* (Georgakopoulou, et al., 2007), and rearing larvae in silos with shaded walls reduced jaw malformation in Atlantic halibut (Morrison and MacDonald, 1995). Supplementation of larval diets with vitamin C reduced opercular malformations in larval milkfish *Chanos chanos* (Gapasin, et al., 1998), while the provision of phospholipids in diets reduced spinal malformations for ayu *Plecoglossus altivelis* (Kanazawa, et al., 1981) and sea bass (Cahu, et al., 2003b; Villeneuve, et al., 2005b). Appropriate levels of vitamin A and vitamin A metabolites in larval diets have been established to reduce jaw malformation in Japanese flounder *Paralichthys olivaceus*, and sea bass (Dedi, et al., 1995; Takeuchi, et al., 1998; Villeneuve, et al., 2005a).

Early rearing trials with striped trumpeter larvae in clearwater and greenwater with low prey density (5 rotifers.ml^{-1}, as described in Cobcroft, et al., 2001b) resulted in a very high proportion of the population with jaw malformations from around Day 44 (>10 mm standard length, SL), although the proportion was not quantified due to the coincident low survival (Cobcroft, et al., 2001a). In a recent experiment, jaw malformations were identified in larvae reared under standard 'control' conditions at a younger age but similar size and developmental stage compared with earlier trials, with 18% of fish at Day 23 (9.5 mm SL) affected (Cobcroft and Battaglene, 2009). However, metamorphosed post-larvae from recent production trials have tended to have lower severity and incidence of jaw malformations than in early trials. The onset and severity of jaw malformation in striped trumpeter is correlated with 'walling' behaviour of larvae during the *Artemia* feeding period, which can be manipulated with tank wall colour (Cobcroft and Battaglene, 2009). Physical damage due to contact with the walls, higher energetic requirements, or compromised nutrition could explain the relationship between increased walling and severe jaw malformations. It is unclear if the genesis of jaw malformation occurs earlier in larval rearing partly because the gross identification of malformations may be constrained by the timing of ossification of the jaw elements around metamorphosis (Cobcroft et. Al., 2001). Our study aimed to:

1) determine how early jaw malformations can develop in striped trumpeter larvae
2) induce populations with high and low levels of jaw malformation at a young age,

We did this by manipulating early jaw development of striped trumpeter larvae through tank colour, culture conditions (clear or greenwater) and diet quality (enriched and non-enriched

rotifers and *Artemia*). The experiment was designed to better understand factors affecting malformations that could be applied to improve culture conditions.

18.3 Materials and Methods

18.3.1 *Yolksac larval culture*

Egg incubation and early larval culture protocols followed standard procedures for striped trumpeter production at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories (Morehead and Hart, 2003; Bransden, et al., 2005b; Battaglene and Cobcroft, 2007). Briefly, gametes were collected from wild-caught, acclimated broodstock. Eggs were stripped from one female and fertilised with milt from three males. Fertilised eggs were disinfected with ozone at 1 mg O_3 l^{-1} for 1 min (Battaglene and Morehead, 2006) prior to stocking at a density of 320 embryos l^{-1} into 270-l upwelling incubation tanks supplied with seawater at 150 l h^{-1}. All seawater used for egg incubation and larval rearing was 1 µm filtered and ozonated to > 700 mV ORP for 10 min, followed by UV treatment (254 nm) and carbon filtration prior to delivery to tank systems at 328 ± 27 mV ORP (values are mean ± SD throughout) range 270-390 mV ORP and a salinity of 34.1 ± 0.1 range of 33.3-34.7. Eggs were incubated with a 14 h:10 h L:D photoperiod, at 14.2 ± 0.2 °C and hatched 5 days post-fertilisation. Following hatching, water temperature was gradually increased to 15.9 °C by 1 dph. At 1 dph, 3000 yolksac larvae were stocked into each of twenty four 300-l hemispherical fibreglass tanks (10 larvae l^{-1}). One litre of water from the incubator was added to each tank, prior to stocking larvae, to provide a thin oil film and prevent mortality associated with larvae sticking to a clean water surface. Photoperiod was increased to 16 h:8 h L:D on 1 dph, and light intensity was 9.9 ± 0.8 µmol s^{-1} m^{-2} at the water surface provided by a single 50 W, 12 V halogen light above each tank. Larvae were held in static clear seawater, without aeration until 5 dph.

18.3.2 *Experiment*

Larvae were cultured from 1 to 29 dph with one of six different treatment combinations to examine the effects of three factors, each with two levels; tank wall colour (black - BL, white - WH), live feed enrichment (Algamac - AM, non-enriched - NE), and culture environment (greenwater - GW, clearwater - CW) (Table 18.1). The experiment design was not fully orthogonal due to the limited number of tanks available and the requirement for a minimum of four replicate tanks per treatment.

Table 18.1 Experiment design, indicating the number of tanks for each treatment. Treatment abbreviations are shown in brackets.

Treatment factor		**Culture Environment**	
Tank colour	**Diet**	Greenwater (GW)	Clearwater (CW)
Black (BL)	Algamac (AM)	4 *	4
	Non-enriched (NE)	4	4
White (WH)	Algamac (AM)	-	4
	Non-enriched (NE)	-	4

* Combination of factors that is used as the standard protocol for the culture of larval striped trumpeter.
- Not tested.

The base colour of the 24, 300-l hemispherical fibreglass tanks in the experimental system was a matt black gel-coat. White Alkor adhesive strips (Alkor-Venilia, Germany) were applied to a depth of 40-42 cm around the walls of the "white" treatment tanks, equivalent to 38 cm water depth. The Alkor adhesive had no detrimental effect on performance of striped trumpeter larvae in a previous experiment (Cobcroft and Battaglene, 2009). There were four replicate tanks of each treatment, randomly distributed in the experimental system. Larvae were fed daily at 09:00 with rotifers *Brachionus plicatilis* Austria, either non-enriched or enriched with Algamac 2000 (Aquafauna Biomarine, USA; 0.2 g per 1 000 000 rotifers for 12 h), according to the treatment, at 10 ml^{-1} from 6 to 17 dph, and at 5 ml^{-1} on 18 and 19 dph during transition to *Artemia*. *Artemia* nauplii, either non-enriched or enriched with Algamac 3050 (Aquafauna Biomarine, USA; 0.2 g per 100 000 *Artemia* for 16-20 h) were added twice on 18 and 19 dph at 05:00 and 09:00 at 0.25 ml^{-1}. From 20 dph, enriched *Artemia* were fed four times daily at 0.25 ml^{-1} at 09:00, 13:00, 17:00 and 21:00. From 6 dph, 42-48 l of temperature acclimated, live algae *Nannochloropsis oculata* or filtered seawater was added daily at 08:30, providing a greenwater or clearwater environment, respectively. Turbidity in the greenwater treatment tanks was 3 NTU.

From 6 dph, flow of clear seawater at 113 l h^{-1} (23:00 to 08:30) flushed out uneaten food and provided water exchange during the dark-phase. From 20 dph, an additional 113 l h^{-1} of continuous recirculating flow increased the clearance of uneaten live food. Water quality was monitored daily and temperature was 15.8 ± 0.3 °C (range 15.2-16.5 °C), pH 8.02 ± 0.04 (7.95 – 8.60), and dissolved oxygen 101 ± 5% (84 – 119%). Surface skimmers were applied to remove oily films from the water surface from 8 to 14 dph. Aeration was 200 ml min^{-1} from 6 to 20 dph and was stopped during the dark phase (6 to 15 dph) to facilitate larval surface access for the swim bladder inflation window (Trotter, et al., 2005). Tanks had a central outlet fitted with a 390 µm screen and a submerged side inlet creating a circular flow. Photoperiod was 16 h:8 h L:D until 20 dph, then 22 h:2 h L:D on 21 dph (07:00 to 09:00 dark phase), and continuous light (24 h L) from 22 dph to overcome problems associated with nocturnal downward movement of larvae (Bransden, et al., 2005a). The tanks were spot siphoned at least every three days to remove uneaten feed and faeces and mortalities were counted.

18.3.3 *Morphological sampling*

Larvae were randomly sampled from the incubators at 1 and 5 dph and from each of the 300-l tanks at 10, 15, 20, 24 and 29 dph during the experiment, for analysis of morphometrics. Larvae were anaesthetised in 0.06% 2-phenoxyethanol, and larval length measured using an eyepiece graticule fitted to an Olympus SZ stereomicroscope. Larvae were scored for swim bladder inflation and the appearance of the jaws was rated on a scale of 0 to 3 according to the jaw malformation index described in (Cobcroft and Battaglene, 2009). The index of 0.5 indicated a very minor variation from normal structure that was unlikely to impair larval performance and that would not be considered a malformation from a commercial perspective. Larvae were defined as malformed when the jaw index was 1, 2 or 3. Twenty larvae from each tank were examined on each sample day, except for 15 and 29 dph when n = 50 for swim bladder inflation and jaw assessment, respectively. The dry weight of 30 larvae from each tank was determined for the same sampling days. Final survival was determined by counting the surviving larvae from each tank at the end of the experiment, 29 dph.

18.3.4 *Assessment of walling behaviour*

Larval distribution and behaviour were assessed according to the methods described in (Cobcroft and Battaglene, 2009). Briefly, the estimated proportion of larvae touching the tank walls (from surface to 38 cm depth) was determined by an observer each day from 7 dph and larval behaviour described.

18.3.5 *Feed intake*

Feed intake was assessed on 14 and 23 dph, during rotifer and *Artemia* feeding stages, respectively, using a modification of the technique described by (Cobcroft, et al., 2001b) from 3-l vessels to 300-l culture tanks. Commencing at 09:00 h, live feed were added to two 300-l tanks at a time, with three minutes between prey addition to each successive pair of tanks to allow time for collection of larvae at the end of the feeding period. Larvae were allowed a 2 h feeding time on 14 dph and 30 min on 23 dph, to account for increasing prey capture ability by older larvae, after which 20 larvae were siphoned from each tank. Sampled larvae were immediately anaesthetised in 0.06% 2-phenoxyethanol, then fixed in 10% neutral buffered formalin. A squash preparation was made of preserved larvae on a glass slide and the number of prey in the gut was counted. Anaesthesia and fixation of striped trumpeter larvae did not cause gut evacuation. Morphometric assessment of larvae sampled at a similar time of day indicated that there was no residual prey in the guts of larvae on 15 and 24 dph, except for very low levels in larvae from white tanks on 24 dph, comparable to the start of the feeding time on 14 and 23 dph, respectively. Consequently, all counted prey items were assumed to be ingested during the feed intake assessment time. Feeding was assessed by the proportion of fish feeding and by the feeding intensity calculated as the number of prey consumed per feeding larva per minute.

18.3.6 *Statistical Analysis*

Separate two way ANOVAs were used to test the effects of tank colour (black and white) x rotifer enrichment (AM2000 and non-enriched), and water type (green and clear) x rotifer enrichment (AM2000 and non-enriched), on larval performance indicators (jaw malformation, length, dry weight, swimbladder inflation, proportion feeding and feeding intensity), survival and walling behaviour. Percentage data were transformed to achieve homogeneity of variance and normal distribution of residuals by arcsin $\sqrt{p}$, where p is the proportion. Where significant treatment effects were detected, a Tukey test was used to determine differences between means. Linear regression was used to describe the relationship between the proportion of fish walling (mean of whole tank observations to the end of the experiment, 29 dph) and the proportion of fish with jaw malformation at 29 dph. Statistical analyses were performed with JMP 5.1 (SAS Institute Inc.) and SPSS 14.0 (SPSS Inc.). Results are reported as mean ± SD and significance accepted as $P < 0.05$.

18.4 Results

18.4.1 *Jaw malformation*

The incidence and severity of jaw malformation increased with age from 15 to 29 dph (Figure 18.1). All fish had normal jaw structure, with no malformations, on 10 dph. By 24 dph, a pattern had emerged with the highest incidence and severity of jaw malformations in the clearwater and enriched treatments, which persisted in larvae on 29 dph. Excluding greenwater tanks, there was a significant effect of tank colour ($P = 0.005$), enrichment ($P = 0.0004$) and the interaction tank colour x enrichment ($P = 0.0412$). Jaw malformation was

significantly higher in larvae from WH CL AM (70 ± 15%) than all other clearwater treatments, where there was no significant difference between means, BL CL AM (26 ± 15%), WH CL NE (17 ± 12%) and BL CL NE (11 ± 10%). Excluding white tanks, there was no significant interaction between water type and enrichment (P = 0.5575), although jaw malformation was significantly higher (P = 0.0413) in clearwater (19 ± 14%) than greenwater tanks (7 ± 6%) and significantly higher (P = 0.0315) in larvae fed enriched diets (18 ± 14%) than in those fed non-enriched diets (8 ± 8%). Most of the jaw malformations observed were resistance to closing the mouth ('open' jaw), with thickening of lower jaw elements and short lower jaws (Figure 18.2). The most severe malformations occurred in fish from WH CL AM tanks where > 10% of malformed fish had wide open mouths that were unable to close (Figure 18.2C).

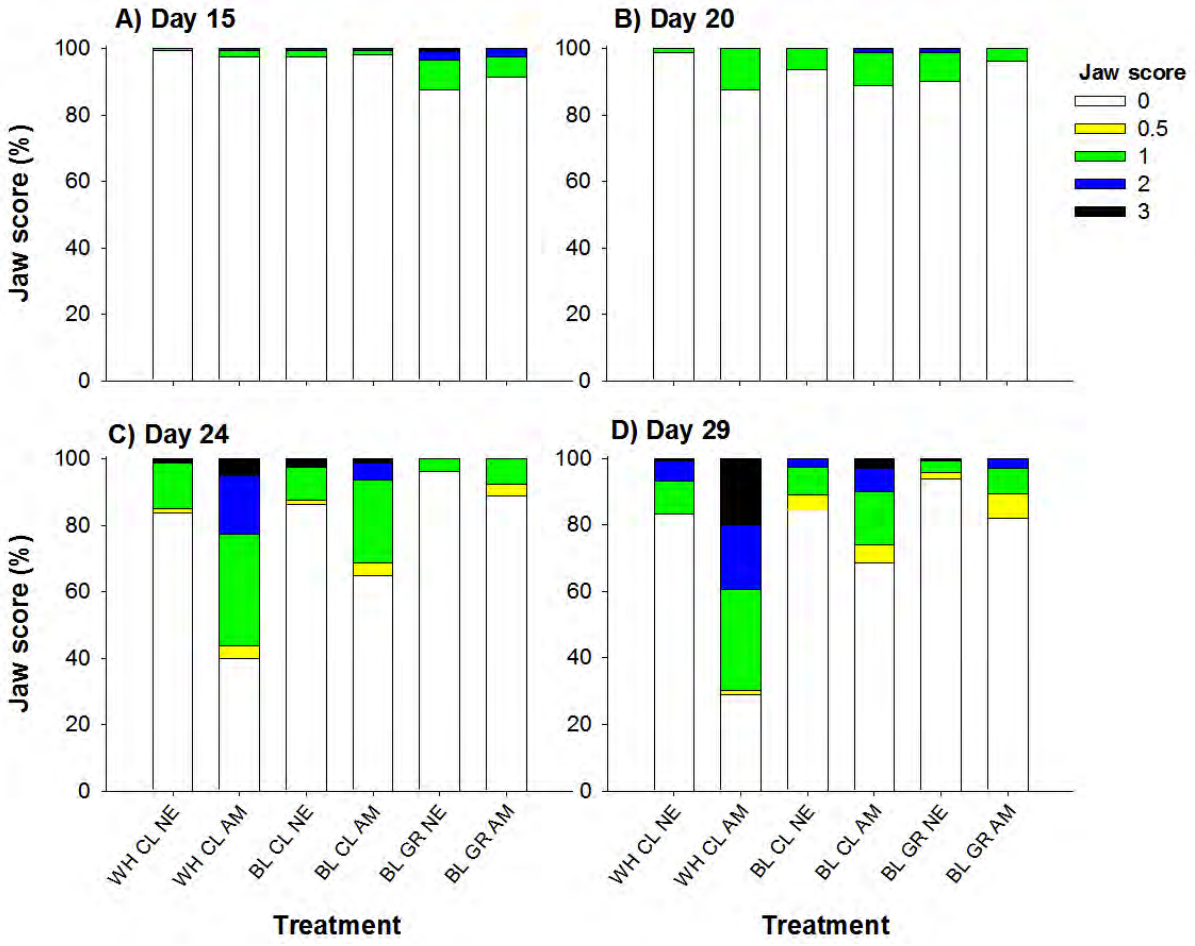

Figure 18.1 Incidence, severity and progression of jaw malformation in striped trumpeter larvae and post-larvae reared in tanks with different culture environment and diet with age. Four replicate tanks per treatment, n = 50 fish per tank on 15 and 29 dph and n = 20 on 20 and 24 dph, except on 29 dph for two white tanks (n =38 WH CL NE, and n = 30 WH CL AM).

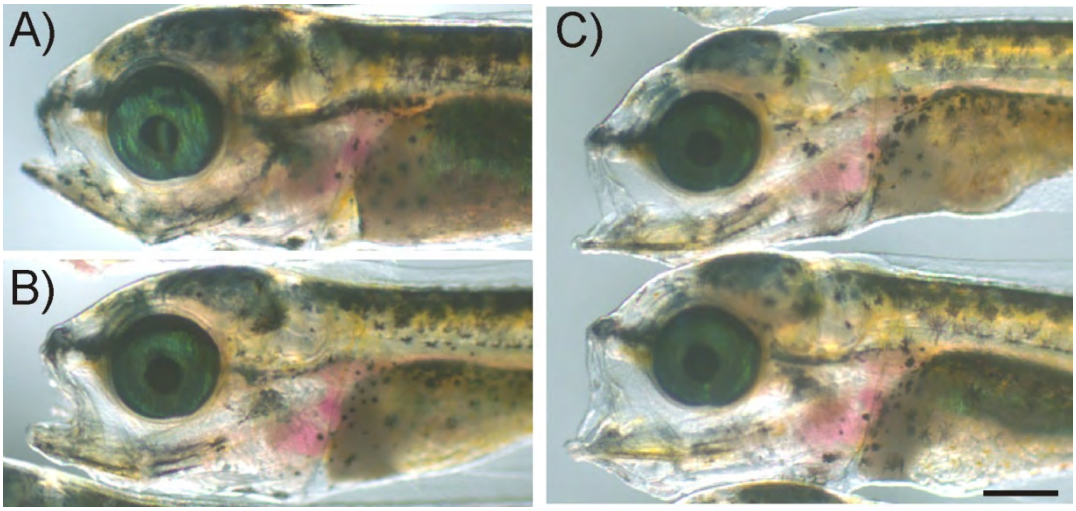

Figure 18.2 Striped trumpeter larvae at 29 dph reared in tanks with different culture environment, A) normal jaw structure, and B) and C) malformed jaws. B) limited movement to close mouth and thickening of the lower jaw structures, particularly the dentary, and C) wide open with no movement to close the mouth. Scale bar is 0.5 mm.

18.4.2 *Larval growth, performance and survival*

At the end of rotifer feeding (20 dph), excluding greenwater tanks, larval length and dry weight were significantly higher ($P < 0.0001$ for both) in black tanks (8.44 ± 0.42 mm, 0.79 ± 0.09 mg) than white tanks (7.51 ± 0.52 mm, 0.55 ± 0.06 mg), and there were no significant effects of enrichment or the interaction between tank colour and enrichment on length or weight (Figure 18.3). Excluding white tanks, water type and the interaction of water type and enrichment had significant effects on larval length ($P < 0.0001$ and $P = 0.0487$, respectively), although there was no significant effect of enrichment on length to 20 dph ($P = 0.6538$). Larvae from greenwater tanks were significantly ($P < 0.05$) longer (BL GR AM 8.77 ± 0.25 mm, and BL GR NE 8.71 ± 0.20 mm) than larvae from clearwater tanks (BL CL AM 8.39 ± 0.46 mm, and BL CL NE 8.49 ± 0.37 mm). Larval dry weight in black tanks on 20 dph was not significantly affected by water type, enrichment or the interaction of water type and enrichment ($P = 0.1167$, $P = 0.5363$, $P = 0.1301$, respectively).

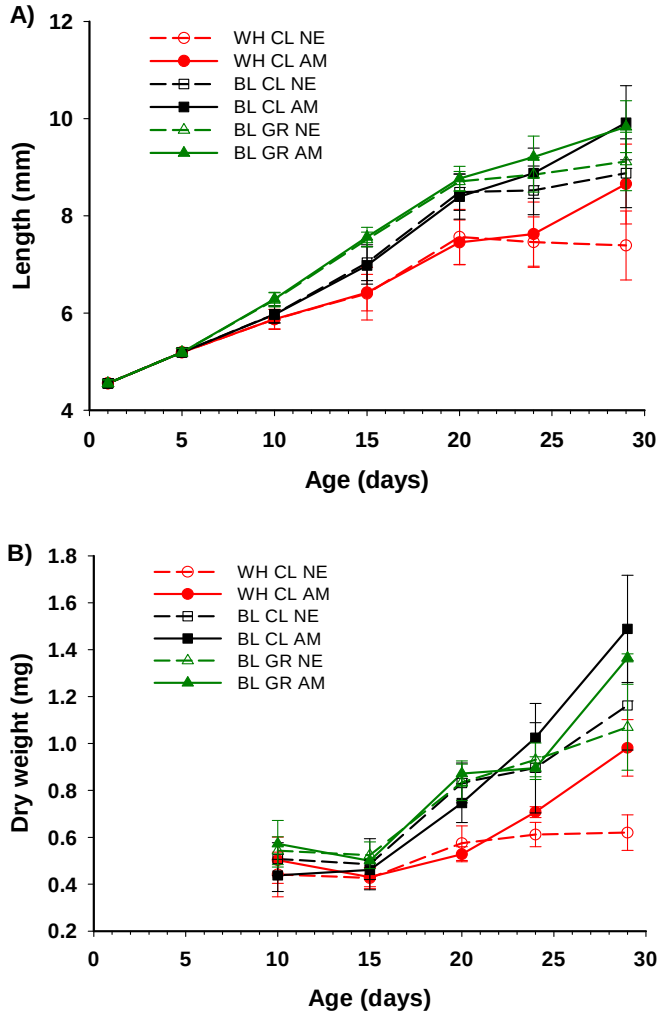

Figure 18.3 Changes in A) standard length and B) dry weight of striped trumpeter larvae grown in tanks with different culture environment and diet with age. Values are mean ± SD, for length from 10 dph n = 20 larvae from 4 replicate tanks, and weight is average of 30 larvae per tank except for 29 dph where too few larvae were surviving for dry weight assessment in one WH CL AM and two WH CL NE tanks. Treatment codes indicated in text. See text for statistical analyses and mean comparisons.

During the *Artemia* feeding phase, after 20 dph, growth slowed in all non-enriched treatments and remained high in larvae fed enriched *Artemia*. Excluding greenwater tanks, there was no significant effect of the interaction between tank colour and enrichment on larval length at 29 dph, and fish cultured in black tanks (9.4 ± 0.9 mm) were significantly ($P < 0.0001$) longer than those cultured in white tanks (8.0 ± 1.0 mm), while larvae fed enriched diets (9.3 ± 1.0 mm) were significantly ($P < 0.0001$) longer than those fed non-enriched diets (8.1 ± 1.0 mm). A similar pattern was observed in dry weight, although this was not analysed statistically due to missing data for some white tanks with low survival. Excluding white tanks, there was a significant interaction between water type and enrichment on larval length ($P = 0.0285$) but not dry weight ($P = 0.8656$) at 29 dph. There was no significant effect of water type on either length ($P = 0.2790$) or dry weight ($P = 0.2396$), while live feed enrichment had a significant effect on length ($P < 0.0001$) and dry weight of larvae ($P = 0.0040$). Again, larvae fed enriched diets (1.43 ± 0.16 mg) were significantly heavier than

those fed non-enriched diets (1.12 ± 0.18 mg), although the difference in mean length with enrichment was less pronounced in greenwater compared with clearwater tanks.

Swimbladder inflation at 15 dph, at the end of the swimbladder inflation window (Trotter, et al., 2005), was significantly affected by environmental culture conditions. Excluding greenwater tanks, swimbladder inflation was significantly ($P = 0.0144$) higher in black (50 ± 20%) than in white (25 ± 11%) tanks, and there were no significant effects of enrichment or the interaction between tank colour and enrichment ($P = 0.8205$ and $P = 0.8804$, respectively). Excluding white tanks, swimbladder inflation was significantly ($P < 0.0001$) higher in greenwater (95 ± 5%) than in clearwater (25 ± 11%) tanks, and there were no significant effects of enrichment or the interaction between water type and enrichment ($P = 0.4093$ and $P = 0.3563$, respectively). Tail flexion had commenced in all treatments by 20 dph, although at a very low incidence in white tanks, and proceeded to a more advanced developmental stage in the black compared with white tanks by 29 dph.

Survival from stocking at 1 dph to the end of the experiment 29 dph was highest in the current 'standard' rearing conditions for striped trumpeter, with black tanks, greenwater and Algamac enrichment of live feed (BL GR AM, 62 ± 2%). Intermediate survival occurred in BL CL AM (34 ± 11%) and BL GR NE (30 ± 9%) treatments and was lower in BL CL NE (18 ± 11%) and WH CL AM (14 ± 14%), with lowest survival in the WH CL NE treatment (5 ± 3%). Excluding greenwater tanks, there was no significant effect of the interaction between tank colour and enrichment ($P = 0.6645$) and survival was over twice as high ($P = 0.0083$) in black than in white tanks (Figure 18.4A), and twice as high ($P = 0.0468$) in larvae fed enriched diets compared with non-enriched diets (Figure 18.4B). Excluding white tanks, there was no significant effect of the interaction between water type and enrichment ($P = 0.2565$) and survival was significantly higher ($P = 0.0017$) in greenwater than in clearwater tanks (Figure 18.4C), and twice as high ($P = 0.0005$) in larvae fed enriched diets compared with non-enriched diets (Figure 18.4D).

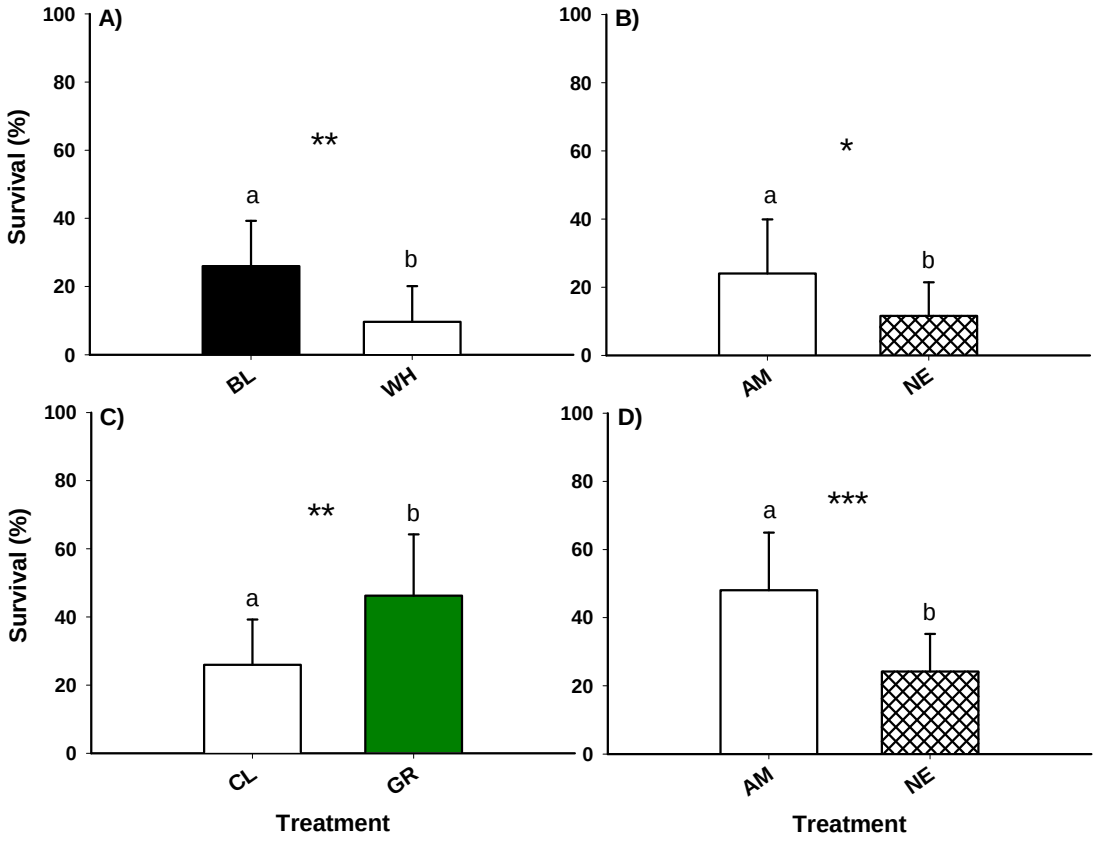

Figure 18.4 Survival of striped trumpeter larvae at 29 dph reared from 1 dph in tanks with different culture environment and diet. In clearwater tanks, the effects of A) tank colour and B) enrichment. In black tanks, the effects of C) clearwater versus greenwater and D) enrichment. Values are mean ± SD, n = 8. Different letters indicate significant differences between means and significance level indicated by * = $P < 0.05$, ** = $P < 0.01$, and *** $P < 0.001$.

Cumulative mortality of larvae up to 15 dph was highest in white tanks, followed by black clearwater, then black greenwater treatments (Figure 18.5). No spot siphons were made from 16 to 21 dph because of the high number of live fish at the bottom of the tanks in some treatments and low numbers of mortalities during that time. From 22 dph, following transition to *Artemia* feeding, 24 h light photoperiod and start of flexion in most treatments, mortality increased in all treatments at a higher rate in tanks provided non-enriched compared with AM enriched *Artemia*. Average cumulative mortality in non-enriched tanks exceeded that in enriched tanks earlier for white tanks (25 dph) than for black tanks (27 dph).

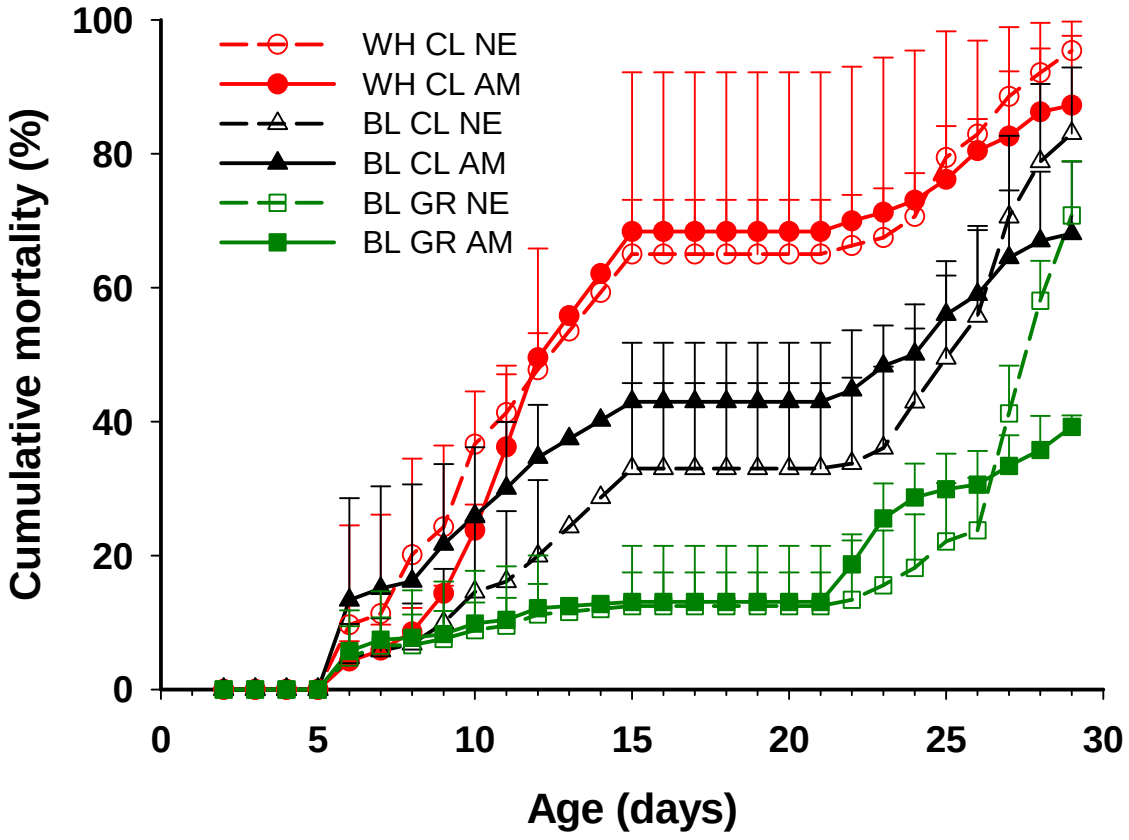

Figure 18.5 Cumulative mortality of striped trumpeter larvae from 2 to 29 dph reared in tanks with different culture environment and diet. Values are mean + SD, n = 4.

18.4.3 *Distribution and behaviour*

Larval walling behaviour was significantly affected by culture environment and enrichment. Excluding greenwater tanks, the average proportion of fish walling from 7 to 28 dph in white tanks (52 ± 7%) was significantly higher ($P < 0.0001$) than in black tanks (15 ± 3%), while walling in larvae fed enriched diets (38 ± 21%) was significantly higher ($P = 0.0003$) than in non-enriched treatments (30 ± 19%) (Figure 18.6). There was no significant effect of the interaction between tank colour and enrichment on the proportion of fish walling. Excluding white tanks, water type, enrichment and the interaction of water type and enrichment had significant effects on the proportion of fish walling ($P < 0.0001$, $P = 0.0074$, $P = 0.0135$, respectively). In black clearwater tanks, walling was significantly ($P < 0.05$) higher in the enriched (18 ± 2%) than the non-enriched treatment (13 ± 2%), which was in turn significantly higher than both greenwater treatments (BL GR AM 3 ± 1%, BL GR NE 3 ± 1%).

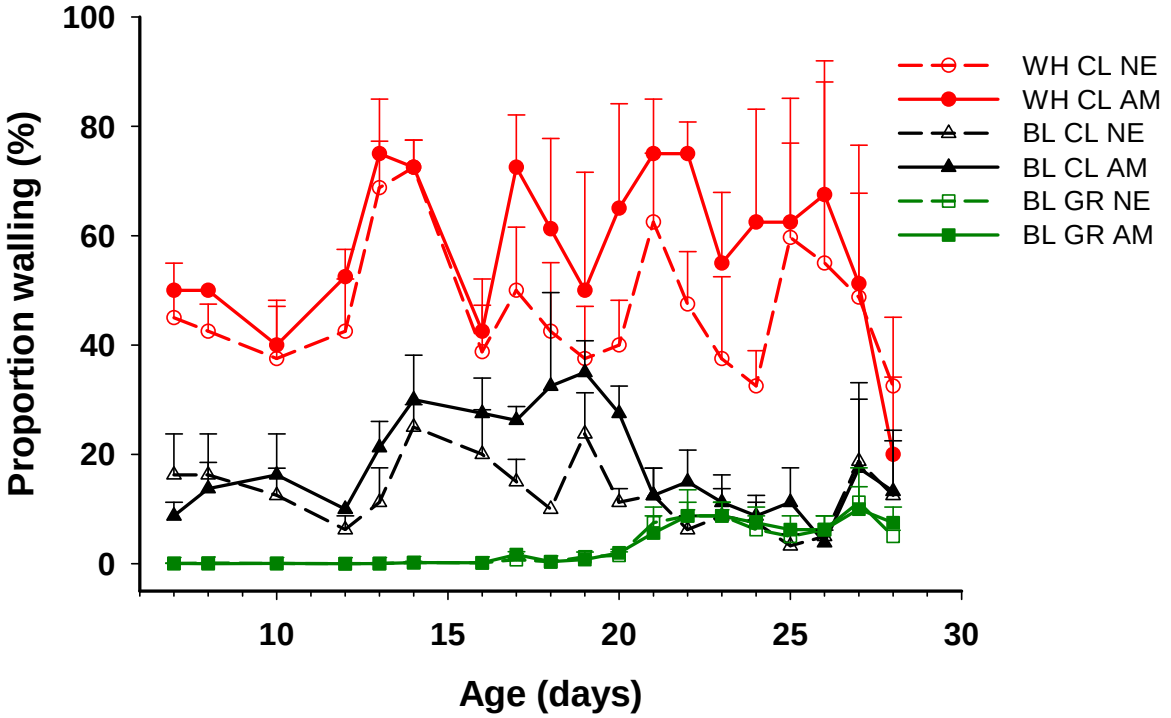

Figure 18.6 Proportion of striped trumpeter at different ages exhibiting walling behaviour in tanks with different culture environment and diet. Values are mean + SD, n = 4 tanks each day.

18.4.4 *Relationship between walling behaviour and jaw malformation*

There was a significant positive relationship between the average proportion of fish walling in each tank fed enriched diets and the incidence of jaw malformation at 29 dph (jaw scores 1 + 2 + 3) ($y = 6.56 + 1.11 x$, $r^2 = 0.830$, $P < 0.001$) (Figure 18.7). However, there was no significant relationship between walling and jaw malformation in fish fed the non-enriched diets ($r^2 = 0.197$, $P = 0.148$).

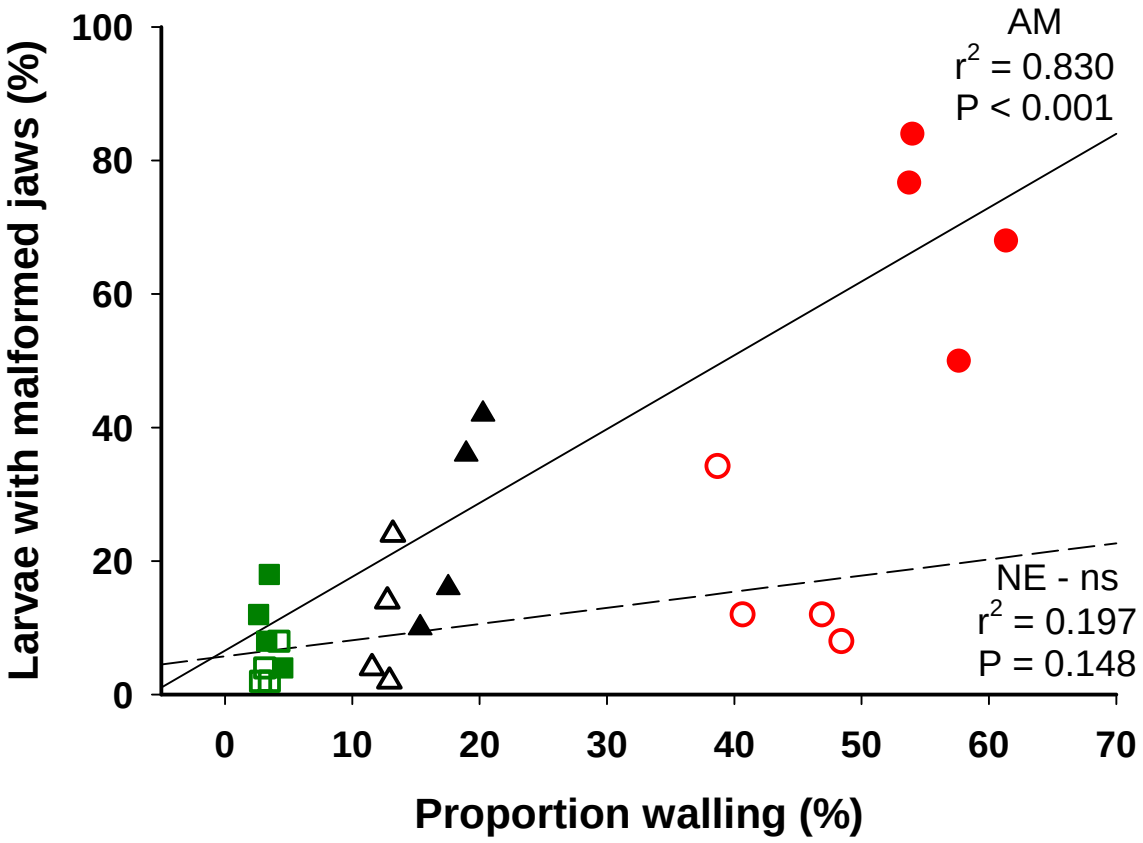

Figure 18.7 Relationship between the average proportion of larvae walling and jaw malformation in striped trumpeter larvae on day 29. Values are for each tank, and treatment is represented by symbol either black triangles (black tanks, clearwater), green squares (black tanks, greenwater) red circles (white tanks) and symbol fill either closed (AM enriched, solid line regression) or open (NE non-enriched, dashed line regression). Walling behaviour is a mean of the proportion of fish walling (assessed once daily) up to the 29 dph. Malformation assessment of n = 50 fish per tank except for two white tanks (n =38 WH CL NE, and n = 30 WH CL AM). ns indicates not significant.

18.4.5 *Feed intake*

In clearwater tanks, the proportion of fish feeding was affected by tank colour on both 14 and 23 dph ($P < 0.0001$ and $P = 0.0029$, respectively), being higher in black than white tanks (Figure 18.8A). There were no significant effects ($P > 0.05$) of enrichment or the interaction between tank colour and enrichment. In black tanks, there was no effect of water type, enrichment or the interaction of water type and enrichment on the proportion of fish feeding. The influence of culture environment on feeding intensity changed between the rotifer and *Artemia* feeding stages. In the rotifer phase, 14 dph, in clearwater feeding intensity of larvae was twice as high in black compared to white tanks ($P = 0.0009$) (Figure 18.8B), with no significant effects ($P > 0.05$) of enrichment or the interaction of tank colour and enrichment. In black tanks, feeding intensity was higher in larvae fed non-enriched than those fed enriched rotifers ($P = 0.0084$) (Figure 18.8C), with no significant effects ($P > 0.05$) of water type or the interaction of water type and enrichment. In the *Artemia* phase, 23 dph, in clearwater there was a significant effect of the interaction of tank colour and enrichment ($P = 0.0011$). In clearwater black tanks, larvae fed enriched *Artemia* had double the feeding intensity of those fed a non-enriched diet and of larvae from clearwater white tanks fed

either diet (Figure 18.8D). In black tanks, again feeding intensity was higher in larvae fed non-enriched than those fed the enriched diet (P = 0.0015) (Figure 18.8E), and feeding intensity was higher in larvae from greenwater tanks than those in clearwater (P = 0.0058) (Figure 18.8F), with no significant effect of the interaction of water type and enrichment.

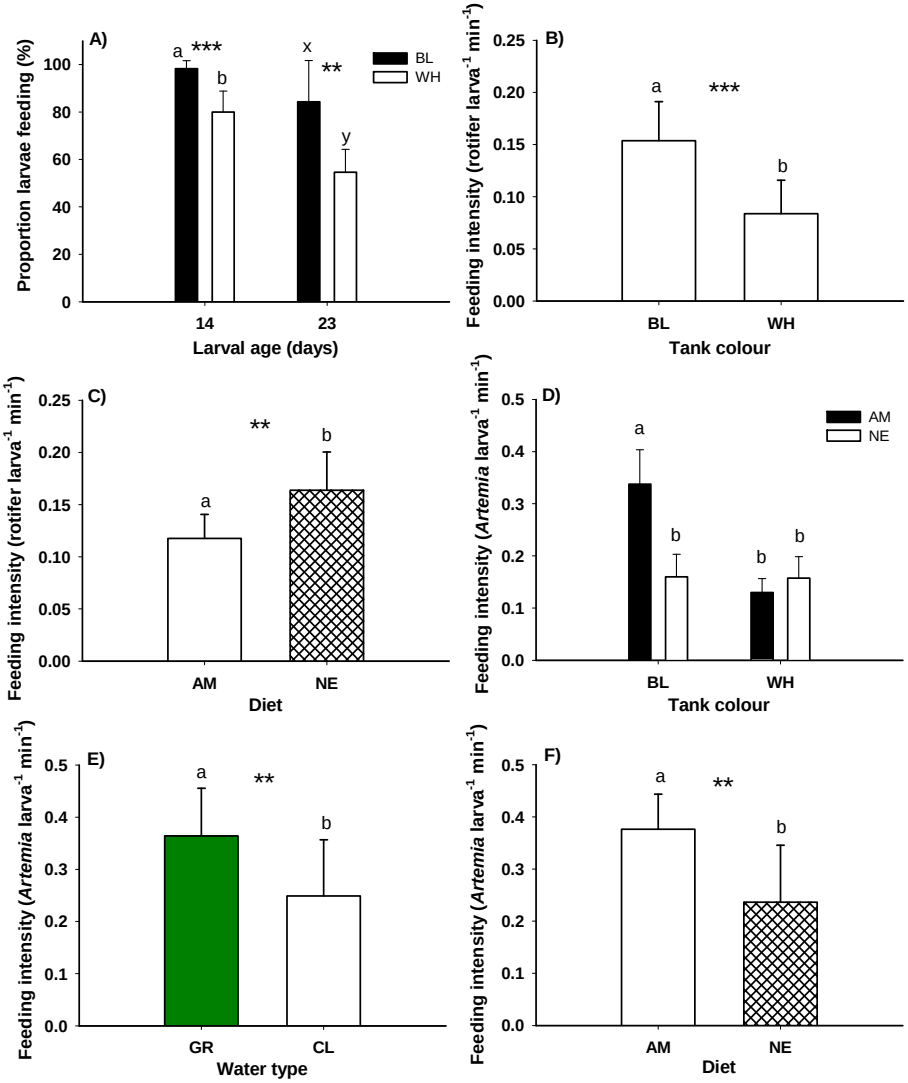

Figure 18.8 Proportion and intensity of striped trumpeter larvae feeding in different environmental conditions and with either enriched or non-enriched live food. A) Proportion of larvae feeding in clearwater tanks on 14 and 23 dph. Feeding intensity, prey consumed per feeding larva per minute, on 14 dph, B) in clearwater tanks and C) in black tanks, and on 23 dph, D) in clearwater, E) and F) in black tanks. Abbreviations: AM, enriched live food; BL, black tanks; CL, clearwater; GR, greenwater; NE, non-enriched live food; WH, white tanks. Values are mean ± SD, n = 8 tanks, except for D) where n = 4. Different letters indicate significant differences (P < 0.05) between means and significance level indicated by ** = P < 0.01, and *** P < 0.001.

18.5 Discussion

Jaw malformation was detected in striped trumpeter larvae from as early as 15 dph (7.5 mm SL) and was affected by tank colour, culture water type and live feed enrichment. Fish cultured in white tanks, or with clearwater or with enriched live feeds had higher levels of

jaw malformation than fish from black tanks, greenwater and fed a non-enriched diet, respectively. The finding that white tanks and clearwater were associated with jaw malformations was consistent with a previous study investigating a range of tank colours with the same species (Cobcroft and Battaglene, 2009). In contrast with previous studies with other species, where addition of dietary HUFA, phospholipids, vitamins and minerals (Zn and Mn) reduced skeletal malformations (Kanazawa, et al., 1981; Gapasin, et al., 1998; Cahu, et al., 2003b; Mazurais, et al., 2008; Nguyen, et al., 2008), our study demonstrated a higher level of malformation with larvae fed enriched diets. This has also occurred in studies testing the effects of high levels of vitamins (Mazurais, et al., 2008) and different phospholipid classes (Geurden, et al., 1998). However, in our study the enrichments used were the 'standard' diet and are considered to meet the nutritional requirements of striped trumpeter (Bransden, et al., 2005b; Battaglene and Cobcroft, 2007). It is hypothesised that the high incidence of jaw malformation in striped trumpeter was not caused directly by the diet enrichment (e.g. lipid in excess of the requirement) but rather caused by the different behaviour of the fish fed the two diets. Larvae fed non-enriched diets tended to be less active, with some larvae observed floating on the water surface on their sides, and less walling behaviour occurred in these tanks. This observation is in agreement with a previous study with striped trumpeter (Bransden, et al., 2005b), and with other species including herring, *Clupea harengus* (Bell, et al., 1995), yellowtail kingfish, *Seriola lalandi* (Masuda, et al., 1998; Masuda, et al., 1999), gilthead seabream, *Sparus aurata* (Benitez-Santana, et al., 2007), Pacific bluefin tuna *Thunnus orientalis* (Seoka, et al., 2008), where larvae and juveniles exhibited aberrant behaviour or a reduced visual response with low lipid, particularly DHA, enrichment. Diets with inadequate lipid content alter neural development and function in fish larvae (Sargent, et al., 1999). Conversely, striped trumpeter that were fed enriched diets were more active and had a higher incidence of walling. Walling behaviour in striped trumpeter is an active swimming of larvae and postlarvae into the tank wall, described in more detail in Cobcroft and Battaglene (2009). We hypothesis that the larvae fed enriched prey were healthier and exhibited normal behaviour which led to their higher level of walling behaviour.

A correlation between walling behaviour and jaw malformation was previously demonstrated in *Artemia*-feeding striped trumpeter larvae (Cobcroft and Battaglene, 2009), and the present study found a similar relationship in younger larvae. The slope of the relationship at 29 dph in the current study (1.11) was similar to that at 37 dph (1.03) and 44 dph (1.22) in the previous study (Cobcroft and Battaglene, 2009). In agreement with the previous study, larvae exhibited more walling behaviour in white than in black tanks. We believe by exposing larvae to the white wall environment from 1 dph, where a high proportion of larvae were walling from early in development, this induced the early appearance of jaw malformations. The intensity, and potentially spectrum, of reflected light from the white tanks appears to cause a phototactic response of larvae toward the wall. A lower level of walling behaviour occurred in black tanks and this was further reduced by greenwater culture, which is in agreement with previous studies of larval fish in turbid conditions (Bristow and Summerfelt, 1994; Rieger and Summerfelt, 1997; Shaw, et al., in review-a). It is postulated that light scattered by algal cells or other particles in the culture water reduces reflection from tank walls and encourages a more even distribution of larvae across the tank (Bristow and Summerfelt, 1994; Naas, et al., 1996). However, greenwater was not effective in reducing walling in black tanks in *Artemia*-fed larvae (> 20 dph), likely due to their increased visual and swimming capabilities (Blaxter, 1986; Cobcroft and Pankhurst, 2006). Consequently, alternative methods such as a combination of the visual environment (tank colour and light intensity), water flow dynamics and tank shape, must be

investigated to reduce walling in older fish in order to decrease the incidence of jaw malformation.

The present study demonstrated that in white tanks with a high incidence of walling behaviour there was a coincidently lower incidence of feeding and lower feed intake in the rotifer and *Artemia* feeding phases. This suggests that larval nutrition was compromised by the lower feed intake in fish in white tanks and this may have been a contributing factor to increased jaw malformation in white tanks. Likewise, larvae in non-enriched treatments consumed more prey per minute than enriched treatments in black tanks in the rotifer phase, and subsequently had a lower incidence of jaw malformation. However, larvae cultured in black tanks with Algamac enrichment had higher feed intake than in larvae fed a non-enriched diet in the *Artemia* feeding phase, and this treatment exhibited a higher rate of jaw malformation at the end of the experiment. Consequently, the link between feed intake and jaw malformation is not as clear as the relationship between larval walling behaviour and jaw malformation.

Regardless of the potential impact on jaw malformation, the visual environment of the different treatments affected larval feeding. In terms of tank colour, this could be explained by a potentially higher prey contrast of rotifers and *Artemia* against a black wall background compared with the white wall background (Browman and Marcotte, 1987; Ostrowski, 1989; Utne-Palm, 1999). Feeding intensity was significantly higher in greenwater tanks compared with clearwater tanks in the *Artemia* feeding phase and there was a similar trend in the rotifer feeding phase. This result was in agreement with previous studies with larvae of striped trumpeter (Cobcroft, et al., 2001b; Shaw, et al., in review-b) and other species such as walleye, *Sander vitreus*, formerly *Stizostedion vitreum* Bristow, et al., 1996, Atlantic halibut, Naas, et al., 1992, and turbot, *Scophthalmus maximus* Øie, et al., 1997). It is suggested that increased feed intake in greenwater is also associated with increased prey contrast, due to the higher scattering of light in greenwater compared with clearwater environments (Naas, et al., 1992; Miner and Stein, 1993; Cobcroft, et al., 2001b). The overall increase in feeding intensity between the rotifer and *Artemia* feeding phases can partly be explained by the increase in the visual field of larvae with age and development (Easter and Nicola, 1996; Job and Bellwood, 1996; Hunt von Herbing and Gallager, 2000; Cobcroft and Pankhurst, 2006), that improves their ability to detect and capture prey in both clearwater and greenwater. In the rotifer feeding phase, feed intake was higher in non-enriched than in enriched treatments, potentially as a compensatory mechanism to increase intake of a nutritionally deficient diet in order to meet requirements. In contrast with the rotifer feeding phase, *Artemia*-fed larvae consumed fewer prey per minute in non-enriched than in enriched treatments in black tanks. This was likely related to a combination of lower larval activity in the non-enriched treatments, as discussed previously in relation to lower walling behaviour in the non-enriched treatments, and to larger fish in enriched treatments having higher feed intake than smaller fish in non-enriched treatments. Prey consumption rates between 0.07 and 0.17 rotifers.larva^{-1}.min^{-1} and 0.14 and 0.41 *Artemia*.larva^{-1}.min^{-1} were similar to those previously observed in striped trumpeter (Cobcroft, et al., 2001b; Bransden, et al., 2005b; Shaw, et al., in review-b) and were higher than in fat snook, *Centropomus parallelus* (Temple, et al., 2004) but lower than in anchovy, *Engraulis mordax* (Hunter and Thomas, 1974) and Atlantic cod, *Gadus morhua* (Puvanendran and Brown, 2002; Puvanendran, et al., 2002).

The performance of larvae in different coloured tanks is species specific since similar beneficial effects (growth, swimbladder inflation and survival) of dark tanks, as found in this study, were observed in yellow perch, *Perca flavescens* (Hinshaw, 1985), mahi mahi (Ostrowski, 1989), striped bass, *Morone saxatilis* (Martin-Robichaud and Peterson, 1998)

and Eurasian perch, *Perca fluviatilis* (Jentoft, et al., 2006), whilst white tanks produced larger larvae with higher survival in grouper, *Epinephelus suillus* (Duray, et al., 1996) and haddock, *Melanogrammus aeglefinus* (Downing and Litvak, 1999). Striped trumpeter larvae from greenwater treatments were longer than clearwater-reared larvae at the end of the rotifer phase, regardless of live feed enrichment, but there was no significant effect of water type on growth at the end of the *Artemia* phase suggesting that the visual environment (Naas, et al., 1992) and beneficial nutritional effects (Reitan, et al., 1997) of microalgae were only important for striped trumpeter growth in the rotifer-phase. In contrast, in clearwater, enrichment had a significant effect on growth of larvae in both feeding phases, with larvae fed enriched live prey being larger, which is in agreement with other studies of striped trumpeter (Bransden, et al., 2004; Bransden, et al., 2005a) and other species (reviewed in (Sargent, et al., 1999).

18.6 Conclusion

Jaw malformation in striped trumpeter was induced in younger larvae than in previous studies and production trials by rearing them in white tanks with clearwater. Feeding larvae with enriched diets resulted in higher growth and survival, but also an increase in walling behaviour, particularly in inappropriate visual environments, coincident with a higher incidence of jaw malformations. Whilst walling and jaw malformation may be reduced in early larval culture through greenwater and tank colour, alternative strategies must be investigated for older striped trumpeter postlarvae. The implications of walling behaviour on cranial malformations in other susceptible species remain to be investigated.

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19 PURIFICATION AND PARTIAL CHARACTERISATION OF STRIPED TRUMPETER (*LATRIS LINEATA*) SYSTEMIC IMMUNOGLOBULIN FOR THE PURPOSE OF POLYCLONAL ANTI-SERUM PRODUCTION

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19.1 Abstract

The striped trumpeter (*Latris lineata*) has been identified as a new species for diversification of the Tasmanian finfish culture industry. It is a deep water species with an unusually long, oceanic post-larval developmental stage and therefore, has not been easy to culture. Recent break throughs in the hatchery phase of culture have enabled the first sea cage grow-out trials. As the culture of striped trumpeter moves towards commercial-scale grow-out, knowledge of the immune system as it relates to disease resistance and vaccination is becoming more important. This study began at the basic level of immunoglobulin (Ig) characterisation and then moved onto the creation of anti-serum, which was used as an immunological tool to investigate the onset of the antibody response in the striped trumpeter. Similar to many other teleost species, striped trumpeter Ig is composed of a light chain of *Mr* 28 ± 3 kDa and a dominant heavy chain of *Mr* 86 ± 7 kDa. As seen in many other species of teleosts, these heavy and light chains form a tetrameric molecule weighing approximately 926 kDa. Purified striped trumpeter Ig was used to create polyclonal anti-serum directed against the light chain. The anti-serum was then used to investigate the ontogeny of the antibody response. Using Western blot analysis, Ig could not be detected until larvae were 225 days post-hatch (dph). This is later in terms of days post-hatch than other fish examined and could affect future husbandry and vaccination practices for this species.

Keywords: immunoglobulin; polyconal antibody; protein A; *Latris lineata*; ontogeny

19.2 Introduction

The striped trumpeter (*Latris lineata*) has been under investigation as a new species for culture in Tasmanian waters since the 1980s (Searle, *et al.*, 1994). Much progress has been made in understanding reproduction, larval culture, and identification and control of disease (Battaglene, *et al.*, 2006). However, successful culture on a large-scale requires an understanding of the immune response. As the culture of striped trumpeter continues to expand, health issues will inevitably arise. Basic knowledge of the antibody response in terms of characterisation of the immunoglobulin molecule and timing of its production will contribute to disease prevention strategies for this species.

The survival strategies of freshwater and saltwater teleosts vary greatly and impact the early developmental stages of larvae. Compared to freshwater species, saltwater fish lay larger numbers of smaller eggs that consequently hatch when less developed (Fuiman, 2002). This in turn has implications for the time post-hatch that each group of fish is without an antibody response. In some freshwater spawning species, such as rainbow trout, antibody molecules can be detected shortly after hatching (4 dph) (Zapata, *et al.*, 2006). Investigation into the ontogeny of the antibody response in temperate marine Perciformes, such as sea bream, has found that antibody molecules can first be detected at approximately 50 dph; the timing of which coincides with weaning and subsequent transformation into juveniles (Breuil, *et al.*, 1997). The striped trumpeter differs from most cultured species in that it has an extended post-larval or 'paperfish' stage lasting approximately 9 months (Battaglene, *et al.*, 2007). After this point, the larvae become striped and metamorphose into juveniles (Tracey, *et al.*, 2005). If the ontogeny of the antibody response is linked to metamorphosis, the striped trumpeter would have to survive without a humoral adaptive immune response for an extended period of time. Such a scenario could affect the early rearing and vaccination strategies required to culture the striped trumpeter on a commercial scale.

This study was undertaken to isolate, purify and partially characterise the striped trumpeter immunoglobulin molecule for the purpose of creating a polyclonal anti-serum. The anti-serum provides an immunological tool to investigate the onset of the antibody response. This information can offer valuable insights into the timing of vaccination as well as mitigation strategies for any health issues that may develop.

19.3 Materials and methods

19.3.1 *Fish*

Fish were housed at the Marine Research Laboratory (Tasmanian Aquaculture and Fisheries Institute), Taroona, Tasmania. Adult striped trumpeter were maintained in 25000 l tanks at densities of approximately 5.0 kg m^{-3}. Ambient seawater was drawn from the Derwent River where temperatures ranged from 9-20 °C and salinity from 27 to 35 ppt. All fish were fed a semi-moist diet at 3 % body weight once daily (Brandsen, *et al.*, 2007).
Larval fish were reared in filtered, ozonated and UV-treated water. Water temperatures ranged between 14-18 °C and salinity 27–35 ppt. From the time of first feeding (approximately 6 dph) until approximately 16 dph, the larvae were maintained on rotifers and from 16-50 dph they were fed on *Artemia*. After this time the post-larvae were given a formulated diet dispensed at 2-5 % body weight per day (Battaglene, *et al.*, 2007).

19.3.2 *Serum Collection*

Serum was collected from cultured adult striped trumpeter (2-6 year old fish) hatched and reared on site. Five fish weighing 2.5-3.0 kg were anesthetized in 200 l of 0.02 % 2-phenoxyethanol. Blood was drawn from the caudal vein using a 5 ml syringe and 21G needle. Approximately 3 ml of blood was taken from each individual. The blood was transferred to 15 ml centrifuge tubes and placed at 4 °C overnight to allow clot formation. The blood was centrifuged at 1000 x *g* for 10 min. Serum aliquots of 1 ml were stored at -20 °C.

19.3.3 *Purification of striped trumpeter Ig*

Striped trumpeter serum immunoglobulin (STIg) was single-step purified using a Staphylococcal protein A (SpA) affinity chromatography kit (Bio-Rad) following the

methods described by Bollag *et al.* (1996). The column was pre-packed with 5 ml of affi-gel SpA coupled to agarose beads. It was washed with 5 column volumes of elution buffer (0.1 M glycine-HCl, pH 2.5). This was followed by re-equilibration with 10 column volumes of starting buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 7.5). Striped trumpeter serum was diluted 1:1 in starting buffer. The mixture was centrifuged at 10000 x *g* for 10 min at 4 °C to remove any particulate material before 2 ml were overlaid onto the column and left for 1 h at room temperature (RT). Additional starting buffer (10 column volumes minimum) was used to wash the column. The effluent was collected and its absorbance was measured at 280 nm (UV-VIS, Shimadzu). The column was washed with starting buffer until the absorbance returned to the background level. The immunoglobulin was released using 5 column volumes of elution buffer. One ml aliquots of eluent were collected in 1.5 ml tubes containing 100 µl of neutralizing buffer (1M Tris-HCl, pH 8.0). Fractions with A_{280} >0.50 were pooled before they were transferred into exchange buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.5) and concentrated using an ultra-15 centrifugal filter device (Amicon).

19.3.4 *Protein concentration*

The concentration of the purified Ig was determined using a BCA™ protein concentration kit (Pierce) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as a standard with eight concentrations ranging from 25-2000 µg ml^{-1} employed to generate a standard curve. Standards and unknown samples were assessed in triplicate in a flat-bottomed microtitre plate (Sarstedt) using a Tecan thermo (Rainbow spectrum) plate reader set to 562 nm. The assay was performed on serial dilutions (up to 1:32) of purified STIg.

19.3.5 *Gel filtration chromatography*

The molecular weight of native, purified STIg was estimated using gel filtration chromatography. A 90 x 1.8 cm glass column was packed with Sephacryl 300 HR (Sigma). A flow rate of 10.5 ml h^{-1} and a temperature of 4 °C were maintained throughout. Fractions of 2 ml were collected using a Bio-Rad 2128 fraction collector and analysed using a spectrophotometer at an absorbance of 280 nm. The void volume (V_o) was determined using 2% blue dextran in TBS. The molecular weight standards used to calibrate the column were BSA (66 kDa; Sigma), alcohol dehygrogenase (150 kDa; Sigma), apoferritin (443 kDa; Sigma), thyroglobulin (669 kDa; Sigma), and human IgM (970 kDa; donated by the Immunobiology Unit UTS, Sydney, Australia). The equation of the line was calculated using the method described by Crosbie and Nowak (2002) (Table 19.1).

Table 19.1 Molecular weight (MW) of STIg estimated from a standard curve[a] of the logarithm of the MW of standard proteins plotted against the elution volume (V_e) divided by the void volume (V_o) of blue dextran after gel filtration.

Standard	V_e	V_e/V_o	Log MW	MW (kDa)
Bovine serum albumin	138	1.550	1.820	66
Alcohol dehydrogenase	124	1.390	2.176	150
Apoferritin	110	1.230	2.646	443
Thyroglobulin	102	1.140	2.825	669
Human IgM	88	1.000	2.987	970
ST peak 1	93	1.040	2.983	962
ST peak 2	116	1.300	2.408	256

[a]The equation for the standard curve was y=-2.2262x + 5.3003, r^2=0.978 and V_o was 89.25 ml.

19.3.6 *Determination of Ig purity and molecular weight*

A Mighty Small II SE 250 vertical electrophoresis unit (Hoefer) was used to determine the purity and molecular weights of the heavy and light chains of the STIg molecule. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 5% stacking gel over a 10% resolving gel for reductive electrophoresis and 5% stacking gel over a 4% resolving gel for native electrophoresis (Bollag, *et al.*, 1996). The STIg molecule was reduced by boiling in sample buffer (0.06 M Tris-HCl; pH 6.8) containing 25% (v v^{-1}) glycerol, 2% (w v^{-1}) SDS, 14.4 mM 2-mercaptoethanol and 0.1% (w v^{-1}) bromophenol blue for 3 min. Under native conditions, samples were prepared in 0.125 M Tris-HCl (pH 6.8) sample buffer containing 20% (v v^{-1}) glycerol and 0.1% (w v^{-1}) bromophenol blue. Broad range molecular weight standards (BioRad) were included on reduced gels and high molecular weight standards (BioRad) were included on native gels. Samples were electrophoresed in running buffer (0.025 M Tris, 0.192 M glycine, 0.1% w v^{-1} SDS) until the dye front reached the end of the gel (approximately 1 h at 200 V, 60 mA). For native gels, SDS was omitted from the running buffer. Gels were fixed and protein bands visualised with either neutral silver staining (Harlow, *et al.*, 1988) or Coomassie Brilliant Blue (Bollag, *et al.*, 1996). Molecular weight estimation of heavy and light chains was made using a minimum of 5 gels via the method described by Hames and Rickwood (1990). Molecular weight estimations were expressed as the mean ± standard error of the mean.

19.3.7 *Polyclonal antisera production*

Rabbits were housed at the Central Animal House of the University of Tasmania, Sandy Bay Campus, Hobart. Two New Zealand white semi lop-eared rabbits were inoculated with purified STIg light chain (STIgL). STIgL was used rather than whole STIg, as the presence of IgL is the last step in the formation of functional surface Ig (Karasuyama, *et al.*, 1996), and therefore a more accurate measure of functional Ig. Fifty µg of purified STIgL was excised from SDS-PAGE and homogenized in 0.5 ml sterile PBS, which was in turn emulsified in 0.5 ml Freund's Complete Adjuvant (FCA; Sigma) for the primary injection. Three subsequent booster inoculations were given at two week intervals, with the STIgL emulsified in Freund's Incomplete Adjuvant (FIA; Sigma). All inoculations were administered intra-muscularly. Blood samples were taken prior to the initial injection (naïve

serum) and second and third boosters. The rabbits were euthanized and bled out two weeks after administration of the third booster.

19.3.8 *Determination of specificity and cross reactivity of polyclonal antisera*

The specificity and cross reactivity of the rabbit anti-STIgL serum were determined using Western blot analyses. Striped trumpeter serum, SpA affinity purified STIg and heterologous sera were electrophoresed under reducing conditions as previously described and subsequently electrotransferred (SemiPhor TE70; Hoefer) to a 0.45 μm nitrocellulose membrane (Hydrabond C; Pharmacia Biotech) under semi-dry conditions (Bjerrum, *et al.*, 1986). Protein transfer was confirmed using Ponceau (Sigma) as a non-specific protein stain. During the Western blot development procedure, all washes and incubations were performed with rocking at RT. Firstly, the membrane was washed in TBS and blocked with 1% non-fat skimmed milk in TBS (SM-TBS) for 60 min. Membranes were rinsed in TBS, tween 20-TBS (TBS-T; 0.1% v v^{-1}), TBS for 5 min each. The membranes were then probed with rabbit anti-STIgL diluted 1:500 – 1:15000 in TBS for 30 min. Membranes were washed as described above and incubated with alkaline phosphatase conjugated sheep anti-rabbit IgG (Chemicon) diluted 1:3000 in TBS for 30 min. Bands were developed in the dark, using BCIP/NBT (Sigma) until a colour reaction took place (approximately 5 min). The process was stopped by rinsing the membrane with distilled water. Initially, negative controls were included on the membranes to rule out non-specific binding. These included membranes not incubated with rabbit anti-STIgL, sheep anti-rabbit IgG or neither. Additionally, membranes were probed with pre-bleed (naïve) rabbit serum to ensure there were no natural rabbit antibodies against the STIgL.

The same electrophoresis and probing protocols were used to assess the cross-reactivity of rabbit anti-STIgL with the Ig light chain of other species. These included snapper (*Pagrus auratus*), barramundi (*Lates calcarifer* Bloch), southern bluefin tuna (*Thunnus maccoyii* Castelnau), and human (*Homo sapien*) IgM.

19.3.9 *Larval lysates*

Whole larvae (up to 116 dph) and pooled heart, kidney and spleen (116-290 dph) were homogenized on ice using a rotor stater in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, pH 7.5) with protease inhibitor cocktail (100 μl 20 mg^{-1} tissue; Sigma). Homogenates were centrifuged at 16000 x *g* for 30 min, before the supernatant was removed and its protein concentration estimated using a BCA™ kit (Pierce) as per manufacturer's instructions. During 10% SDS-PAGE, the same amount of protein (approximately 7.5 μg) was added to each well. After electrotransfer, the blots were probed with rabbit anti-STIgL at a concentration of 1:1000. Head kidney lysate from a 10 year old striped trumpeter was used as a positive control and the pyloric caeca from the same fish was used as a negative control on each blot.

19.4 Results

19.4.1 *SpA purification of Striped Trumpeter Ig*

The SpA column was used to extract STIg from pooled serum. As a single pass through the column was enough to yield significant amounts of STIg, multiple applications were not necessary. Nine, 1 ml fractions of the highest absorbance (A_{280} > 0.50) were pooled,

yielding approximately 0.45 mg of STIg from 1 ml of serum. The elution profile revealed that the Ig was released within 15 ml of elution buffer.

19.4.2 *Ig purity and structure*

The purity and structure of the SpA derived STIg was determined using SDS-PAGE. A total of 7 gels were analysed to estimate the molecular weights of the heavy (STIgH) and light (STIgL) chains of the striped trumpeter immunoglobulin molecule. One dominant STIgH as well as two smaller, less prominent bands and one STIgL band were seen. The molecular weight of the dominant STIgH was 86 ± 7 kDa, with two smaller, less prominent bands weighing approximately 77 ± 5 kDa and 67 ± 5 kDa. Polyclonal rabbit anti-snapper whole Ig antibody bound to both of the smaller STIgH chains, indicating that they were Ig and not contamination (data not shown). The single STIgL band had a molecular weight of 28 ± 3 kDa (Figure 19.1A).

Native gel electrophoresis revealed a high molecular weight band of STIg; presumably in tetrameric form. Additionally, there was a 670 kDa band present, which was presumed to be degradation product created during the purification of the STIg (Figure 19.1B). Molecular weight estimation of these entities was performed using gel filtration chromatography.

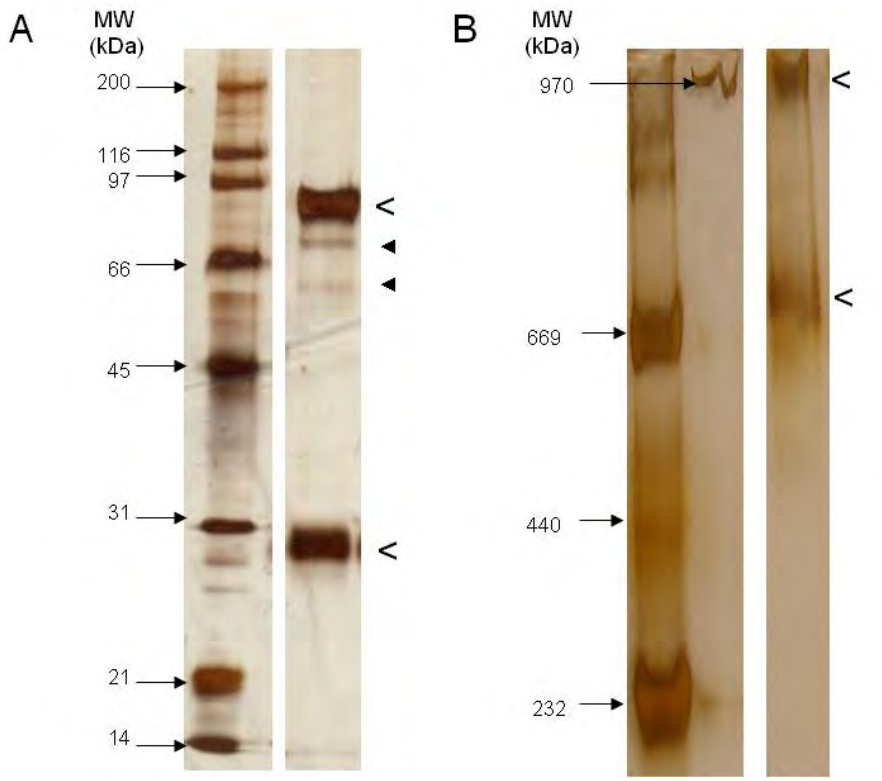

Figure 19.1 . (A) Neutral silver stained 10% SDS-PAGE gel showing STIgH and STIgL chains. The predominant STIgH chain and the STIgL chain (<) have approximate molecular weights of 86 kDa and 28 kDa respectively. The two lesser variants of the STIgH chain (◄) weigh approximately 77 kDa and 67 kDa. (B) Neutral silver stained 4% native PAGE showing putative tetrameric IgM at approximately 970 kDa as well as a presumed trimeric degradation product at approximately 670 kDa (<).

19.4.3 *Gel filtration chromatography*

Two distinct Ig molecules were detected by gel filtration chromatography. However, instead of weighing 970 kDa and 670 kDa, as predicted by native gel electrophoresis, they weighed 926 kDa and 256 kDa. Using the molecular weights of the STIgH and STIgL, it could be discerned that the molecular weight of tetrameric STIg should be approximately 928 kDa, and a monomer approximately 232 kDa. These projected weights were a close fit to the molecular weights estimated from the fractions of peak absorbance (A_{280}). Western blot analysis revealed that the eluent corresponding to each increase in absorbance contained STIgL (Figure 19.2B). Whether or not this putative monomeric STIgM molecule existed naturally in serum or was a product of degradation was investigated further. It was found that in fresh serum subjected to native gel electrophoresis and electrotransfer, only the tetrameric molecule was present (Figure 19.2C). However, when purified STIg was subjected to Western blot analysis, it was shown that there were in fact three distinct populations of Ig weighing ~930 kDa, ~700 kDa and ~240 kDa (Figure 19.2D). Therefore, it appears that the putatively monomeric molecule was an artefact of gel filtration chromatography rather than a true native form found in serum. The additional band revealed by both native gel elecrophoresis and Western blotting is presumed to be a product of tetrameric Ig degradation. In fact, its molecular weight of approximately 670 kDa is similar to that of a trimeric molecule (684 kDa).

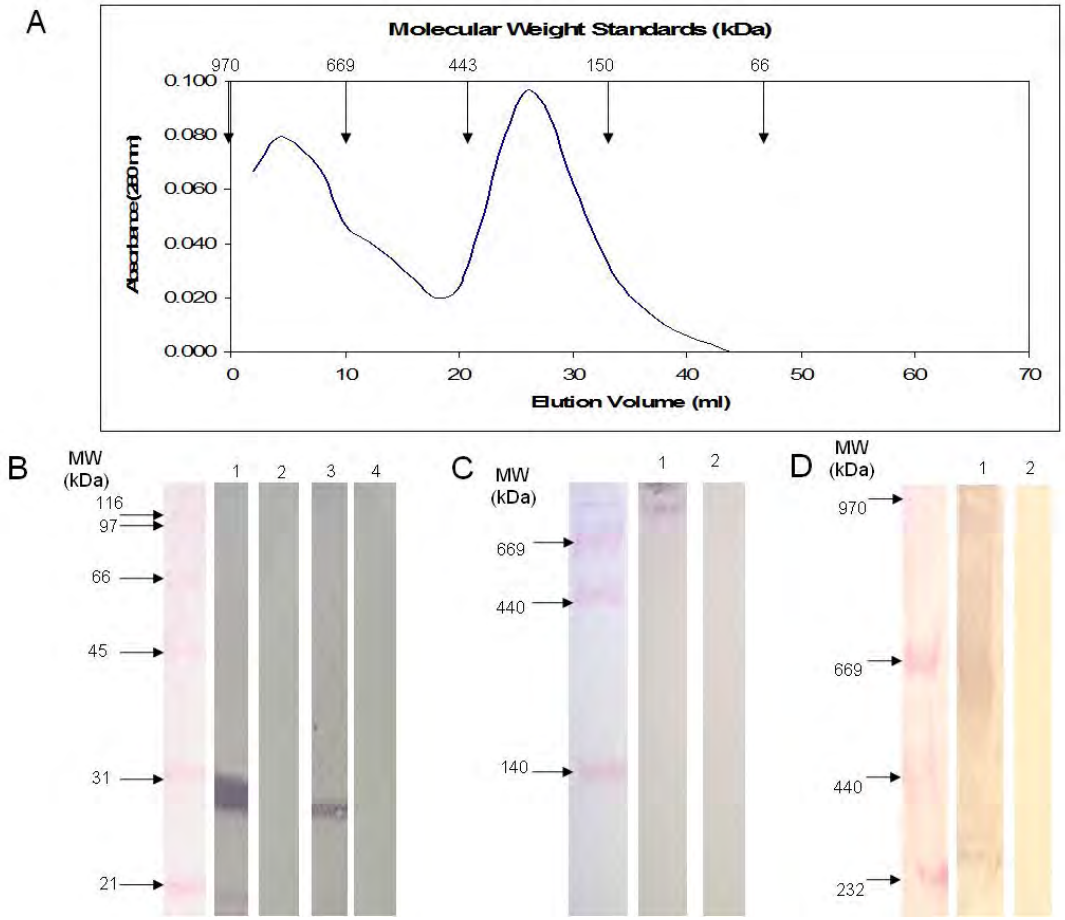

Figure 19.2 (A) Gel filtration chromatography of SpA purified ST Ig. The molecular weights of the two fractions were determined to be 962 and 256 kDa. (B) Reduced Western blot illustrating the presence of STIgL in the high molecular weight and low molecular weight fractions of peak absorbance (A_{280}). Lane 1- 926 kDa fraction incubated with rabbit anti-serum, lane 2 - 926 kDa fraction incubated with naïve rabbit serum, lane 3 – 256 kDa fraction incubated with rabbit anti-serum, lane 4 – 256 kDa fraction incubated with naïve rabbit serum. (C) Native Western blot showing that STIg was present as a tetrameric molecule in serum. Lane 1 – striped trumpeter serum incubated with rabbit anti-serum, lane 2 – striped trumpeter serum incubated with naïve rabbit serum. (D) Native Western blot revealing the three populations present in purified striped trumpeter Ig. Lane 1 – purified STIg incubated with rabbit anti-serum. The three STIg antibody populations (~930 kDa, ~700 kDA and ~240 kDA) are arrowed. Lane 2 – purified STIg incubated with naïve rabbit serum. Western blots presented in B-D were incubated with rabbit anti-STIgL and developed using BCIP/NBT.

19.4.4 *Titration of rabbit-anti striped trumpeter Ig antisera*

Western blots of reduced STIg probed with rabbit anti-serum showed that serum from both rabbits contained IgG that was strongly reactive with only the STIgL band at dilutions exceeding 1:15000 (Figure 19.3). Neither of the naïve rabbit antisera showed any non-specific binding.

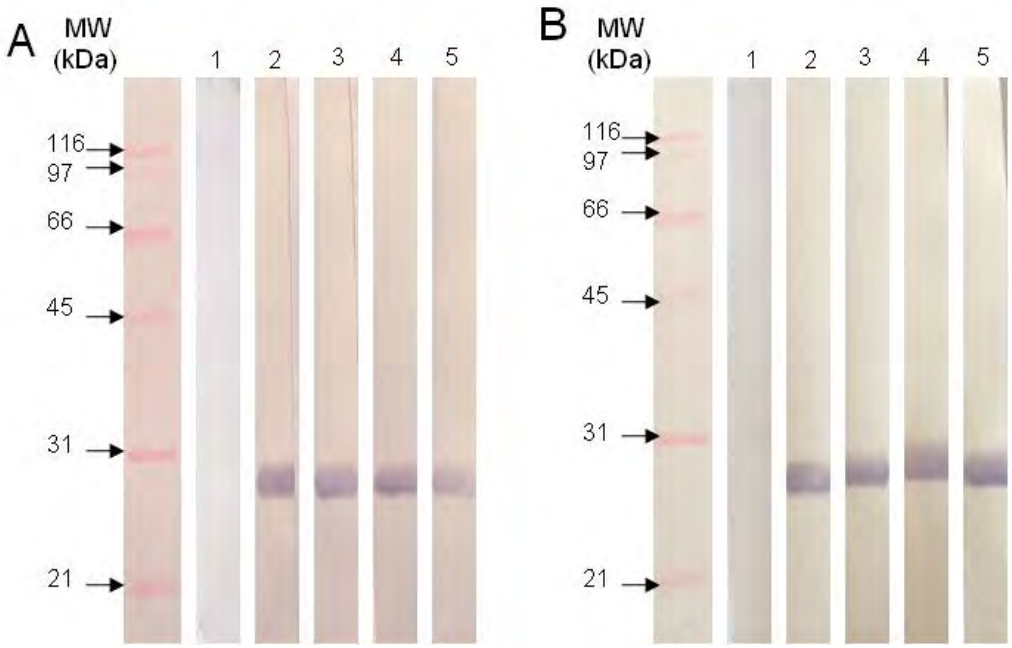

Figure 19.3 Western blot analysis of rabbit anti-STIgL antisera (A) Rabbit A and (B) Rabbit B showing reactivity to reduced SpA-purified STIg. Lane 1 – naïve serum (1:200), lane 2 – rabbit anti-STIgL 1:3200, lane 3 – rabbit anti-STIgL 1:5000, lane 4 – rabbit anti-STIgL 1:10000, lane 5 – rabbit anti-STIgL 1:15000.

19.4.5 *Cross-reactivity of polyclonal antisera with heterologous Ig*

Rabbit anti-STIgL was tested for cross-reactivity with purified Ig from other teleost species as well as human IgM. Electrotransfers were probed with 1:1000 dilution of rabbit B anti-serum, which reacted with all three teleost species tested, but not the human IgM (Figure 19.4). The fact that there was cross-reactivity with all the teleost species tested indicates that the polyclonal antibodies are targeted to the conserved regions of the STIgL chain; possibly the constant domain. It should be noted that although the rabbit anti-STIgL antiserum was cross-reactive with a number of teleost species, it was still specific for the IgL chain. Non-specific binding was not observed in any of the reduced Ig or serum samples.

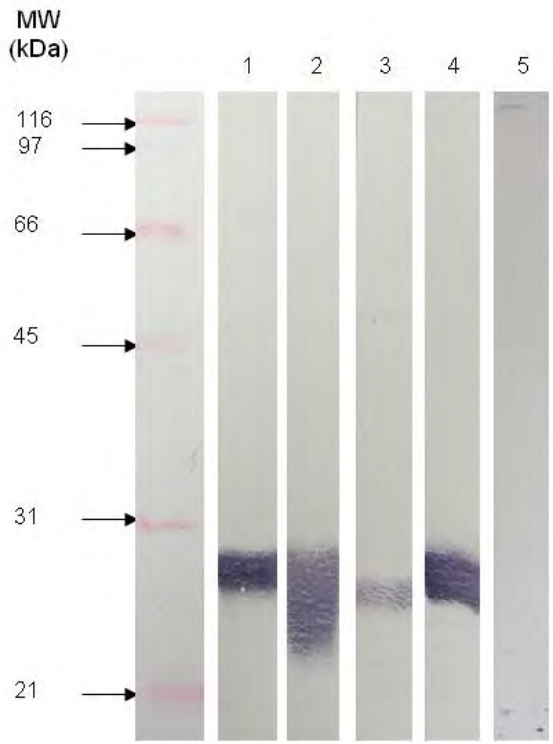

Figure 19.4 Cross-reactivity of rabbit anti-STIgL antisera with purified heterologous fish and mammalian Ig. Lane 1 – striped trumpeter Ig, lane 2 – snapper Ig, lane 3 – barramundi Ig, lane 4 – southern bluefin tuna Ig, Human IgM., lane 5 – negative control. Each lane was loaded with 7.5 μg of protein.

19.4.6 *Ontogeny of the antibody response in larval striped trumpeter*

The polyclonal rabbit anti-STIgL antiserum was used as an immunological tool to investigate the onset of the antibody response in larval striped trumpeter. When using Western blot analysis of lysates from whole pooled larvae (up to 116 dph) and pooled heart, kidney and spleen of larvae (116-290 dph), STIgL did not reach detectable levels until 225 dph (Figure 19.5)

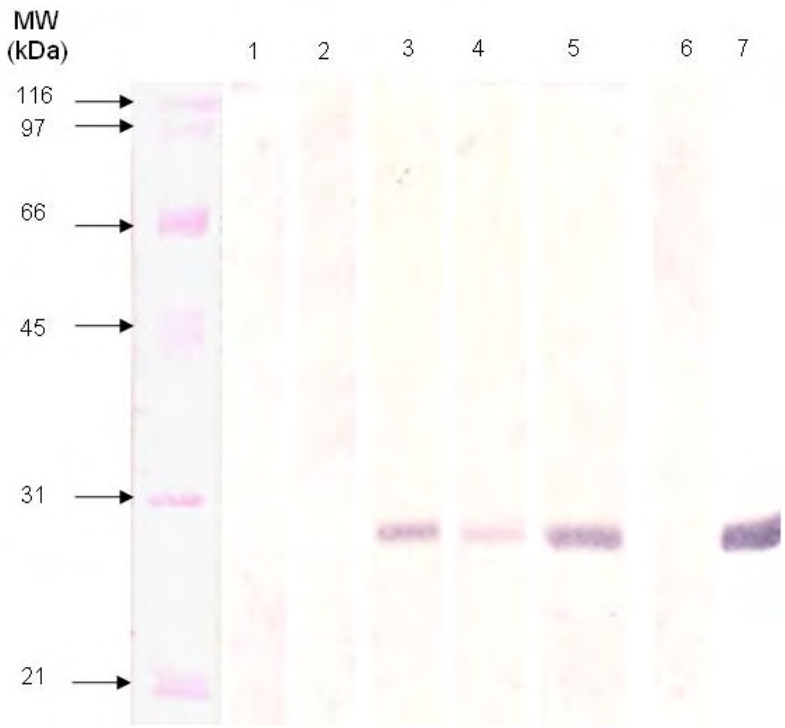

Figure 19.5 Western blot analysis of pooled larval heart, kidney and spleen lysates (equal amount of protein added to each lane) using rabbit B anti-STgL antiserum at a dilution of 1:1000. Lane 1- 175 dph, lane 2 - 190 dph, lane 3 - 225 dph, lane 4 - 249 dph, lane 5 - 290 dph, lane 6 – pyloric caeca lysate (negative control), lane 7 – head kidney (10+ year fish) lysate (positive control).

19.5 Discussion

As the culture of striped trumpeter moves towards commercial scale grow-out, it is important to gain a basic understanding of its immune response. This study sought to characterise the antibody molecule of the striped trumpeter and to create anti-serum to be used as an immunological tool to investigate the onset of the antibody response in this species. It was found that the antibody molecule of striped trumpeter is similar to those of other teleost species in that it naturally occurs in serum as a tetramer. Unlike other teleost species studied to date, the onset of the antibody response appears to be delayed until 225 dph. This finding may have implications for future vaccination and husbandry practices applied to this species.

Although the striped trumpeter Ig molecule was able to bind to the SpA column with relatively high yield, this ability varies depending upon the species in question. Successful, high yield purification has been attained using serum from the common carp (*Cyprinus carpio*), goldfish (*Carassius auratus*), yellowtail (*Seriola quinqueradiata*) and red sea bream (*Pagrus major*) (Suzuki *et al.*, 1990) as well as barramundi (*Lates calcarifer*) (Bromage, *et al.*, 2004; Crosbie, *et al.*, 2002), striped bass (*Morone saxatilis*), Mozambique tilapia (*Oreochromis mossambicus*) and Nile tilapia (*O. niloticus*) (Bromage, *et al.*, 2004). There has been less success with turbot (*Scophthalmus maxima*) (Estevez, *et al.*, 1993), where it is estimated that only 25% of the total Ig is bound by protein A. With snapper (*Pagrus auratus*), it was found that Ig yield could be increased by up to 73% through repeated application (5x) of the serum (Morrison, *et al.*, 2001). This was also the case for striped bass, Mozambique tilapia and Nile tilapia (Bromage, *et al.*, 2004). Although an SpA column can be an effective method for IgM extraction, some authors have had less success with this method using sera from rainbow trout (*Oncorhynchus mykiss*) (Bromage, *et al.*,

2004; Estevez, *et al.*, 1993; Suzuki, *et al.*, 1990), Atlantic salmon (*Salmo salar*) (Bromage, *et al.*, 2004) and halibut (*Hippoglossus hippoglossus*) (Grove, *et al.*, 2006). In the case of striped trumpeter, one step SpA affinity purification appears to be an appropriate method for the extraction of IgM, as yields of 0.45 mg were obtained from a single application of 1 ml of serum. Despite the relatively high and pure yield of STIg using this method, the possibility remains that only a fraction of the Ig population with the ability to bind to SpA was extracted and that other Ig molecules without specificity for SpA were excluded.

When examining the antibody molecules with the ability to bind the SpA, SDS-PAGE revealed that there was one dominant STIg heavy chain, two smaller, less prominent bands and only one STIg light chain. Western blots using anti-snapper whole Ig antibodies showed that these less prominent bands were reactive, indicating they contained H chain protein and were not a result of contamination. The fact that the bands were reactive in both serum and purified striped trumpeter immunoglobulin, suggests that these H chains were not a result of degradation during the purification process and are naturally present in serum. It is possible that the multiple STIgH bands represent isotypes or variants of the IgH chain that are a result of differential glycosylation. In fact, IgH isotypes have been reported in a number of other species, including channel catfish (*Ictalurus punctatus*) (Lobb, *et al.*, 1988), rainbow trout (Sanchez, *et al.*, 1989) and the more advanced redfin perch (*Perca fluviatilis*) (Whittington, 1993) and fugu (*Fugu rubripes*) (Savan, *et al.*, 2005). Although IgL chain variants with distinct molecular weights have been described in teleost species, such as the channel catfish (Lobb, *et al.*, 1984), none were observed here.

SpA purified STIg was also examined in its quaternary form using gel filtration chromatography, which revealed the presence of two distinct molecules. Unlike pentameric mammalian IgM, teleost Ig described to date has been in tetrameric form (Flajnik, 2005). Given the estimated molecular weight of the STIg fractions, it is highly probable that they were the tetrameric $[(H_2L_2)_4]$ and monomeric (H_2L_2) forms of the STIg molecule. Although there have been reports of systemic monomeric IgM molecules in catfish (*Ictalurus punctatus*) (Kaattari, *et al.*, 1998), it is assumed that the monomeric STIg seen here was a by-product of tetrameric Ig degradation. Native PAGE using STIg revealed the presence of both tetrameric and trimeric molecules. However, whole serum contained only one immunoglobulin (tetrameric) band with no evidence of a naturally occurring tri, di or monomeric STIg molecules. Bromage *et al.* (2004) showed that SpA had a greater affinity for reduced forms of the IgM molecule (tri-, di- and monomers) than for the completely polymerized tetramers. If this holds true for the striped trumpeter, it is likely that a serum derived, monomeric Ig molecule would have been bound by the SpA and detected.

Following characterisation of the reduced and native forms of the purified STIg molecule, anti-serum was created to investigate the onset of the antibody response. The rabbit anti-STIgL anti-sera showed an extremely high titre and had strict specificity for the STIgL chain. Despite its stringent specificity for STIgL, the anti-serum was able to bind to the IgL chain of every teleost species tested. This suggests that the polyclonal antibodies were targeted to conserved regions of the IgL chain. As barramundi, snapper and southern bluefin tuna all belong to the same order (Perciformes) as the striped trumpeter, it may not be all that surprising that there is cross-reactivity with the anti-serum.

When the rabbit anti-STIgL was employed to investigate the ontogeny of the antibody response, it was found that STIgL did not reach levels detectable by Western blot analysis until 225 dph, when the fish weighed ~3 g and were ~6.5 cm in length. A prominent STIgL positive band was seen at days 225 and 290 with a less reactive band at day 249, despite

equal amounts of protein being added to each lane. It is possible that the weaker signal at day 249 is the result of natural variation within the population, but it may also be due to differences in the efficiency of tissue excision, homogenate preparation and protein quantification. Nonetheless, the first observation of Ig at day 225 is very late when compared to other marine species. For instance, IgM positive lymphocytes were first detected in Atlantic cod (*Gadus morhua*) at 56-70 dph (Schroder, *et al.*, 1998). In a more closely related species such as sea bass (*Dicentrarchus labrax*), mature B-cells and strong IgM synthesis were detected at 50 dph by both enzyme-linked immunosorbent assay (ELISA) and fluorescence-activated cell sorting (FACS) and the lymphoid organs were fully developed by this point in time (Breuil, *et al.*, 1997). However, it is now accepted that the appearance of lymphoid cells does not coincide with immunocompetence (Zapata, *et al.*, 2006). In fact, a functional adaptive response develops sometime afterwards, likely when a critical number of immunocompetent cells have produced enough IgM (Nakanishi, 1986). Surface IgL is one of the last steps in the production of functional surface IgM in mammals. Pre-B cells are capable of expressing IgH chains on their surface, but at this stage no functional IgM is present. It is the replacement of the surrogate IgL with IgL that completes the formation of functional surface IgM (Karasuyama, *et al.*, 1996). As this study was aimed at identifying the time at which antibodies become functional, rather than the development of lymphoid organs, the anti-serum was directed against the IgL chain.

Some possibilities as to why the antibodies did not reach detectable levels until 225 dph include the extended larval phase characteristic of this species as well as the sensitivity of the detection method. The striped trumpeter is different from most other commercially cultured species in that it has a long larval phase, lasting approximately 9 months. At this point in time, the larvae take on their characteristic striped appearance and metamorphose into juveniles (Tracey, *et al.*, 2005). It is possible that the functionality of the adaptive immune response coincides with major changes in life stage. In fact, it has been suggested that the appearance of IgM coincides with the first feeding (Doggett, *et al.*, 1991) and/or yolk sac absorption (Nagae, *et al.*, 1993; Razquin, *et al.*, 1990). Therefore, the extended larval stage of the striped trumpeter may account for the delay in the onset of the antibody response. Sea bass absorb their yolk sac relatively early, at about 10 dph, and are fed live *Artemia* from 7 dph. It is around 40 dph that they are first exposed to a pelleted feed. The increase in the IgM levels, therefore coincides with the weaning period of ~30-50 dph. It is at 50 dph that Breuil *et al*. (1997) suggest the sea bass becomes immunocompetent. This hypothesis is supported by successful vaccination at this stage. Despite this evidence, it was suggested that immunological maturity is not reached until 137-145 dph (dos Santos, *et al.*, 2000). As the sea bass is one of the more closely related species to the striped trumpeter, it may follow a similar schedule of IgM production. However, given their extended larval period, it is entirely possible that striped trumpeter antibodies are not present until 225 dph, with immunocompetence following thereafter.

19.6 Conclusion

This preliminary investigation has shown that the ontogeny of the antibody response in larval striped trumpeter may occur later than in other marine teleosts. It is hypothesised that the unusually long larval stage of this species may play a part in the delayed onset, as the production of antibodies may be linked to developmental changes. Work to develop sensitive RT-PCR primers and *in situ* hybridisation probes targeted to recombination activating gene (RAG) and Ig molecules is currently underway. It is hoped that such studies can further elucidate the timing of a functional antibody response in the striped trumpeter. This information can then be applied to husbandry and vaccination strategies for this species.

19.7 Acknowledgements

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20 CLONING AND EXPRESSION ANALYSIS OF THREE STRIPED TRUMPETER (*LATRIS LINEATA*) PRO-INFLAMMATORY CYTOKINES, TNF-A, IL-1B AND IL-8 IN RESPONSE TO INFECTION BY THE ECTOPARASITIC, *CHONDRACANTHUS GOLDSMIDI.*

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20.1 Abstract

This study reports the cloning and sequencing of three striped trumpeter (*Latris lineata* Forster) pro-inflammatory cytokines, TNF-α, IL-1β and IL-8, as well as their differential expression in response to an infection by the ectoparasite *Chondracanthus goldsmidi*. The striped trumpeter TNF-α transcript consisted of 1093 bp, including a 759 bp ORF which translated into a 253 aa transmembrane peptide. The sequence contained a TACE cut site, that would produce a 167 aa soluble peptide containing the TNF ligand family signature. The IL-1β sequence consisted of 963 bp, including a 774 bp ORF which translated into a 258 aa protein. The protein lacked both a signal peptide and an ICE cleavage site, but did contain the IL-1 family signature. The sequence for the chemokine IL-8 contained 906 bp, with an ORF of 297 bp, which translated into a 99 aa protein. The protein lacked an ELR motif as is common with many teleost IL-8 sequences. The differential expression of the three cytokine genes in parasitized fish was investigated via quantitative real-time PCR. A significant up-regulation of all three pro-inflammatory cytokines was found in the gills, which were the site of parasite attachment. Examination of head kidney cells revealed a significant up-regulation of TNF-α, but not IL-1β or IL-8. Conversely, the spleen cells showed significant up-regulation of both IL-1β and IL-8, but not TNF-α. These findings allow for more detailed investigations of the striped trumpeter immune response.

Keywords: inflammatory response, TNF-α, IL-1β, IL-8, ectoparasite

20.2 Introduction

The striped trumpeter (*Latris lineata* Forster) has been under investigation as a new species for culture in Tasmanian waters since the 1980's [1]. During that time, much progress has been made in understanding reproduction, larval culture, and identification and control of disease [2]. However, as the culture of striped trumpeter continues to expand, health issues will inevitably arise; making an understanding of the immune response crucial to any

mitigation strategies that may be employed. The importance of such knowledge has recently been highlighted with the appearance of a new species of Chondracanthid parasite, *Chondracanthus goldsmidi*, that has the ability to infect cultured striped trumpeter [3]. There is now a real need to investigate the striped trumpeter responses to this parasite and examine the types of immune response it elicits. This information will help estimate the level of damage that may occur in the event of an epizootic affecting commercially cultured fish and will be a first step towards developing possible mitigation strategies.

Pro-inflammatory cytokines have been proven to be especially useful when looking at the inflammatory responses of fish following pathogenic infection [4]. However, to date, no cytokine sequences from the striped trumpeter are available. An understanding of the involvement of cytokines in immune regulation within this species would allow immune responses to be studied in more detail. Inflammatory cytokines can be divided into two groups; those that promote inflammation, the pro-inflammatory cytokines, and those that suppress inflammation, the anti-inflammatory cytokines. Three important pro-inflammatory cytokines that are also good markers of an inflammatory response are: tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-8 (IL-8). TNF-α is a member of the TNF ligand superfamily [5] and an important inflammatory cytokine, as it is a strong inducer of gene expression, and possesses the ability to provoke apoptosis and necrosis [6]. These properties make it a crucial driver of the mammalian immune response against both intracellular bacteria and some viral infections [7], although recent work seems to suggest that this may not be the case in teleosts [8]. In mammals, homotrimeric TNF is released from the membrane of macrophages, monocytes, neutrophils, NK-cells and T-cells by a protease from the metalloprotease/disintegrin/ cysteine-rich family called TNF-alpha converting enzyme (TACE) [9]. In bony fish, TNF-α was first discovered in Japanese flounder [10] and rainbow trout [11] but has since been characterised in a number of other species [12-19].

IL-1β is a member of the β-trefoil cytokines as it contains 12 β-sheets within the mature protein, and folds to create a trefoil-like structure [20]. In mammals, IL-1β lacks a signal peptide, but is found as an inactive pre-cursor that must be cleaved by the IL-1β converting enzyme (ICE) to form a biologically active mature peptide [21]. IL-1β is a pro-inflammatory cytokine involved in the initiation and/or increase of a wide variety of non-structural, function-associated genes expressed during inflammation. IL-1β is produced by many cell types, but predominantly blood monocytes and tissue macrophages. It is important in the host's response to microbial invasion, tissue injury and immunological reactions including autoimmune diseases via its ability to enhance phagocytic activity, lysozyme synthesis, macrophage proliferation and leucocyte migration [21]. In addition to humans [22] and mice [23], IL-1β has been characterised in large number of non-mammalian vertebrates, such as, *Xenopus* [24], chicken [25], cartilaginous fish [24, 26] and bony fish [24, 27-37].

IL-8, also known as CXCL8, is a member of a multifunctional chemokine family comprised of around 40 small cytokines with the ability to induce the migration of cells to sites of infection or injury [38]. These proteins are defined by 4 cysteine residues, which form disulfide bonds as part of the primary sequence structure. Many of the CXC chemokines, which includes IL-8, contain a three amino acid residue motif, ELR, prior to the first cysteine (ELR+ CXC). The ELR motif is believed to play a critical role in the effectiveness of IL-8, with a change in any of the three amino acids leading to a dramatic decrease in the ability to recruit leucocytes, particularly neutrophils, to the site of inflammation [39]. ELR+ CXC chemokines are produced by a wide variety of cells in response to many stimulants, particularly the pro-inflammatory cytokines IL-1β and TNF-α. IL-8 was first characterised

within the lamprey [40], a fish that belongs to the most primitive vertebrate group, the agnathans. More recently, it has been discovered in the cartilaginous banded dogfish [41] and silver chimaera [42] as well as a number of teleosts [27, 43-45]. Until recently, all known fish IL-8 sequences lacked the ELR motif typical of mammalian IL-8 [41, 42, 44], however, both the haddock [27] and cod IL-8 (CAD59734) sequences have now been shown to possess this motif.

The aim of this investigation was to clone the first cytokine molecules within the striped trumpeter. Here we describe the sequences of three pro-inflammatory cytokines, TNF-α, IL-1β and IL-8, and compare them to known sequences from other vertebrate species. In addition, the expression of these genes was used to examine the inflammatory response of striped trumpeter to the ectoparasite *C. goldsmidi*. Quantitative real-time PCR was carried out on gill, head kidney and spleen tissues from parasitized and non-parasitized striped trumpeter and transcript expression levels were normalised against those of the reference genes β-actin, EF1-α and RPS20. Lastly, histological sections were examined for parasite-induced differences. This information will provide valuable insights into the level of host response to the parasite, as well as the level of damage inflicted on the host.

20.3 Materials and Methods

20.3.1 *Striped trumpeter (Latris lineata)*

Standard husbandry conditions

Fish were held at the Marine Research Laboratory (TAFI), Taroona, Tasmania. Adult striped trumpeter were maintained in 25 000 l tanks at densities of approximately 5.0 kg/m3. Ambient seawater was drawn from the Derwent river where temperatures ranged from 9-20 °C and salinity from 27 to 35 ppt. All fish were fed a semi-moist diet at 3 % body weight once daily [46]. The fish used for initial cDNA production for the cloning of cytokine genes were killed by anaesthetization in 0.02 % 2-phenoxyethanol, for collection of the head kidney.

Parasite trial

The fish used in the parasite infected pro-inflammatory expression study were 496.5±157 g in weight, 31.8±2.8 cm in length, and approximately 850 days post-hatch (dph). Infection with the ectoparasite, *C. goldsmidi*, was natural, and had been established in the tank for approximately 10 months prior to tissue collection. The number of parasites on each infected fish ranged from 1-6 at the time of sampling. As this was an ongoing trial, the level of infection throughout the previous 10 months is unknown. All fish were held under standard husbandry conditions throughout.

Fish were examined for secondary infections both visually and histologically. None of the fish used in this experiment exhibited any signs of secondary infection via external and internal visual examination. Although the myxozoan, *Kudo neurophila*, is known to infect striped trumpeter held at this facility, histological examination of the brain did not reveal any evidence of its presence. Although bacteriological sampling was not conducted, at no time during this trial were there any signs of such an infection.

20.3.2 *cDNA production*

The head kidney was aseptically extracted from striped trumpeter and placed in 10 ml of ice-cold Liebovitz-15 (L-15) media supplemented with 2% foetal calf serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), and heparin (10 IU/ml). The organ was then dissociated by passing it through a 20 µm nylon mesh. The mixture was transferred to a 15 ml tube and allowed to settle for 5 min. The supernatant was decanted into a new 15 ml tube and LPS (Sigma-Aldrich, USA) was added at 10 µg/ml. Cells were transferred to a sterile T-15 tissue culture flask (Iwaki, Japan), which was placed in an 18 °C humidity chamber for 4 h. Cells were subsequently harvested and centrifuged at 440 x *g* for 5 min at 4 °C. The pellet was resuspended in RLT buffer (Qiagen Inc., CA, USA) and stored at -80 °C until use.

Total RNA extraction was performed using an RNeasy kit (Qiagen Inc., CA, USA) according to the manufacturer's instructions. cDNA was produced with a GeneRacer kit (Life Technologies, Invitrogen, USA), following the manufacturer's instructions. Briefly, RNA was dephosphorylated and decapped before an adaptor oligonucleotide was ligated to the 5` end. Reverse transcription was carried out using the superscript III kit component.

Table 20.1 Oligonucleotide primers used to amplify striped trumpeter TNF-α, IL-1β and IL-8 cDNA for cloning and oligonucleotide primers used to amplify striped trumpeter target genes TNF-α, IL-1 β, IL-8 and reference genes β-actin, EF-1 and RPS20 for quantitative real time PCR expression studies.

Primer name	5`-3` sequence	Information
PERCTNFα F1	ATC AGC AGC AAA GCC AAG GCA GC	Primers used to obtain initial fragments
PERCTNFα R1	GAA GGT CTT GCC CTC GTC TGT CTC CAG	
PERCIL-1β F1	GCT CCA CGC ATG ATG CTG CAG GGA GG	
PERCIL-1β R1	GCT GTG CTG ATG TAC CAG	
PERCIL-8 F1	CGA GTG TAA CTG CAC TGC CGC TGC	
PERCIL-8 R2	TTC AGA GTG GCA ATG ATC TC	
LaliTNF R1	GTA GAC GAA CTA GAG GCC GGT TTG	Primers for 5` RACE
LaliTNF R2	TCT TGT TCT CCA CCA GTT TGA AGC	
LaliIL-1 R1	TTT GTG TCC TTG ATG CCC AGA GC	
LaliIL-1 R2	TCA GAT GAA CTT TGC GGC TGT C	
LaliIL-8 R1	AGT GCG AGT TGG CGG GAA TCA G	
LaliIL-8 R1	AAT CAG CTC CAC CTT CCC GAT GTG	
LaliTNF F1	ATT TAT CTG GGA GCA GTG TTT CAG	Primers for 3` RACE
LaliTNF F2	AAC AAA GGG GAC ACG CTG TGG AC	
LaliIL-1 F1	TCT GGA GAC CGT TGA GGA CAA AGA C	
LaliIL-1 F2	ATT TCT CTT CTA CAA ACA GGA CAC C	
LaliIL-8 F1	ACA TCG GGA AGG TGG AGC TGA TTC	
LaliIL-8 F1	ATT CCC GCC AAC TCG CAC TG	
LaliTNF F	GCG TCC TGC TGT TTG CTT GGT	Target gene primers for quantitative real time expression studies
LaliTNF R	GGC TGC CTT GGC TTT GCT G	
LaliIL-1 F	CGA CAG CAG GAA GAG GAG C	
LaliIL-1 R	TGC CCA GAG CCA CGG T	
LaliIL-8 F	GTG CTC CTG GCG TTC CT	
LaliIL-8 R	GCT CCA CCT TCC CGA TG	
Laliβ-actin F	GGG AAA TCG TGC GTG AC	Reference gene primers for quantitative real time expression studies
Laliβ-actin R	AGG AAG GAA GGC TGG AAG	
LaliEF1 F	CCC TTA CAG CCA GAG CCG T	
LaliEF1 R	GAT CTT CCA ACC CTT GAA CCA T	
LaliRPS20 F	CTC ACC AGC CGC AAC G	
LaliRPS20 R	CAC CGC AGG GAG TCT TTC T	

20.3.3 *Cloning and sequencing of TNF-α, IL-1β and IL-8 cDNA*

The initial fragments of striped trumpeter TNF-α, IL-1β and IL-8 genes were amplified using perciform specific primer pairs (PERCTNF-F1/R1, PERCIL1-F1/R1, and PERCIL8-F1/R1 respectively), which were designed based on the conserved regions of known perciform cytokine sequences (Figure 20.1, Table 20.1). All three fragments were cloned, sequenced and subsequently used to design striped trumpeter-specific primers for use with the GeneRacer 5` and 3` adaptor oligonucleotide primers (Figure 20.1, Table 20.1).

Each PCR was run in standard 25 µl reactions combining 1 µl of each of the primers (10 µM), 1 µl of cDNA, along with 2.5 µl 10x PCR buffer, 0.5 µl MgCl2 (50 mM), 0.5 µl dNTP (2.5 mM each), 18.35 µl nuclease-free water and 0.15 µl Taq DNA polymerase (5 u/µl). The cycling protocol was as follows: an initial denaturation step of 3 min at 94 °C followed by 25 amplification cycles of 94 °C for 30 sec, annealing at primer specific temperatures (50-55 °C) for 30 sec and extension at 72 °C for 60 sec. The reaction was finished with a 72 °C extension period lasting 10 min. All PCR products were visualized in 1.5 % agarose gels containing ethidium bromide (100 ng/ml). Products of the expected size were ligated into pGEM®-T Easy Cloning vector (50 ng/µl) (Promega, Madison, WI). Following transfection of TOP10F` One Shot® (Invitrogen, BV, Netherlands), recombinant cells were grown on MacConkey agar (Sigma-Aldrich, USA). Colonies containing the insert of interest were grown overnight in 5 ml of Luria-Bertani (LB) medium with 2.5 µl ampicillin (100 mg/ml) in a shaking incubator at 37 °C. Plasmid DNA from at least 3 different colonies was purified using a QIAprep® Plasmid DNA Miniprep kit (Qiagen Inc., CA, USA) following the manufacturer's instructions. The purified plasmid was then sequenced using M13 forward or M13 reverse vector-specific primers with an ABI automated 377 Sequencer. Sequences were analysed for similarity with other known sequences using the Basic Local Alignment Search Tool (BLAST) program [47]. Comparisons between more than two species were carried out using CLUSTAL W v1.60 multiple sequence alignment package [48]. Phylogenetic trees were drawn from CLUSTAL-generated alignments using the TreeView v1.6.1 program [49] and confidence limits added [50]. Predicted amino acid sequences were analysed by SignalP version 3.0 [51] and the hydrophobicity profile was determined using Kyte and Doolittle plots [52]. Transmembrane domain predictions were made using HMMTOP [53]. Estimations of the molecular weights of the mature proteins were made using the ExPASY compute Mw tool [54]. Protein family signatures were obtained from the PROSITE database [55] and glycosylation sites were predicted using the NetNGlyc 1.0 Server [56].

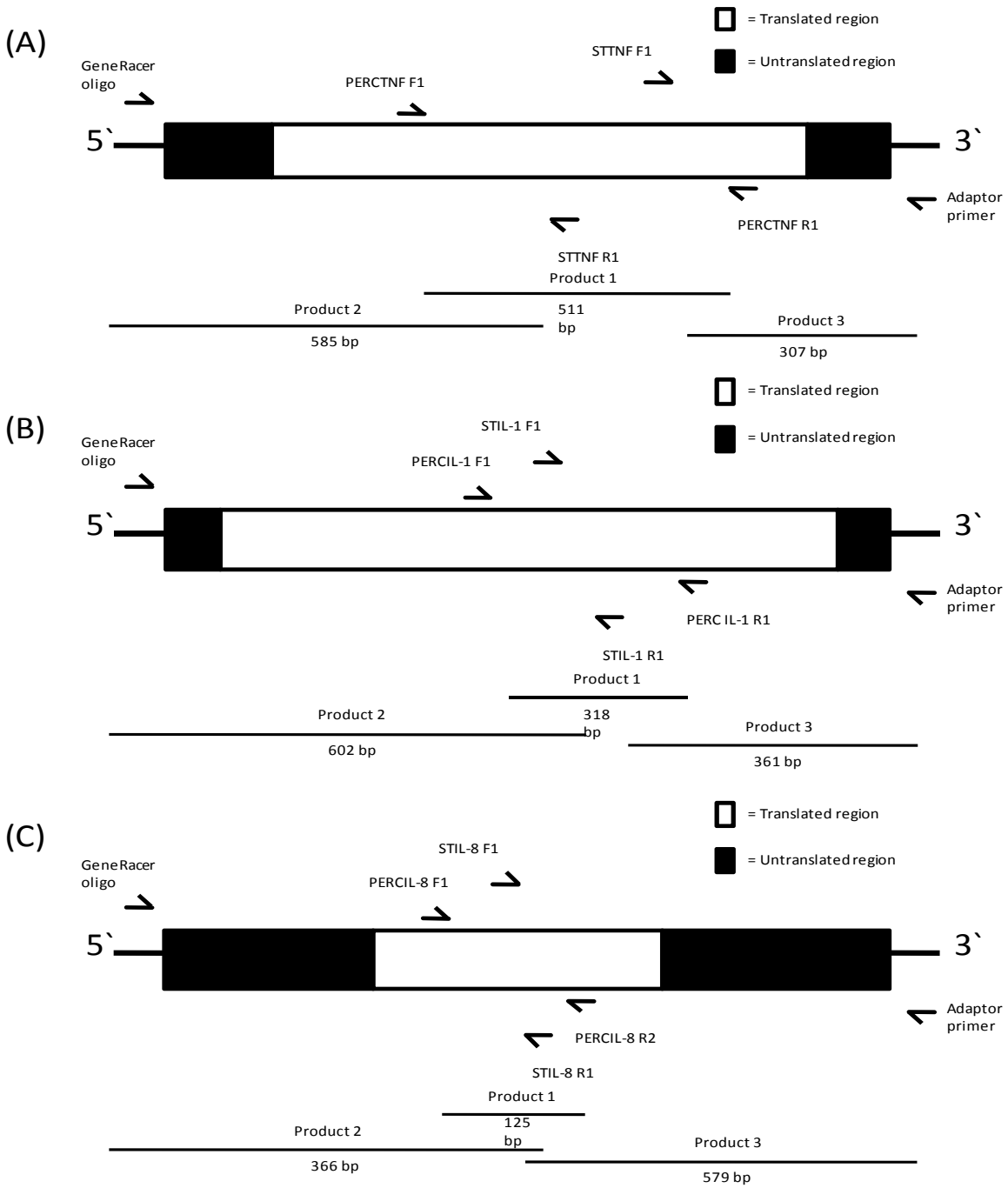

Figure 20.1 Localisation of primers used for the cloning and sequencing of striped trumpeter (A) TNF-α, (B) IL-1 β and (C) IL-8 genes and the sizes of the products obtained.

20.3.4 *Expression studies*

Gill, head kidney and spleen samples from six *C. goldsmidi* parastized and six non-parasitized striped trumpeter were collected and stored in RNAlater (Sigma-Aldrich, USA) at -20 °C. Total RNA extraction and cDNA production were carried out according to section 2.2. Additionally, TURBO DNAse (Ambion, CA, USA) was used to remove any contaminating genomic DNA from the RNA following the manufacturer's instructions. Reverse transcriptase-free controls were included to ensure the absence of genomic DNA. Once the full length TNF-α, IL-1β and IL-8 sequences were obtained, striped trumpeter specific qPCR expression primers were designed for each gene and the reference genes β–

actin (EF571597), EF-1 (Cobcroft, Zambonino and Le Gall, pers. comm.), and RPS20 (FJ532284), which are listed in Table 20.1. Each PCR was run in a 96-well plate (Bio-Rad, CA, USA) combining 1µl of forward primer (5 µM), 1 µl of reverse primer (5 µM), 11 µl of SensiMixPlus SYBR (Quantace, UK), 7 µl of nuclease free water (Ambion, CA, USA) and 2 µl of cDNA (diluted 1:25 in nuclease-free water).

PCRs were performed using an ICycler iQ5 (Bio-Rad, CA, USA) under the following conditions: initial denaturation cycle at 95 °C for 8 min, followed by 40 amplification cycles of 95 °C for 15 sec, annealing for 30 sec at 55 °C and extension for 25 sec at 72 °C. At the end of each PCR, melt curve analysis was performed to ensure that only one product was amplified. The products were also visualised on a 1.5 % agarose gel containing ethidium bromide (100 ng/ml) to ensure they were of the correct size. Additionally, one product from each tissue for each gene from each group was sequence verified.

The results were analysed using qBase relative quantification framework software v 1.3.5 [57]. Primer efficiencies, where $E=10^{(-1/slope)}$, were determined by analysis of 10-fold serial dilutions of pooled cDNA and averaged 2.0±0.1 for all genes across all tissues. The stability of β-actin, EF-1 and RPS20 as reference genes was measured via the qBase GeNorm application [58]. All three reference genes were deemed to be stable across the two groups, and therefore used to calculate the mean normalised relative quantities (MNRQ) of target gene transcripts. Statistical analysis between the two groups was made using a Mann-Whitney Wilcoxon (MWW) test, where $p<0.05$ was considered significant.

20.3.5 *Histological sampling*

At the time of tissue collection for cytokine gene expression, samples were also collected for histological analysis. Gill, head kidney and spleen tissue samples were collected from parasitized and non-parasitized striped trumpeter. Gill samples were fixed in seawater Davidson's for 24 h, while head kidney and spleen samples were fixed in 10% neutral buffered formalin for 24 h. Next, all samples were transferred to 70% ethanol for 24 h, and subsequently paraffin-embedded. Tissue blocks were cut into 5 µm sections and stained with hematoxylin and eosin. Stained slides were examined for cellular evidence of a host inflammatory response.

20.4 Results

20.4.1 *Cloning and sequencing of striped trumpeter TNF- α*

PCR amplification of cDNA from LPS stimulated head kidney cells, using perciform specific TNF-α primers, provided an initial fragment of 511 bp (Figure 20.1A). The fragment was cloned, sequenced and used to design subsequent striped trumpeter TNF-α primers. 5` RACE performed using a striped trumpeter gene specific primer (LaliTNF- R1) and the GeneRacer 5` adaptor primer resulted in a 585 bp product (Figure 20.1A). 3` RACE performed using a striped trumpeter gene specific primer (LaliTNF-F1) and the GeneRacer 3` adaptor primer amplified a fragment of 307 bp (Figure 20.1A). Both the 5` and 3` ends were cloned and sequenced. The three fragment sequences were assembled to form a continuous sequence of 1093 bp (Figure 20.2). The 5` untranslated region (UTR) consisted of 144 bp, with the 3` UTR containing 190 bp, leaving an open reading frame (ORF) of 759 bp. The 3` UTR did not contain any instability motifs (ATTTA), or a polyadenylation signal (AATAAA), indicating that it may not be complete. The coding sequence translated into a 253 aa peptide that contained the TNF family signature, [LV] - x - [LIVM] - x(3) - G -

[LIVMF] - Y - [LIVMFY](2) - x(2) - [QEKHL] - [LIVMGT] - x - [LIVMFY], in the form IvIpqtGLYFVysQAsF and a transmembrane domain A^{35}SGALLAVALCIGGVLL^{51} (Figure 20.2). A multiple alignment with other known teleost and human TNF-α sequences (Figure 20.3) revealed areas of good amino acid conservation not only within the family signature, but also with two conserved cysteines (positions 144 and 175 in human) important for disulphide bridge formation and ultimately correct folding of the mature protein. Conserved threonine-leucine residues consistent with those used by mice as the cleavage site for TNF-α converting enzyme (TACE) were found at positions 86 and 87, creating a soluble, mature peptide of 167 aa, with a molecular weight of ~18.26 kDa. Striped trumpeter TNF-α shared the highest nucleotide and amino acid identity (Table 20.2) with sea bass at 57.2% and 83.8% respectively, with the other amino acid identities ranging between 13.4% (chicken) and 79.1% (sea perch). Phylogenetic analysis grouped the striped trumpeter TNF-α sequence with those of other percifiormes and the close identity to the sea bass sequence was maintained (data not shown).

Table 20.2 Amino acid and nucleotide homology of striped trumpeter TNF-α with other known vertebrate TNF-α sequences. Values in bold show the highest homology to the striped trumpeter sequence. GenBank accession numbers of the TNF-α genes not included in the multiple alignment are: carp (AJ417565), chicken (AY65397), mouse (AB185895), plaice (AJ416943), sea bass (DQ200910), sea perch (AY376595) and *Xenopus* (AB298595).

Species	Nucleotide identity (%)	Amino Acid similarity (%)	Amino acid identity (%)
Human	25.7	49.4	32.4
Mouse	27.1	31.2	23.1
Chicken	27.7	21.7	13.4
Xenopus	47.6	24.4	47.4
Zebrafish	39.5	54.5	39.4
Carp	40.8	58.5	40.3
Salmon	43.9	64.8	50.6
Trout	43.9	65.2	51.0
Plaice	17.5	28.1	23.8
Flounder	46.4	71.5	57.4
Fugu	21.1	77.5	65.2
Tilapia	50.9	81.8	72.4
Black seabream	55.2	85.8	78.3
Sea perch	55.5	86.2	79.1
Sea bass	57.2	90.9	**83.8**

```
1    gaaatcactccaacacaatagcaacacaaagagggagaagcgtcaccactggacacacct   60
61   gaagacaaggacccagcatccagtgttcagttggtagatattttcagcagaaggaaaact  120
121  cagagctcagacttgttgcacagtatggtggcgtacacaaccgcacaaggtgacgtggag  180
                              M  V  A  Y  T  T  A  Q  G  D  V  E    12
181  atgggtcttgaggagaggacggtggtgctggtcgaaaagaagtcatctgcgggccggata  240
      M  G  L  E  E  R  T  V  V  L  V  E  K  K  S  S  A  G  R  I    32
241  tggaaggcgtccggggcccttctcgctgtggccctttgtatcggaggcgtcctgctgttt  300
      W  K  A  S  G  A  L  L  A  V  A  L  C  I  G  G  V  L  L  F    52
301  gcttggtgctggagcggaaggccagaaatgacgacgcaatcaggccaaacagaagcacta  360
      A  W  C  W  S  G  R  P  E  M  T  T  Q  S  G  Q  T  E  A  L    72
361  atcaagaaggacactgctgagaaaacagatccccattccacgctgaggcgaatcagcagc  420
      I  K  K  D  T  A  E  K  T  D  P  H  S  T  L  R  R  I  S  S    92
421  aaagccaaggcagccatccatttagaaggtagctacgacgacggcgagtgttcgaaaggc  480
      K  A  K  A  A  I  H  L  E  G  S  Y  D  D  G  E  C  S  K  G   112
481  cagctggagtggagaaacggccagggccaggcgttcgcccagggcggcttcaaactggtg  540
      Q  L  E  W  R  N  G  Q  G  Q  A  F  A  Q  G  G  F  K  L  V   132
541  gagaacaagatcgtcatcccacaaaccggcctctacttcgtctacagccaggcgtcgttc  600
      E  N  K  I  V  I  P  Q  T  G  L  Y  F  V  Y  S  Q  A  S  F   152
601  agagtctcctgcagcgacggcgacgaggaggggcgggaaaacgcctcacacctctcagc  660
      R  V  S  C  S  D  G  D  E  E  G  A  G  K  R  L  T  P  L  S   172
               ▲
661  cacaggatctggcgctattcagactccgtaggcaacaaggcctccctgatgagcgccgtg  720
      H  R  I  W  R  Y  S  D  S  V  G  N  K  A  S  L  M  S  A  V   192
721  aggtcggcgtgccagaacacagcccaggaggacggctacaatgccggacacggctggtac  780
      R  S  A  C  Q  N  T  A  Q  E  D  G  Y  N  A  G  H  G  W  Y   212
               ▲
781  aacgccatttatctgggagcagtgtttcagctgaacaaaggggacacgctgtggacggaa  840
      N  A  I  Y  L  G  A  V  F  Q  L  N  K  G  D  T  L  W  T  E   232
841  accaaccagccgtcggagctggagaccgatgagggcaagactttctttggtgtgtttgca  900
      T  N  Q  P  S  E  L  E  T  D  E  G  K  T  F  F  G  V  F  A   252
901  cttgaaaatgactcttcgatgtgatgccagcagagaagacgctctgcacagtgccatgag  960
      L  -                                                        253
961  tttgggctttttctttaacattatgtacgtattgtttcatctttattattcatcgtcat 1020
1021 ggtgatgtagaaatgtttaaatctcaatggagataaatgcttgtgcctgaaaaaaaaaaa 1080
1081 aaaaaaaaaaaaa                                               1093
```

Figure 20.2 The full cDNA sequence of striped trumpeter TNF-α with the 5` and 3` UTR in italics. Features highlighted include the start and stop codon (boxed), the TNF family signature (light grey), the putative TNF-alpha converting enzyme (TACE) cleavage site for the mature peptide (dark grey), the predicted transmembrane domain (bold and underlined) and the two cysteine residues involved in quaternary structure formation (▲).

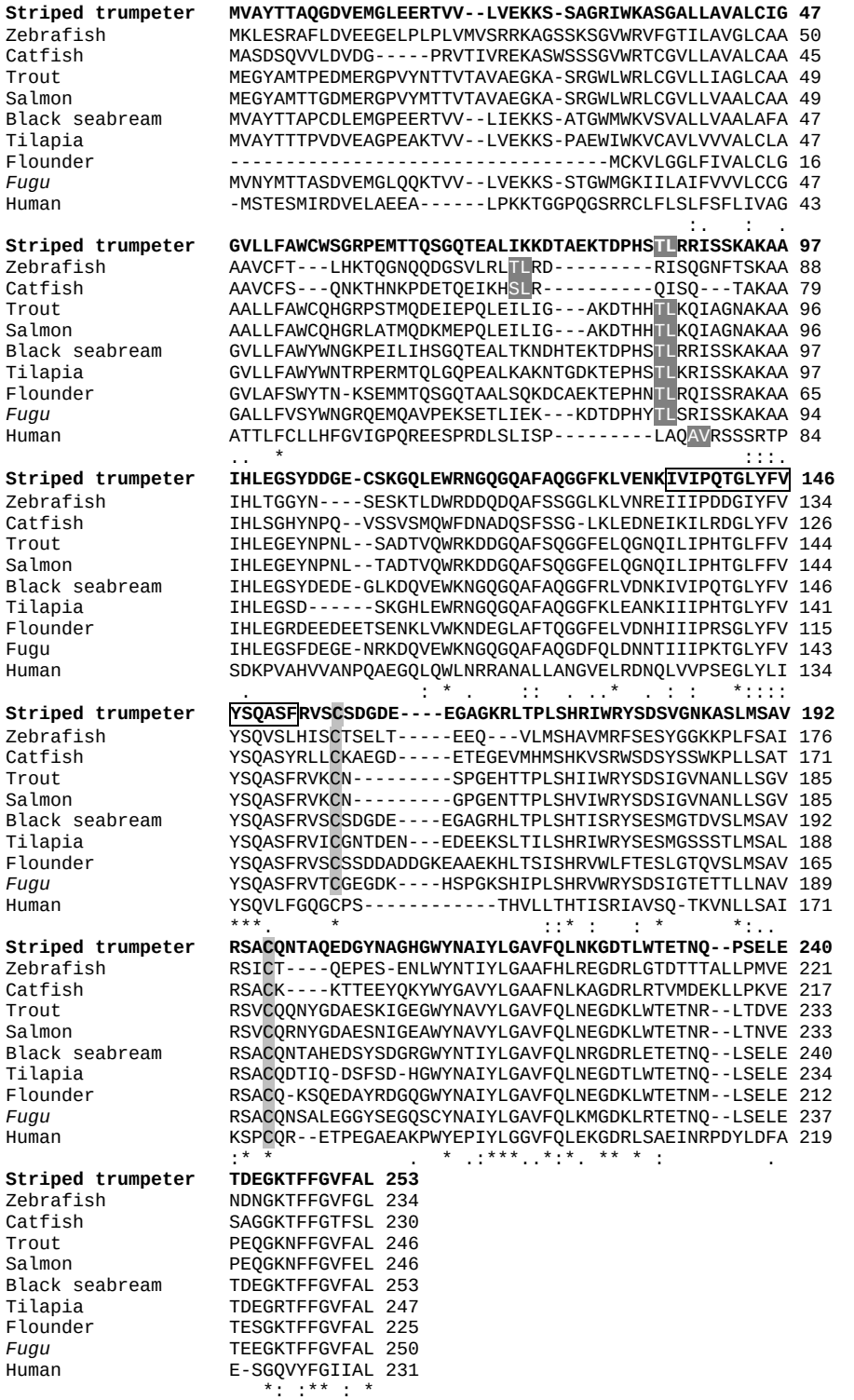

Figure 20.3 Multiple amino acid alignment of striped trumpeter TNF-α (bold) with other known vertebrate TNF-α genes. Identical (*, highlighted) and similar (:, .) residues identified by CLUSTAL W are shown. The TNF family signature and the conserved cysteine residues are highlighted in light grey. The putative TNF-alpha converting enzyme (TACE) cleavage site is highlighted in dark grey. The GenBank accession numbers for the TNF-α genes are: black seabream (AY335443), catfish (CAD10389), flounder (AB040448), Fugu (DQ393274), human (AL662847), mouse (AAA40458), salmon (AY929385), striped trumper (FJ532281),tilapia (AY428948), trout (AJ278085) and zebrafish (AB183467).

20.4.2 *Cloning and sequencing of striped trumpeter IL-1β*

PCR amplification of cDNA from LPS stimulated head kidney cells, using perciform specific IL-1β primers, provided an initial fragment of 318 bp (Figure 20.1B). The fragment was cloned, sequenced and used to design subsequent striped trumpeter IL-1β primers. 5` RACE performed using a striped trumpeter gene specific primer (LaliIL1-R1) and the GeneRacer 5` adaptor primer resulted in a 602 bp product (Figure 20.1B). 3` RACE performed using a striped trumpeter gene specific primer (LaliIL1-F1) and the GeneRacer 3` adaptor primer amplified a fragment of 361 bp (Figure 20.1B). Both the 5` and 3` ends were cloned and sequenced. The three fragment sequences were assembled to form a continuous sequence of 963 bp (Figure 20.4). The 5` UTR consisted of 96 bp, with the 3` UTR containing 93 bp, leaving an ORF of 774 bp. The 3` UTR contained one region of AT repeats and one instability motif (ATTTA), but no discernable polyadenylation signal (AATAAA), indicating that it may not be complete. The coding sequence translated into a 258 aa peptide that contained two predicted N-glycosylation sites and a clearly identifiable IL-1 family signature, [FC] - x - S - [ASLV] - x(2) - P - x(2) - [FYLIV] - [LI] - [SCA] - T - x(7) - [LIVM], in the form LvSVafSdwYISTtednkpvE (Figure 20.4). A multiple alignment with other known teleost IL-1β, as well as cartilaginous fish, and human sequences, revealed areas of good amino acid conservation, particularly within the predicted 12 β-sheets (Figure 20.5). As with all other known teleost IL-1β sequences, there was an absence of both an IL-1β converting enzyme (ICE) cleavage site and a signal peptide (data not shown). Striped trumpeter IL-1β shared the highest nucleotide and amino acid identity with the Chinese perch at 64.4% and 74.9% respectively (Table 20.3), with the other amino acid identities ranging between 22.9.% (Leopard shark) and 68.9% (sea bass). Phylogenetic analysis grouped the striped trumpeter IL-1β sequence with those of other perciformes and the close identity to the Chinese perch sequence was maintained (data not shown).

Table 20.3 Amino acid and nucleotide homology of striped trumpeter IL-1β with other known vertebrate IL-1β sequences. Values in bold show the highest homology to the striped trumpeter sequence. GenBank accession numbers of the IL-1β genes not included in the multiple alignment are: black seabream (AY669059), cat shark (AJ251201), catfish (DQ160229), chicken (AY15006), Chinese perch (AY647430), goldfish (AJ419848), haddock (AJ550166), red seabream (AY257219), salmon (AY617117), sea perch (AY385480) and *Xenopus* (AJ314758).

Species	Nucleotide identity (%)	Amino acid similarity (%)	Amino Acid identity (%)
human	42.2	42.0	24.8
chicken	45.6	44.4	29.0
xenopus	47.6	46.3	28.0
leopard shark	48.8	47.6	22.9
catshark	44.8	44.1	23.9
goldfish	46.8	45.7	26.9
zebrafish	47.1	46.2	28.1
catfish	47.7	46.8	28.5
carp	45.6	45.0	29.1
salmon	51.5	49.9	51.3
trout	51.5	50.3	51.3
haddock	49.4	48.5	49.8
cod	50.1	49.3	51.7
black seabream	58.8	58.3	60.5
seabream	61.1	58.7	60.5
Japanese flounder	56.3	55.6	61.1
red seabream	59.9	59.4	63.0
sea perch	62.2	61.2	66.5
turbot	59.9	59.4	67.9
seabass	61.4	58.4	68.9
Chinese perch	**64.4**	**64.2**	**74.9**

```
1    cgactggagcacgaggacactgacatggactgaaggagtagaaaaggcataacaacactg    60
61   acaggacaactgctgggatcttaaccaaacacactaacactctactaaccttaacagatc   120
121  tttcttaattcaaagaaaaagatggaatccgagatgagatgcaacgtgagcgagatgtgg   180
                          M  E  S  E  M  R  C  N  V  S  E  M  W     13
181  agccccaagatgcccaaggggctggacttggagattacccatcatccgctgacaatgaga   240
      S  P  K  M  P  K  G  L  D  L  E  I  T  H  H  P  L  T  M  R     33
241  cgcgtggtcaacctcatcgtcgccatggagaggttgaagggcagcgagtcagcgctgagc   300
      R  V  V  N  L  I  V  A  M  E  R  L  K  G  S  E  S  A  L  S     53
301  accgagctcagagacgaaaacctgctcaacttcatgctggagagcatagtggaagagcaa   360
      T  E  L  R  D  E  N  L  L  N  F  M  L  E  S  I  V  E  E  Q     73
361  attgtgttcgagcgcagttcagctccaccagtgcaattccgctgcacgggcgtggaccag   420
      I  V  F  E  R  S  S  A  P  P  V  Q  F  R  C  T  G  V  D  Q     93
421  tgcagcgtgaccgacagcaggaagaggagcttagttctggtccaggacagcatggagctc   480
      C  S  V  T  D  S  R  K  R  S  L  V  L  V  Q  D  S  M  E  L    113
481  cacgcagtgatgctgcagggaggcgctgacagccgcaaagttcatctgaacatgtcgacc   540
      H  A  V  M  L  Q  G  G  A  D  S  R  K  V  H  L  N  M  S  T    133
541  tacgtgcaccctgcacccagctccgaggccagaaccgtggctctgggcatcaaggacaca   600
      Y  V  H  P  A  P  S  S  E  A  R  T  V  A  L  G  I  K  D  T    153
601  aagctctacctgtcttgccacaaggatggtgacaaagtgaccttgcatctggagaccgtt   660
      K  L  Y  L  S  C  H  K  D  G  D  K  V  T  L  H  L  E  T  V    173
661  gaggacaaagacagtctggtgaggatcggctcggacagcgacatggtgcgatttctcttc   720
      E  D  K  D  S  L  V  R  I  G  S  D  S  D  M  V  R  F  L  F    193
721  tacaaacaggacaccgggctgaacatcagcaccctcgtgtccgtcgccttcagcgactgg   780
      Y  K  Q  D  T  G  L  N  I  S  T  L  V  S  V  A  F  S  D  W    213
781  tacatcagcacaacagaggacaacaagccggtggaaatgtgcctggagagcgcccaaagc   840
      Y  I  S  T  T  E  D  N  K  P  V  E  M  C  L  E  S  A  Q  S    233
841  taccggaccttcagcatccagaatctgccgaggacgaccagaatgggctgtggatctgca   900
      Y  R  T  F  S  I  Q  N  L  P  R  T  T  R  M  G  C  G  S  A    253
901  tccgggcgtcaagcttaagaagaatatttgagtgtaaaatatactgcacttcagcttttt   960
      S  G  R  Q  A  -                                             258
961  tgatttaaaaagcactactactgtctgcaaaaaaaaaaaaaaaaaaaa              1008
```

Figure 20.4 Full length cDNA sequence of striped trumpeter IL-1β with the 5` and 3` UTR in italics. Features highlighted include the start and stop codon (boxed), the family signature (light grey), predicted N-glycosylation sites (dark grey) and an mRNA instability motif (bold).

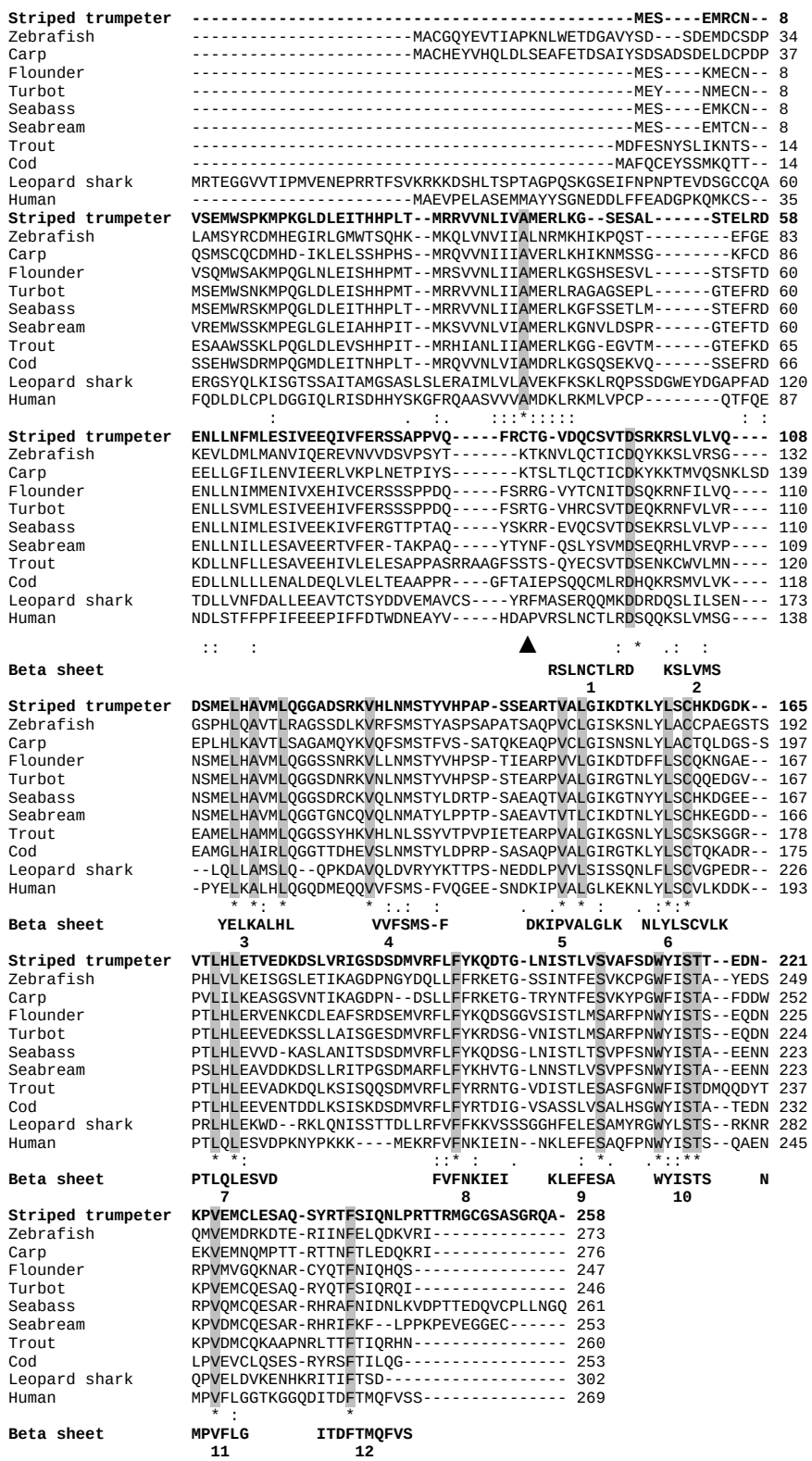

Figure 20.5 Multiple amino acid alignment of known cartilaginous fish, teleost and human IL-1β sequences. Areas of high conservation are mostly centered around the 12 β-sheets (indicated below the alignment). Identical (*, highlighted) and similar (:, .) residues identified by CLUSTAL W are shown. The mammalian ICE cut site is indicated (▲). GenBank accession numbers of the IL-1β genes are: carp (AB010701), cod (AJ535730), flounder (AB070835), human (M15330), leopard shark (AB074142), sea bass (AJ269472),

seabream (AJ277166), striped trumpeter (FJ532282), trout (AJ223954), turbot (AJ295836) and zebrafish (AY340959).

20.4.3 *Cloning and sequencing of striped trumpeter IL-8*

PCR amplification of cDNA from LPS stimulated head kidney cells, using perciform specific IL-8 primers, provided an initial fragment of 125 bp (Figure 20.1C). The fragment was cloned, sequenced and used to design subsequent striped trumpeter IL-8 primers. 5` RACE performed using a striped trumpeter gene specific primer (LaliIL8- R1) and the GeneRacer 5` adaptor primer resulted in a 366 bp product. 3` RACE performed using a striped trumpeter gene specific primer (LaliIL8-F1) and the GeneRacer 3` adaptor primer amplified a fragment of 579 bp. Both the 5` and 3` ends were cloned and sequenced. The three fragment sequences were assembled to form a continuous sequence of 906 bp (Figure 20.6). The 5` UTR consisted of 185 bp, while the 3` UTR contained 424 bp, leaving an ORF of 297 bp. The 3` UTR contained four mRNA instability motifs (ATTTA), and a polyadenylation signal (AATAAA) directly upstream from the poly-A tail. The coding sequence translated into a 99 aa peptide with a putative signal peptide cleavage site between amino acid positions 23 and 24 and contained the CXC subfamily signature, C - x - C - [LIVMS] - x(4,6) - [LIVMFY] - x(2) - [RKSEQNA] - x - [LIVM] - x(2) - [LIVMA] - x(5) - [STAG] - x(2) - C - x(3) - [EQ] - [LIVM](2) - x(9,10) - C - [LV] - [DN], in the form CrCIqteskpIgRhIgkVelipaNshCeetEIIatlkktgqevCLD (Figure 20.6). Multiple alignment with other known IL-8 sequences, revealed areas of amino acid conservation (Figure 20.7) and it was shown that all sequences contained four cysteines, two of which were separated by a single amino acid, as is typical of CXC chemokines. Unlike mammals, but like most teleosts, the striped trumpeter did not contain an “ELR” motif, but instead had an “ELH” tri-peptide in its place. Striped trumpeter IL-8 shared the highest nucleotide identity with Japanese flounder at 35.4% and amino acid identity with *Fugu* at 91.9% (Table 20.4), with the other amino acid identities ranging between 57.6% (human) and 90.1% (haddock). Phylogenetic analysis showed the striped trumpeter IL-8 sequence most closely grouped with the *Fugu* and black seabream (data not shown).

Table 20.4 Amino acid and nucleotide homology of striped trumpeter IL-8 with other known vertebrate IL-8 sequences. Values in bold show the highest homology to the striped trumpeter sequence. GenBank accession numbers of the IL-8 genes not included in the multiple alignment are: chicken (DQ393272).

Species	Nucleotide identity (%)	Amino Acid similarity (%)	Amino acid identity (%)
Human	21.3	37.6	57.6
Chicken	30.3	40.6	60.2
Leopard shark	31.3	42.7	64.4
Carp	28.7	57.6	78.8
Flounder	**35.4**	66.4	78.9
Trout	29.0	59.6	79.8
Black seabream	35.3	**85.9**	88.9
Haddock	30.9	71.3	90.1
Fugu	33.4	82.8	**91.9**

```
1    ggacactgacatggactgaaggagtagaaagctagattcaaacagcagagtgagaaggca   60
61   gctgaaccaagaaaaggaaaagtagagagacgcaactgagcgagagaactaagagaagta  120
121  gaagaaaaaactctgtttcaggcttcatcagacggctttctgaaggacattcttatcctt  180
181  tagtgttaattgttgcagaatttgtaaaaggcaaaatgaagagcagcagagtcatcgtca  240
                                        M  K  S  S  R  V  I  V      8
241  cctctattgtggtgctcctggcgttcctggccatcagtgaagggatgagcctgaggagcc  300
     T  S  I  V  V  L  L  A  F  L  A  I  S  E  G  M  S  L  R  S   28
301  tgggagtggagctgcactgccgctgcatccaaacagagagcaaacccattggccgccaca  360
     L  G  V  E  L  H  C  R  C  I  Q  T  E  S  K  P  I  G  R  H   48
361  tcgggaaggtggagctgattcccgccaactcgcactgcgaggagactgagatcattgcaa  420
     I  G  K  V  E  L  I  P  A  N  S  H  C  E  E  T  E  I  I  A   68
421  ctctgaaaaagacaggccaagaggtttgcctggacccagaggctccctgggtgaagaggg  480
     T  L  K  K  T  G  Q  E  V  C  L  D  P  E  A  P  W  V  K  R   88
481  ttattcagaagatcctgtccaacagaagacgctgaacagagcgggagggatgtgtttcat  540
     V  I  Q  K  I  L  S  N  R  R  R  -                          99
541  gagtctgagctgtttactggaaagacaaatgaaaagtactgaaaagtatttgttggataa  600
601  aagggtgaagccatcatgctctcgaggtcagcttaatgtcccgatcaactgttcaacaga  660
661  attaacccagtaatgttatgtttgtatcaaacatgttatttattgacaagtctgtgcttg  720
721  tttgtacatcacttgtgtttgttgtggccagttactctctgatgtaatttggctttttct  780
780  gtagtttgtaaaagtgcatcactgttgtcttgtctataacttattagttatttaaaattt  840
841  attgatgtatttatgacttgtaagtgtgtctgcattcaattctttacctttgctgaaaac  900
901  gtatggattgattaataaaaaaaaaaaaaaaaaa                            936
```

Figure 20.6 The full cDNA sequence of striped trumpeter IL-8. Features highlighted include the start and stop codon (boxed), the CXC family signature (light grey), an ELH motif (boxed), 4 mRNA instability motifs (bold) and the polyadenylation signal (bold and underlined). The putative signal peptide is shown in italics and underlined (cleavage site between amino acid positions 23 and 24).

```
Striped trumpeter   --MKSSRVIVTSIVVLLAFLAISEGMSLRSLGVELHCRCIQTESKPIG-R 47
Black seabream      ---MSSRVFVATIVGLLAFLAISE----ASLGVELHCRCIQTESKPIG-R 42
Fugu                ---MCSRVFLTSLVVLLAFLAISNGMSLRSLGVEQHCRCIQTESRPIG-R 46
Haddock             MKMTSGKIPISSLLVLLVLLSITEGKSLRGLGMELRCRCIQTESRPIG-R 49
Cod                 MKMTSGKIPISSLLVLLVLLTITEGISLRGLGMELRCRCIQTESRQIG-R 49
Flounder            ---MSSRVIVVAVMVLLASLAISEAVSLRSLGVSLHCRCIETESRPIG-R 46
Carp                ---MHFKIFS--VIVFLGFLTIGEGMSLRGLGVDPRCRCIETESQRIG-K 44
Trout               ---MSIRMSASLVVVLLALLTITEGMSLRGMGADLRCRCIETESRRIG-K 46
Leopard shark       ---MNSKVILAVLALFILYLASTQAASLRHAGVSLRCQCIKTNSKFIHPR 47
Human               ---MTSKLAVALLAAFLISAALCEGAVLPRSAKELRCQCIKTYSKPFHPK 47
                          ::    :  ::   :  :       . . :*:**:* *: :  :

Striped trumpeter   HIGKVELIPANSHCEETEIIATLKKTGQEVCLDPEAPWVKRVIQKILSNR 97
Black seabream      HIEKVELIPANSHCEETEIIATLKRTGQEVCLDPEAPWVKKVIQKILSNA 92
Fugu                HIGKVELIPPNSHCEETEIIATLKMSGQEVCLDPKAPWVKKVINKIMSSR 96
Haddock             HIGKMEIIPANSHCEESEIIATLKRTGQEVCLDGEAPWVKRLIAKMMSSR 99
Cod                 HIGKLEIIPANSHCEETEIIATLKRTGQEVCLDADAPWVKNVIERMISSR 99
Flounder            YIKSVEIISPNSHCDKTEIIATLKDTGVELCLDPEAPWVKRVINKLISKR 96
Carp                LIESVELFPPSPHCKDTEIIATLKVSRKEICLDPIAPWVKKVIEKIIANK 94
Trout               LIKKVEMFPPSSHCRDTEIIATLSKSGQEICLDVSAPWVKRVIEKMLANN 96
Leopard shark       RMENIEIFPSGPHCSNVEIIATLKN-GTPVCLNPEAAWVKKIIDMIIKNS 96
Human               FIKELRVIESGPHCANTEIIVKLSD-GRELCLDPKENWVQRVVEKFLKRA 96
                     : .:.:: ...** . ***..*.     :**:    **:.::  ::

Striped trumpeter   RR---------- 99
Black seabream      RR---------- 94
Fugu                QR---------- 98
Haddock             RR---------- 101
Cod                 RH---------- 101
Flounder            RLSRWREMGSEAV 109
Carp                TPAA-------- 98
Trout               K----------- 97
Leopard shark       EKTES------- 101
Human               ENS--------- 99
```

Figure 20.7 Multiple amino acid alignment of known vertebrate IL-8 genes. Identical (*) and similar (:, .) residues identified by CLUSTAL W are shown. The ELR motif (dark grey), signal peptide (italics) and the conserved cysteines (light grey) are highlighted. The GenBank accession numbers for the IL-8 genes are: black seabream (DQ000611), carp (AJ421443), cod (AJ535731), flounder (AF216646), *Fugu* (AB125645), haddock (AJ566335), human (AAH13615), leopard shark (AB063299) striped trumpeter (FJ532283) and trout (AJ279069).

20.4.4 *TNF-α, IL-1β and IL-8 expression in parasitized & non-parasitized striped trumpeter*

In the gills of striped trumpeter, the mRNA expression of all three pro-inflammatory cytokines was significantly up-regulated in the parasitized group when compared to the non-parasitized group (Figure 20.8A). TNF-α expression was approximately 2 times greater (MWW test U=2.000, N_{np}=6, N_p=6, p=0.038), while IL-1β expression was approximately 16 times greater (MWW test U<0.001, N_{np}=6, N_p=6, p=0.010) and IL-8 expression was approximately 4 times greater (MWW test U=3.000, N_{np}=6, N_p=6, p=0.015). There were no significant differences in expression levels of any of the three cytokines when lesion areas from parasitized gill tissue were compared to non-lesion areas from the same individuals (data not shown).

The qPCR analysis of the head kidney (Figure 20.8B) showed that there was a significant increase of approximately 6 times in the expression level of TNF-α in the parasitized group (MWW test U<0.001, N_{np}=6, N_p=6, p=0.002). There were no significant changes in the

expression levels of IL-1β (MWW test U=7.000, N_{np}=6, N_p=6, p=0.177) or IL-8 (MWW test U=7.000, N_{np}=6, N_p=6, p=0.093).

Analysis of the spleen tissue (Figure 20.8C) revealed a significant up-regulation of both IL-1β and IL-8, but not TNF-α (MWW test U=12.000, N_{np}=6, N_p=6, p=1.00). IL-1β expression was approximately 23 times greater (MWW test U<0.001, N_{np}=6, N_p=6, p=0.01) and IL-8 expression approximately 8 times greater (MWW test U<0.001, N_{np}=6, N_p=6, p=0.016) in the parasitized group compared to the non-parasitized group.

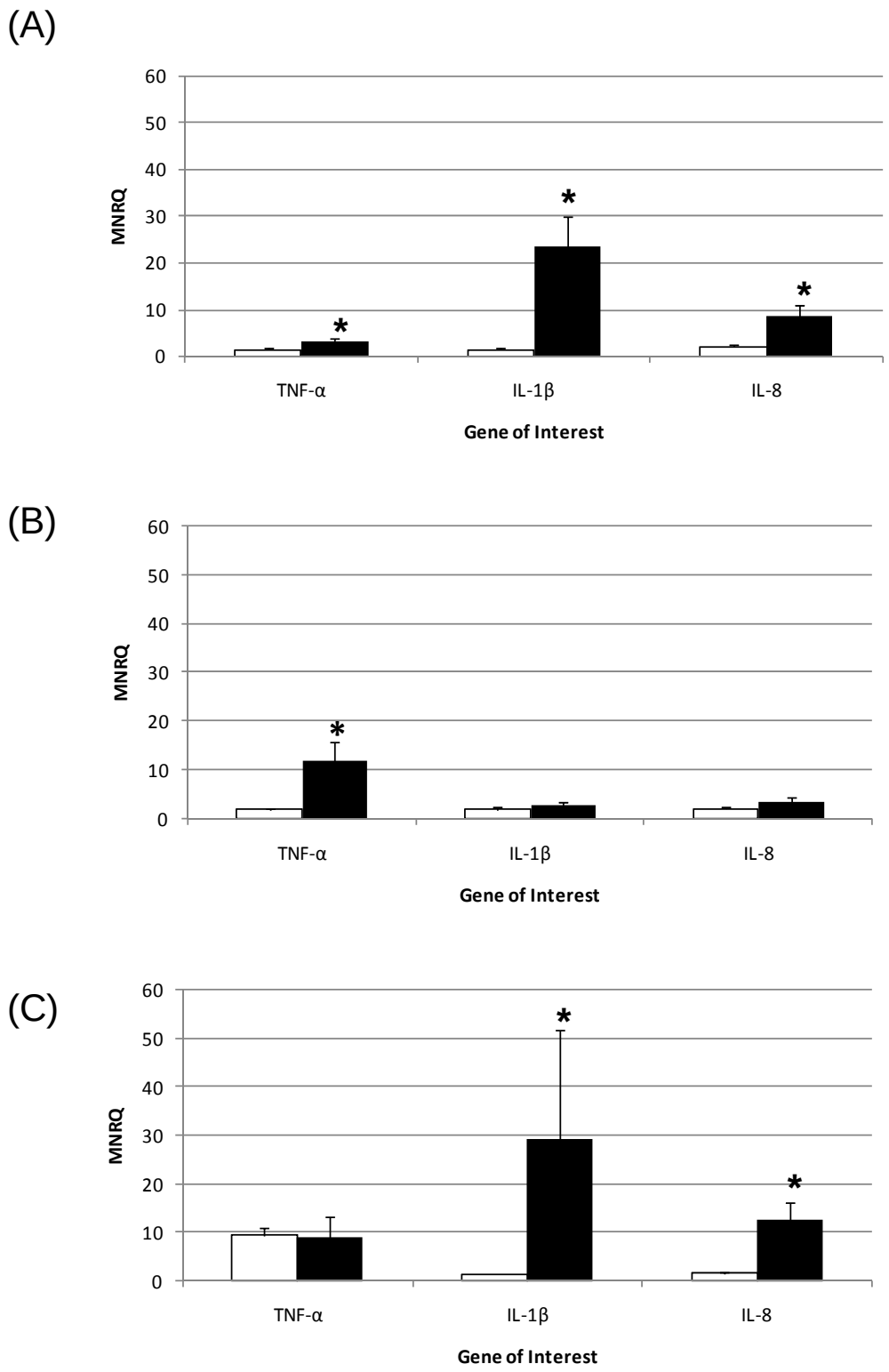

Figure 20.8 Relative expression of IL-1β, IL-8 and TNF-α mRNA in the (A) gill, (B) head kidney and (C) spleen of non-parasitized (□) and parasitized (■) striped trumpeter as analysed by quantitative real time PCR. The 3 different cytokine genes from each group are shown on the X-axis, with their mean normalized relative quantity (MNRQ) of mRNA expression against β-actin, EF-1 and RPS20 represented on the Y-axis. Bars indicate the mean ± standard error. * Denotes significant difference (MWW $p < 0.05$) from MNRQ of the control group.

20.4.5 *Histological analysis of parasitized and non-parasitized striped trumpeter*

Examination of histological slides revealed that the parasite-induced effects were localized to the gill. No differences were apparent in the head kidney and spleen of parasitized and non-parasitized fish. However, at the site of parasite attachment, the striped trumpeter showed a strong host response that manifested as epithelial cell hyperplasia and mucous cell proliferation (Figure 20.9 A and C). Closer examination showed an infiltration of neutrophil-like cells at the base of the gill filaments that were adjacent to the site of attachment (Figure 20.9B, D and E). Brain, liver and intestines were also examined histologically in both control and parasitized fish to rule out any concurrent infections.

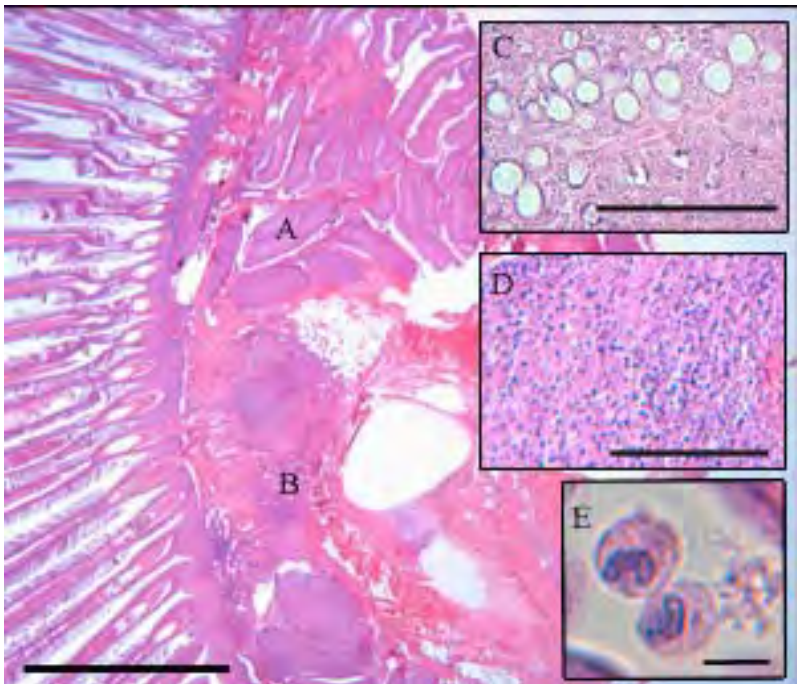

Figure 20.9 Hematoxylin and eosin stained histological section of *Chondracanthus goldsmidi* infected striped trumpeter gill tissue. Gill arch at site of parasite attachment showing epithelial and mucous cell hyperplasia. Scale bar = 2mm. Inset (C) is a magnified view of the area marked by (A), showing mucous cell proliferation. Scale bar = 100 μm. Inset (D) is a magnified view of the area marked by (B) showing the infiltration of inflammatory cells. Scale bar = 100 μm. Inset (E) is a high magnification view of the cell infiltrate (D) showing the presence of neutrophil-like cells. Scale bar = 5 μm.

20.5 Discussion

In this study, we cloned and sequenced the first cytokine genes from the striped trumpeter. In addition, the expression of the pro-inflammatory cytokines, TNF-α, IL-1β and IL-8, was analysed in response to infection by the ectoparasite C. goldsmidi. As the first response of the immune system upon injury or infection is inflammation, monitoring the expression levels of these three cytokines could give some insight into the timing and degree of the parasite-induced changes.

Primers designed to conserved regions of known perciform genes were initially used to clone and sequence fragments of striped trumpeter TNF-α, IL-1β and IL-8. Species specific

primers were then designed to obtain full-length sequences. The isolated striped trumpeter TNF-α cDNA translated into a 253 aa peptide of approximately 27.58 kDa. As with other TNF-α molecules in both mammals and teleosts, a transmembrane domain was predicted to be present and a TACE cleavage site was found after residue 86, between a threonine and leucine [19]. Cleavage at this site creates a 167 aa soluble, mature protein of approximately 18.26 kDa. Overall the TNF family signature is relatively well conserved when compared between vertebrate species. However, many piscine sequences differ in that they contain an isoleucine instead of a leucine or valine at position 1, an isoleucine instead of a valine at position 3 and a phenylalanine instead of a leucine at position 10. In addition, teleost sequences tend to have a phenylalanine at position 9 in place of a tyrosine [59]. The striped trumpeter molecule contains the piscine modifications to the family signature, but not the teleost modification. It does differ in one additional way in that there is an alanine at position 15, making the family signature [LVI] - x - [LIVM] - x(3) - G - [LIVMF] - Y - [LIVMFY](2) - x(2) - [QEKHL] - [LIVMGTA] - x - [LIVMFY]. Despite these changes, the striped trumpeter sequence is still recognized as belonging to the TNF superfamily. The striped trumpeter TNF-α showed good homology with other TNF-α genes, with the highest amino acid identity to sea bass (83.8%), a closely related perciform. Good amino acid homology was found not only within the TNF family signature, but also in the conservation of cysteine residues important for correct folding of the mature protein. The presence of these similarities across such a diverse group of species highlights the importance of the conserved regions to the correct functioning of the protein.

The coding sequence of the striped trumpeter IL-1β translated into a 258 aa peptide that contained a clearly identifiable family signature. The conventional IL-1 family signature has been previously modified to include trout [37], seabass [34] and haddock [27]. If the striped trumpeter is to fit the IL-1 family signature, the motif must be further modified so that only 6 amino acids occur after position 13, not 7, to give [FCL] - x - S - [ASLV] - x(2) – [PRS] - x(2) - [FYLIV] - [LI] - [SCAT] - T - x(6,7,9) - [LIVM]. As with all other known teleost sequences, neither an ICE cleavage site, nor a signal peptide was present. The striped trumpeter sequence showed good homology with other IL-1β genes, with the highest amino acid identity to the closely related perciform, the Chinese perch (74.9%). In addition, areas of high conservation were seen within the family signature and the 12 β-sheets, characteristic of IL-1β molecules. Again, these similarities are conserved across a diverse group of species, highlighting their importance to the function of IL-1β.

The coding sequence of striped trumpeter IL-8 translated into a 99 aa peptide with a putative signal peptide of 23 aa, giving a soluble, mature protein of 76 aa that weighed approximately 8.65 kDa. The mature protein contained a slightly modified CXC chemokine family signature, making the consensus signature C - x - C - [LIVMS] - x(4,6) - [LIVMFY] - x(1,2) - [RKSEQNA] - x - [LIVM] - x(2) - [LIVMA] - x(5) - [STAGN] - x(2) - C - x(3) - [EQ] - [LIVM](2) - x(9,10) - C - [LV] - [DN]. Multiple alignment revealed areas of good homology, which included the conservation of the four cysteines, that are important in disulphide bond formation [38]. In contrast to mammalian IL-8 sequences, and all teleost sequences apart from cod and haddock, the striped trumpeter lacks an ELR motif. Instead, the striped trumpeter contains an ELH tri-peptide prior to the CXC residues that initiate the family signature. In mammals, the ELR motif is thought to play a crucial role in the effective functioning of IL-8. In fact, a change in any of the 3 amino acids leads to a dramatic decrease in the functionality of IL-8 [39]. This motif appears to be less crucial in teleosts, with ELR motif-deficient recombinant rainbow trout IL-8 acting in the predicted biological manner [60]. Therefore, it is expected that the lack of a complete ELR motif will not impair the function of striped trumpeter IL-8.

Damage to host fish via parasitic infection has become a subject of increasing focus over the past several years, as there are many teleost ectoparasites that infect both wild and farmed fish the world over. Possibly the most intensely studied are the effects of sea lice from the genus Lepeoptheirus on salmonids [61, 62]. As with sea lice species, the recently described C. goldsmidi causes host damage through both attachment and feeding. The adult stage of C. goldsmidi is sedentary and in constant contact with host tissue. It is likely that the perpetual movement of the parasite's appendages irritate the surrounding gill and operculum tissue resulting in epithelial cell hyperplasia and increased numbers of mucous cells [63]. However, it is likely that these parasite induced changes vary depending on the site and duration of attachment.

To determine the striped trumpeter's response to C. goldsmidi infection and the damage caused by this parasite, the expression of TNF-α, IL-1β and IL-8 was investigated. A significant up-regulation of TNF-α, IL-1β and IL-8 was seen at the local level within the gill. Regulation of these cytokines at a systemic level was less well defined with head kidney cells showing an up-regulation of TNF-α only, and the spleen cells showing an up-regulation of both IL-1β and IL-8. These results are to some extent consistent with previous studies investigating the host response to ectoparasites, where there is a trend towards a stronger localized pro-inflammatory response at the site of infection or injury [64-66], and a less well defined systemic response [61, 62, 67]. In addition, histological analysis of the site of parasite attachment in the striped trumpeter gill revealed both a hyperplasic thickening of the surrounding tissue as well as an increase in mucous cell numbers, with a substantial infiltration of neutrophil-like cells observed adjacent to the site of attachment. Both the histology and the significant local up-regulation of the pro-inflammatory cytokines would indicate a local inflammatory response was occurring within the gills. No histological changes were observed within the spleen or head kidney despite the significant up-regulation of pro-inflammatory cytokine(s) in these organs.

Previous studies dealing with changes in gene expression in response to ectoparasite infection differ depending on both the host and the parasite species. Perhaps more importantly, they may also vary depending on the amount of damage caused to the host. In a study involving the infection of Atlantic salmon with Lepeoptheirus salmonis, an initial systemic inflammatory reaction was detected, but subsequently resolved within 2 weeks [61]. The authors suggest that the resolution occurred because early life stages of the louse do not cause significant damage to the host. As it is difficult to separate the extent of the pro-inflammatory response that is elicited by the parasite as opposed to the damage the parasite is causing, it is possible that a certain level of purely mechanical damage needs to occur before a pro-inflammatory response is mounted. Therefore, an up-regulation of pro-inflammatory cytokines may be more dependent upon tissue damage than on the source of that damage. A study by Gonzalez et al., [65] showed that mechanical injury, mimicking that caused by an ectoparasitic infection, was enough to trigger a pro-inflammatory response, even in the absence of the parasite. In the study by Fast et al., [61], as the infection progressed, the mobile, adult stages of the parasite elicited a second inflammatory response, which was sustained. The authors speculated that the prolonged response may be a result of the host's inability to clear the parasite. Given the results obtained from the Gonzalez et al., [65] injury mimicking experiment, it follows that an established ectoparasite infection leads to increased host damage which in turn elicits a pro-inflammatory response from the host.

The length of infection in the present study was likely much longer and more chronic than in most other published work, which may have contributed to the systemic up-regulation of the

pro-inflammatory cytokines. In most previous studies, the fish were infected for no longer that 4-6 weeks before end point samples were taken. The fish analysed in this study had been naturally exposed to the parasite for approximately 10 months. This prolonged exposure and/or re-current infection, taken together with its resultant host damage, may explain the systemic up-regulation of the cytokines observed in association with the more complete local response. An additional contributing factor that must be considered is the extent of the local infection. When parasitized areas of gill tissue were compared to non-parasitized areas from the same individual, no differences in cytokine expression levels were seen. This indicates that although some areas of the gill were not directly parasitized, they still underwent an up-regulation of pro-inflammatory cytokines. This is in contrast to similar studies on amoebic gill disease (AGD) where non-parasitized areas of the gill were seen to have cytokine expression levels similar to those of control tissue from non-parasitized individuals [68]. Again, this may be related to the degree of damage inflicted by the parasite, as lesion-associated amoeba induce less histologically identifiable damage than *C. goldsmidi*.

20.6 Conclusions

The cloning of three important pro-inflammatory cytokines in the striped trumpeter allowed investigation of their differential expression in response to an ectoparasitic infection. The strong local inflammatory reaction coupled with a less well defined systemic response, may be the result of a number of contributing factors, including the duration of infection, level of host damage and extent of the local response. As this investigation focused on the up-regulation of cytokines at the mRNA level, it is unknown if these increases were translated to the protein level. Additional work focusing on this, as well as the regulation of the cytokine-associated receptors, would give a more complete view of the biological significance of such up-regulation

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21 IDENTIFICATION AND CONTROL OF PARASITES IN A NEW SPECIES FOR AQUACULTURE: A CASE STUDY WITH STRIPED TRUMPETER.

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21.1 Abstract

This story takes place in Tasmania, but where exactly is Tasmania? It is Australia's southern most island state situated directly on the 'roaring forties', which has a very changeable weather pattern throughout the year with snowfalls not uncommon in summer. This cool to mild climate is ideal for temperate agriculture (stone- and deciduous fruit) and aquaculture. The Tasmanian aquaculture industry began in the mid 1940's with the cultivation of Pacific oysters. It has since expanded to include other shellfish, such as abalone, scallops and mussels. Today the largest Tasmanian aquaculture industry involves the culture of over 18,000 t of Atlantic salmon per year, which are farmed in sea cages. Since the beginning, the salmon industry has experienced various health challenges, including amoebic gill disease (AGD), which is caused by amoebic protozoans. Treatment of this disease using freshwater is relatively simple but expensive and progress is being made in the development of a vaccine. Atlantic salmon are also reared in sea temperatures close to their thermal maxima in late summer and it makes sense for the industry to diversify into other marine species. The industry is particularly interested in developing an alternative native white-fleshed fish species. Striped trumpeter, *Latris lineata*, was identified in the 1980's as a possible aquaculture species because of its tolerance to handling, high stocking density as well as the superior flesh quality, which is high in omega fatty acids and highly regarded as sashimi (Nichols *et al.* 2005).

21.2 Striped Trumpeter: an Overview

Striped trumpeter are a very interesting species with an unusual life history that includes a protracted post-larval stage and, until very recently, little was known of their biology (Tracey and Lyle 2005). They used to be plentiful; however, the population is currently depleted because of the combined effects of fishing and several years of poor recruitment. The wild population will not be able to support higher levels of catch, therefore, aquaculture is seen as the best option to provide striped trumpeter in greater quantity. The species is common around Tasmania, the more southern waters of mainland Australia and around New Zealand and some islands in the Indian Ocean. They are typically found in water ranging between 11 and 18° C and spawn in Tasmanian waters between July and October, depending on latitude (Tracy and Lyle 2005). Adults live in deep water and can reach 1.2m in length with sexual maturity reached at 5-6 years of age (Figure 21.1).

Figure 21.1 Adult striped trumpeter at the Marine Research Laboratories, Tasmania.

Research into the culture of striped trumpeter started in earnest in the 1990's with early emphasis being placed on the reproductive cycle, including broodstock management and egg incubation. Another very important aspect of the project was to develop techniques to improve the survival and quality of the early life stages by developing more efficient hatchery and incubation methods (Morehead and Hart 2003). Despite much research, the species has proven difficult to culture and only recently has reliable hatchery production been achieved following extensive research into nutrition and control of the bacterial environment (Bransden *et al.* 2005, Battaglene *et al.* 2006). These studies have helped overcome many of the problems experienced in larval rearing and the first sea cage trials will take place in the near future.

21.3 Aquaculture and the Problem of Diseases and Parasites

It is a widely accepted that diseases and parasites pose a major problem to aquaculture, with a variety of cultivated marine and freshwater species being affected globally. Examples of problem parasites are the copepods affecting the culture of Atlantic salmon and sea trout in the northern hemisphere, AGD in Atlantic salmon in Australia and the monogenean that affects the tiger puffer in Japan (Hirazawa *et al.* 2003). Emerging new species are particularly vulnerable as can be seen with yellowtail kingfish *Seriola lalandi* in Australia that are affected by the monogenean *Benedenia seriolae*, which causes reduced growth, carcass quality and increased mortalities (Chambers and Ernst 2005). Another new species candidate cultured in Australia is the mulloway, *Argyrosomus japonicus*, which is parasitized by monogeneans and ciliated protozoans.

So the research team working on striped trumpeter are only too aware that when they get their prized juveniles into sea cages the next big challenge is going to be how to keep them healthy. To date one parasite, a myxozoan *Kudoa neurophila* (Grossel *et al.* 2003) has been identified as a problem to the culture of striped trumpeter. This parasite targets the tissues in the central nervous system, which results in behavioral changes and is a possible cause of spinal deformities (Grossel *et al.* 2003, Grossel *et al.* 2005), many infections result in a reduction in flesh quality and, therefore, have potential to cause major losses to the industry. Good detection methods are available, including a polymerase chain reaction (PCR), which is useful in the detection of the parasite in the early stages of the host's life cycle (Grossel 2005). *Kudoa neurophila* appears to be only a problem with larvae and post-larvae but it

remains to be seen if fully scaled juveniles are resistant when kept in high densities. Bacteria are another common problem in most hatchery-reared marine finfish, and striped trumpeter are no different. Both problems have been effectively resolved by treating the hatchery water supply with ozone, this has decreased the bacterial problem and the incidence and severity of infection by the *Kudoa* parasites (Smith *et al*. 2006).

21.4 Striped Trumpeter and Its Uninvited Guests

Very little work has been conducted on the metazoan parasites of wild striped trumpeter outside two surveys off the New Zealand coast (Hewitt and Hine 1972, Hine *et al*. 2000). During these surveys four species of parasite were collected, including two nematode gut parasites and two monogenean gill parasites. So the discovery of two copepod parasite species on cultured striped trumpeter at the Marine Research Laboratories was of considerable interest. The first copepod (Figure 21.2) is a previously undescribed *Chondracanthus* species, no species belonging to this genus has ever before been recorded from any cultured fish species. The parasite attaches to the hosts gills, operculum and nasal cavities (Figure 21.3), where their movement and feeding activity irritate the host tissue, which then becomes swollen and enflamed.

Figure 21.2 Adult *Chondracanthus* sp. parasitising the gills of the striped trumpeter.

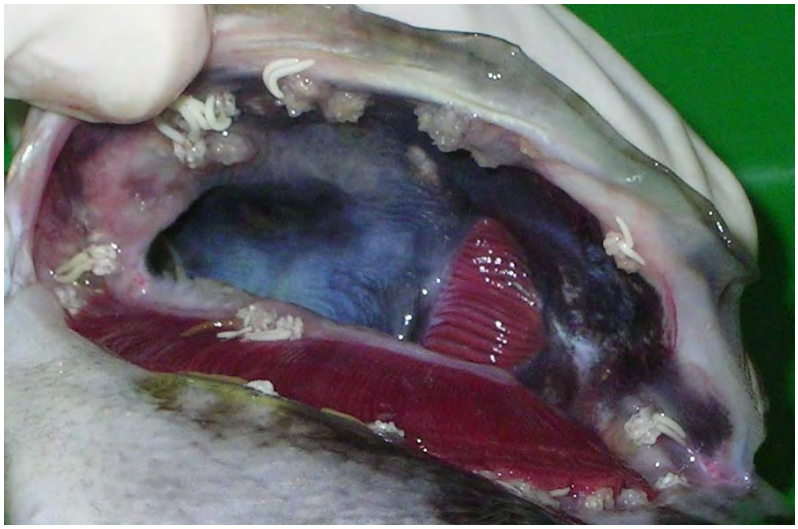

Figure 21.3 Gills and operculum of a striped trumpeter parasitised by the *Chondracanthus* sp.

The second species (Figure 21.4) found on the striped trumpeter belongs to the genus *Caligus*, which is one of the most common groups causing problems in aquaculture. This group includes *Lepeophtheirus salmonis* Krøyer 1838 and *Caligus elongatus* Nordmann 1832, both of which cause major problems in the culture of Atlantic salmon in the northern hemisphere (Costello *et al.* 2004). The new species occurs on the skin where its movement and feeding activity cause discomfort to the fish host, especially when they are found in high numbers. The host tries to relieve its discomfort by rubbing and this causes the formation of lesions, which are then susceptible to secondary infections. Although the species found on striped trumpeter belong to the genus Caligus, 20 years of farming salmonids in Tasmania suggests it does not parasitize Atlantic salmon.

Figure 21.4 Adult *Caligus* sp. parasitising the skin of the striped trumpeter

21.5 Who are the Guests and How Do They Develop?

The discovery of new parasites on striped trumpeter has prompted a variety of descriptive studies. These include detailed descriptions of the male and female for both copepod species. This is a vital step that will aid in future identification if they are ever found parasitizing other fish species. We have also begun to describe their various life stages. Many descriptive studies have been conducted on the developmental stages of various *Caligus* species (MacKinnon and Piasecki1992, Piasecki and MacKinnon 1995), whereas only one study has been performed on those of the *Chondracanthus* species (Izawa 1986). During our study we collect eggs from gravid females, hatch them using water baths and describe each developmental stage as they occur (Figure 21.5). This will allow us to predict the length of the developmental stages, which will assist us in developing effective treatments and preventing or controlling future outbreaks.

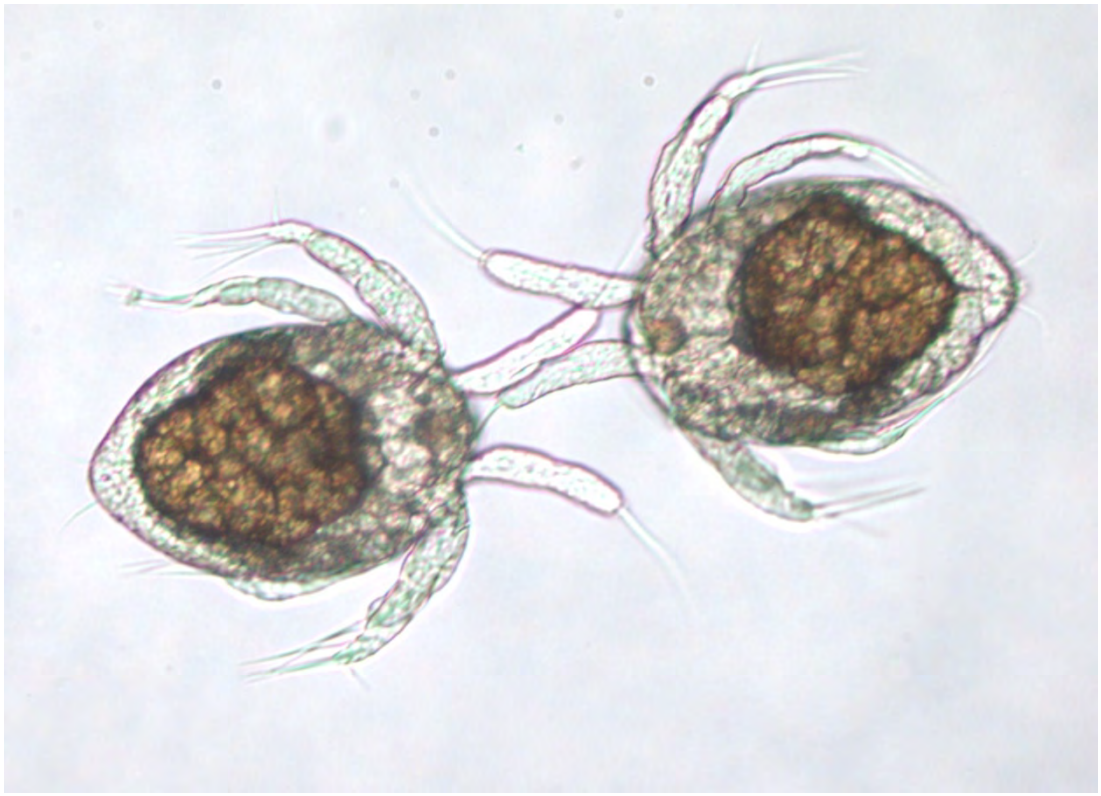

Figure 21.5 Nauplii larval stage of the *Chondracanthus* sp. parasitising the striped trumpeter

21.6 How Will We Control Them?

Many studies have been conducted on the development of treatment and control methods to reduce the impact that parasites have on worldwide aquaculture (Raynard *et al.* 2002, Treasurer 2002, Pietrak and Opitz 2004). Thankfully, neither parasite affecting the striped trumpeter is a major problem when they occur in low numbers, they only become a nuisance when their numbers increase rapidly (Figure 21.3). We will monitor parasite numbers to determine if they are affected by water temperature. Even though the project is in its first year one can see that there is a correlation between the chondracanthid parasite numbers and water temperatures (Figure 21.6). In the case of the striped trumpeter, the lack of knowledge of the parasites affecting this species means that there are no treatments in place if the parasite loads increase. This is why it is vital that we use the knowledge gained by studying the developmental stages to identify those most susceptible to treatment. With the planned start of sea cage trials, there is a need for treatments that are easy, cost-efficient and effective as well as being environmentally sustainable and having minimal detrimental effects on the host. During this study we will examine the effects of freshwater, hydrogen peroxide and Neguvon® treatments on both copepod parasites at a number of concentrations and treatment times. We will conduct *in vitro* trials on the developmental stages for both parasites and determine the effect treatments have on parasite viability. *In vitro* treatment trials for the adult *Caligus* sp. will be conducted, with the most effective treatment then being used to treat parasitized fish. Due to the method of attachment used by the *Chondracanthus* sp. *in vitro* trials aren't possible, therefore, all treatment trials for the adult parasites will be conducted on parasitized fish. Many of the potential treatments to control parasite infections in aquaculture can be quite harmful to the environment. This is why we

hope to develop one that will have minimal impact and could possibly replace other, more harmful, treatment methods.

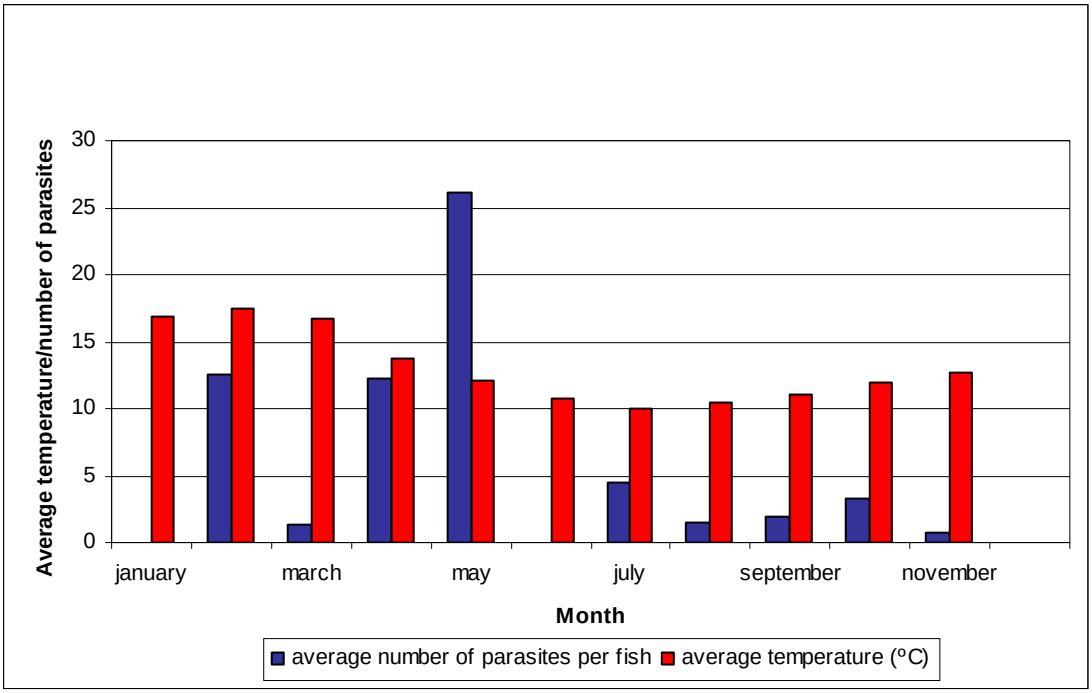

Figure 21.6 Average number of chondracanthid parasites per fish compared to the average ambient temperatures.

21.7 Conclusion

Many parasites pose a considerable threat to aquaculture. The discovery of two previously undescribed copepod species on striped trumpeter is a potential challenge to the successful sea cage culture trials planned for 2007. With both parasites now fully described and their developmental stages being properly identified, we can concentrate on the development of control and treatment methods. We hope to eventually demystify general opinions regarding parasites by increasing our knowledge of their biology and effective control methods.

21.8 Acknowledgments

We gratefully acknowledge the excellent technical assistance of Alan Beech, Ross Goldsmid, Anna Overweter, Gavin Shaw and Bill Wilkinson. Experiments were conducted in accordance with University of Tasmania Animal Ethics approval A0008975. The work forms part of a project of the Aquafin CRC, and received funds from the Australian Government's CRC Program, and the Fisheries Research and Development Corporation and other CRC Participants.

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22 A NEW SPECIES OF COPEPOD (SIPHONOSTOMATOIDA: CALIGIDAE) PARASITIC ON THE STRIPED TRUMPETER, *LATRIS LINEATA* (FORSTER), FROM TASMANIA

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22.1 Abstract

A new species of caligid copepod (Siphonostomatoida), *Caligus nuenonnae* n. sp., is described based on material collected from the body surface of striped trumpeter [*Latris lineata* (Forster)] reared at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories, Australia. *Caligus. nuenonnae* n. sp. is characterised by the following combination of features: 1) female genital complex with a mid-lateral indentation and highly concave posterior margin; 2) 1-segmented abdomen in the female that is about one-third the length of the genital complex; 3) distinctly broader first abdominal somite relative to the second abdominal somite in the male; 4) antenna with a spatulate process on the proximal segment; 5) recurved postantennal process without a basal accessory process; 6) female maxilliped with a proximal ridged protrusion on the corpus; 7) sternal furca with widely separated, apically truncate tines; 8) distal exopodal segment of leg 1 with a lateral flange on each apical spine and an accessory process on apical spines two and three; 9) leg 3 protopod with two adhesion pads on the dorsolateral surface; 10) leg 4 exopod 2-segmented, with I-0; I, III armature; 11) terminal exopodal segment of leg 4 with the outer apical spine being slightly shorter than the middle apical spine; 12) male maxillulary dentiform process with numerous small points embossed on the surface; and 13) male legs 5 and 6 represented by distinct lobate projections.

Key words: *Caligus*, parasitic copepods, marine fish, Australia

22.2 Introduction

Striped trumpeter, *Latris lineata* (Forster), has recently been identified as a future aquaculture species in Australia, with progress being made in the areas of broodstock and hatchery management, egg incubation and larval rearing at the Tasmanian Aquaculture and

Fisheries Institute (TAFI), Marine Research Laboratories, Hobart, Australia (Morehead & Hart 2003; Bransden *et al*. 2005; Battaglene *et al*. 2006; Battaglene & Cobcroft 2007). Nevertheless, knowledge of the parasites and diseases affecting this fish in captivity is relatively limited (Grossel *et al*. 2005). Two parasite species, namely the myxozoan *Kudoa neurophila* (Grossel, Dyková, Handlinger & Munday, 2003) and the copepod *Chondracanthus goldsmidi* Tang, Andrews & Cobcroft, 2007, have been reported thus far from cultured striped trumpeter. Both parasites have been associated with host mortality: *K. neurophila* infects the host's central nervous system, which disrupts the host's swimming and feeding behaviour; the presence of large numbers of *C. goldsmidi* on the gills and inner opercula of striped trumpeter juveniles has been linked to host mortality (Grossel *et al.* 2003; Grossel 2005; Tang *et al.* 2007). Ongoing parasite monitoring of striped trumpeter cultured at TAFI recently revealed numerous individuals of a *Caligus* Müller, 1785 species attached to the body surface of this marine fish. Of the 32 valid *Caligus* species collected from Australian waters (Heegaard 1962; Kabata 1965; Hewitt 1971; Roubal 1981; Roubal *et al*. 1983; Byrnes 1987; Tang & Newbound 2004; Hutson *et al*. 2007; Hayward *et al.* 2008), none bear a resemblance to the species infecting striped trumpeter. Indeed, further comparisons with other congeners revealed that this caligid copepod represents a species new to science. The description of this new *Caligus* species provided herein is important, as a number of caligid copepod species are known to reduce growth rates, reduce the marketability and cause mortality of cultured marine fishes elsewhere (Lin *et al.* 1994; Gonzalez *et al.* 2000; Bravo 2003).

22.3 Materials and Methods

Captive striped trumpeter were removed from rearing tanks at the TAFI, Marine Research Laboratories, anaesthetised with 2-phenoxyethanol at a concentration of 0.2 ml/L and examined for attached copepods. Copepods were removed from the host using fine forceps and preserved in 70% ethanol. Sixteen male and 13 female specimens were cleared in lactic acid for 24 h for further examination. Measurements were made using an ocular micrometer and are given in millimetres as the range followed by the mean in parentheses. Selected specimens were dissected using the wooden slide procedure of Humes & Gooding (1964). Drawings were made using a drawing tube attached to a Wild M5 dissection microscope, with additional details incorporated from images taken from a Nikon Optiphot 2 compound microscope fitted with an Insight Spot 4MP digital camera. All type material was deposited at the Queensland Museum (QM), Brisbane, Australia.
PCR products were purified using Macherey-Nagel NucleoSpin® kit (Macherey-Nagel GmbH, Düren, Germany) and cycle-sequenced from both strands using ABI BigDye™ Version 3.1 (Applied Biosystems, Foster City, USA) chemistry, alcohol precipitated and automated sequences obtained. The contiguous sequences were aligned using Sequencher™ (GeneCodes Corp., Ann Arbor, U. S. A. ver. 4.2). Sequences were aligned using ClustalX (Thompson *et al.* 1997) and further refined by eye. The newly obtained sequence was added to Genbank (http://www.ncbi.nlm.nih.gov/Genbank/) under the accession number EF452642.

22.4 Results

Siphonostomatoida Burmeister, 1835
Caligidae Burmeister, 1835
***Caligus* Müller, 1785**

***Caligus nuenonnae* n. sp.**
(Figure 22.1-Figure 22.3)

Type material. Holotype f# (QM W28171), allotype m# (QM W28172) and 4 paratypes (2m# and 2f#) (QM W28173), ex body surface of *Latris lineata* (Forster) reared at the TAFI Marine Research Laboratories, Tasmania, Australia (43°35'S, 147°35'E), 10 October, 2006, leg. M. Andrews.

Other material examined. 15 m# and 15 f#, ex body surface of *L. lineata* reared at the TAFI Marine Research Laboratories, Tasmania, Australia, 10 October, 2006, leg. M. Andrews.

Description of female. Body as in Figure 22.1. Total length (excluding setae on caudal rami) 4.27–4.82 (4.56), based on 9 specimens. Cephalothoracic shield ovate, marginally wider than long, 2.58–2.88 (2.75) × 2.62–2.94 (2.79) (excluding marginal membrane). Posterior margin of thoracic zone extends past lateral zone. Fourth pedigerous somite wider than long, 0.38–0.58 (0.47) × 0.40–0.64 (0.54). Genital complex indented laterally, almost equal in length and width, 1.29–1.58 (1.43) × 1.29–1.60 (1.46). Dorsal and ventral surfaces with lateral setules; posterior margin concave. Abdomen (Figure 22.1B) 1-segmented, almost equal in length and width, 0.44–0.46 (0.45) × 0.45–0.47 (0.46); with 10 setules on dorsal surface. Caudal ramus (Figure 22.1B) slightly longer than wide, 0.06–0.08 (0.07) × 0.05–0.06 (0.06); with row of setules along medial margin, 1 dorsal setule, and 3 long and 3 short pinnate setae.

Antennule (Figure 22.1C) 2-segmented; proximal segment with 27 setae (18 plumose, 9 naked); distal segment slightly shorter than proximal segment, with 1 subterminal seta on posterior margin, 11 apical setae and 2 apical aesthetascs. Antenna (Figure 22.1D) 3-segmented; proximal segment with large spatulate process; middle segment with adhesion pad on dorsal surface; distal segment uncinate, longer than middle segment, with 1 short proximal seta and 1 long seta at mid-length of claw. Postantennal process (Figure 22.1E) slender, distally curved, pointed at tip, with proximal pair of bisetose papillae and 1 bisetose papilla alongside process. Mandible (Figure 22.1F) modified into stylet, with 12 teeth on medial margin of apex. Maxillule (Figure 22.1G) reduced, with 3 unequal setae anteriorly and slender, bluntly pointed dentiform process posteriorly. Maxilla (Figure 22.1H) 2-segmented, brachiform; lacertus unarmed, broader than brachium; brachium elongate, with triangular flabellum at mid-length and 2 unequal apical elements. Short canna with serrate marginal membranes; longer calamus with smooth marginal membranes. Corpus of maxilliped (Figure 22.1I) large, with proximal ridged protrusion on posterior surface. Subchela with relatively long, straight shaft and sharply pointed claw; shaft with distal hyaline element; claw with basal setiform barb. Sternal furca (Figure 22.1J) with broad box; tines slender, widely separated, apically truncate.

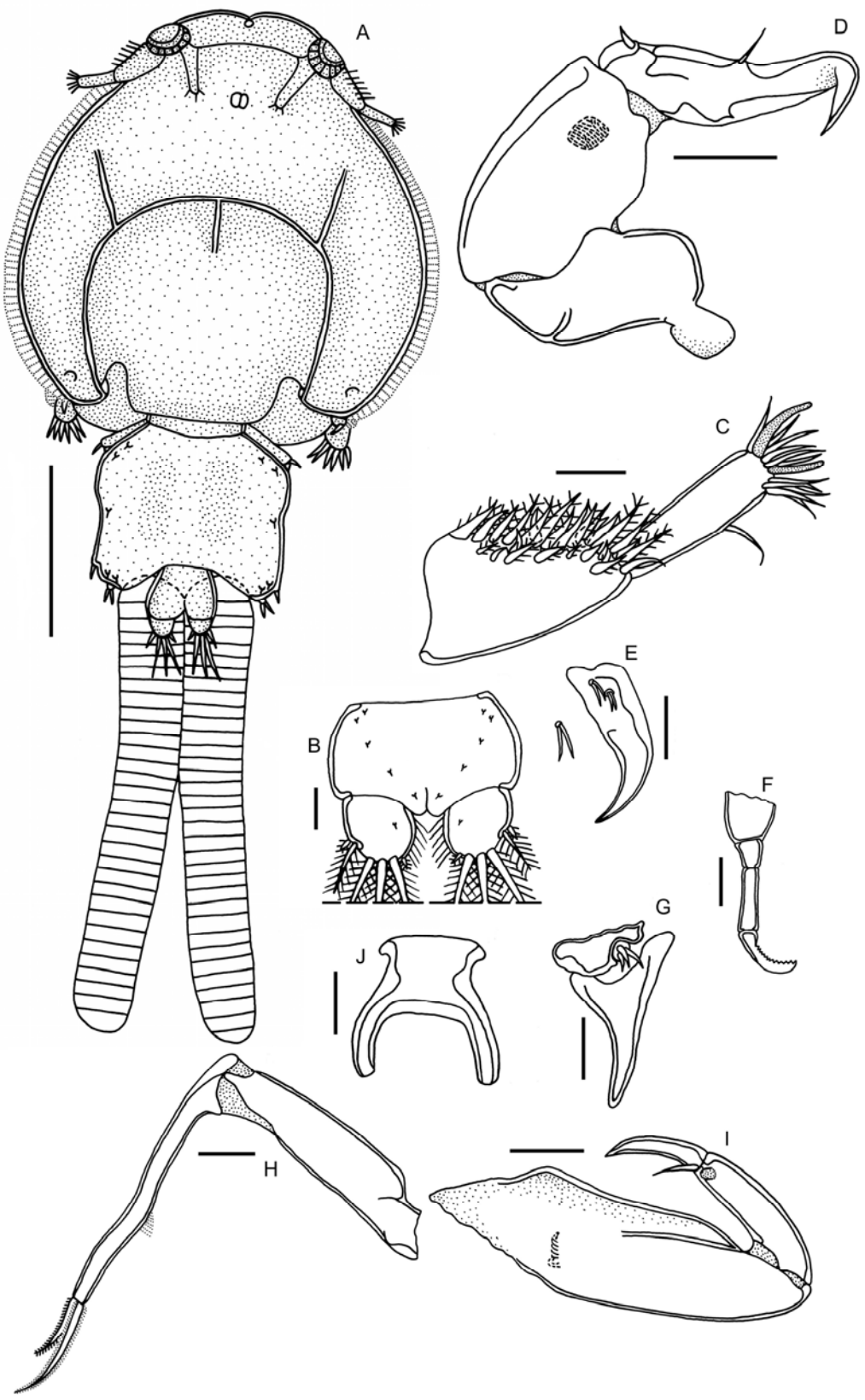

Figure 22.1 *Caligus nuenonnae* n. sp., adult female. (A) habitus, dorsal; (B) abdomen and caudal rami, dorsal; (C) antennule, ventral; (D) antenna, ventral; (E) postantennal process; (F) mandible; (G) maxillule; (H) maxilla; (I) maxilliped; (J) sternal furca. Scale bars: A, 1.00 mm; B–D, H–I, 100 μm; E–G, J, 50 μm.

Armature on rami of legs 1–4 (Figure 22.2A–D) as follows (Roman numerals indicating spines and Arabic numerals setae):

	Exopod	Endopod
Leg 1	I-0; III, 1, 3	vestigial
Leg 2	I-1; I-1; II, 6	0-1; 0-2; 6
Leg 3	I-0; I-1; III, 4	0-1; 6
Leg 4	I-0; I, III	absent

Leg 1 (Figure 22.2A) protopod with 1 outer setule, 1 anterolateral seta, and 1 distal seta. Endopod vestigial, represented by unarmed lobe near joint of sympod and first exopodal segment. First exopodal segment with row of setules along posterior margin and small anterodistal spine. Second exopodal segment with 3 large apical spines (each with lateral flange; inner 2 with secondary process), 1 long apical seta, and 3 long inner setae (each seta with denser row of setules along outer margin).

Leg 2 (Figure 22.2B) coxa with striated membrane on posterior margin, 1 inner setule and 1 large inner plumose seta. Basis with striated membrane on posterior margin, outer naked seta, and small inner setule. First and second exopodal segments with striated membrane on lateral margin. Spine on first exopodal segment with flange on both margins and extends beyond medial margin of second exopodal segment. Spine on second exopodal segment with flange on lateral margin and extends to medial margin of third exopodal segment. Distal exopodal segment bears 1 small and 1 large lateral spine (latter with flange on medial margin) and 6 inner plumose setae. All exopodal segments with row of setules along medial margin; all endopodal segments with row of setules along lateral margin; second endopodal segment with row of setules along medial margin.

Leg 3 (Figure 22.2C) protopod with 2 small adhesion pads on dorsolateral surface (indicated by arrows), striated membrane on lateral and medial margins, 1 short pinnate seta on posterior surface, 1 small setule, and 1 plumose inner seta. First exopodal segment with striated membrane along lateral margin, 1 ventral setule, and 1 large naked spine. Second exopodal segment with row of setules along both margins. Terminal exopodal segment with row of setules along lateral margin. First endopodal segment expanded laterally into velum, with one inner plumose seta. Second endopodal segment with row of setules along lateral margin.

Leg 4 (Figure 22.2D) protopod with plumose outer seta. First exopodal segment with 1 naked distolateral spine. Second exopodal segment with 1 outer, laterally flanged spine situated ⅔ along length of segment and 3 apical, flanged spines; small pectinate membrane present at base of outer spine and apical spines 1 and 3; innermost apical spine about twice as long as middle and outermost apical spines.

Leg 5 (Figure 22.2E) highly reduced, represented by 2 small lobes on genital complex; anterior lobe with 1 pinnate seta; posterior lobe with 2 unequal pinnate setae.

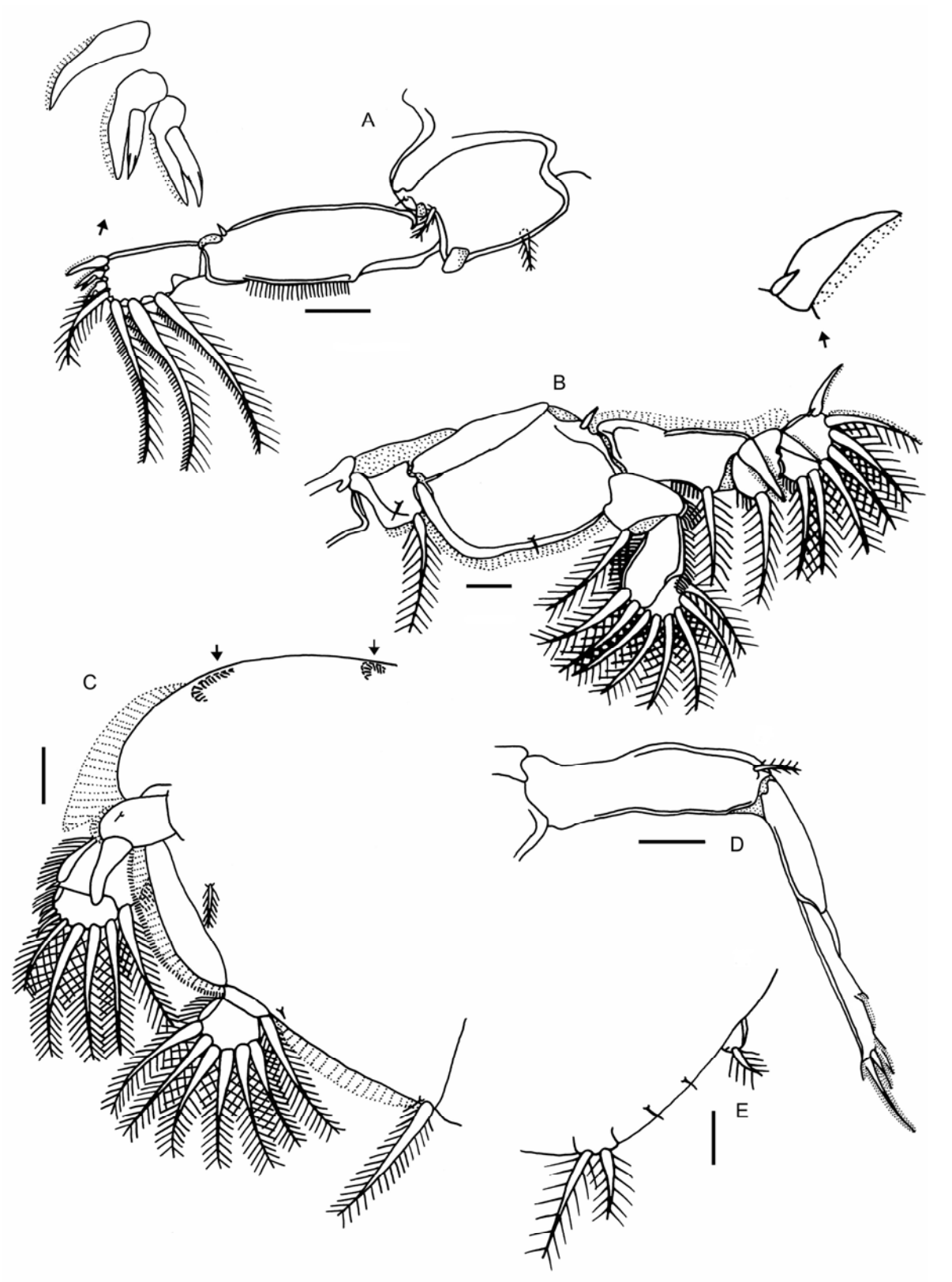

Figure 22.2 *Caligus nuenonnae* n. sp., adult female. (A) leg 1, with enlarged view of apical spines on distal exopodal segment, ventral; (B) leg 2, with enlarged view of outer spines on distal exopodal segment, ventral; (C) leg 3 (arrowheads indicate dorsal adhesion pads), ventral; (D) leg 4, ventral; (E) leg 5, ventral. Scale bars: A–D, 100 μm; E, 50 μm.

Male. Body as in Figure 22.3A. Total length (excluding seta on caudal rami) 3.99–5.26 (4.58), based on 9 specimens. Cephalothoracic shield ovate, marginally longer than wide, 2.56–3.20 (2.82) × 2.38–3.24 (2.70) (excluding marginal membrane). Thoracic zone extends past posterior margins of lateral zones. Fourth pedigerous somite slightly wider than long, 0.33–0.52 (0.42) × 0.43–0.58 (0.51). Genital complex laterally indented, longer than wide, 0.91–1.38 (1.17) × 0.84–1.17 (0.98); with 10 setules on ventral surface.

Abdomen (Figure 22.3B) 2-segmented, both segments wider than long [proximal segment 0.17–0.21 (0.19) × 0.40–0.56 (0.48); distal segment 0.25–0.36 (0.31) × 0.38–0.47 (0.43)]; with 8 setules on dorsal surface. Caudal ramus (Figure 22.3B) longer than wide, 0.075–0.087 (0.080) × 0.055–0.063 (0.059); with row of setules along medial margin, 1 dorsal setule, and 3 long and 3 short pinnate setae.

Antennule (Figure 22.3C) 2-segmented; proximal segment with 29 setae (22 plumose, 7 naked); distal segment longer than that of female, with 1 subterminal seta on posterior margin, 11 apical setae, and 2 apical aesthetascs. Antenna (Figure 22.3D) 3-segmented; proximal segment with large adhesion pad; second segment with 3 corrugated adhesion pads (2 dorsal, 1 ventral); distal segment uncinate, with 1 large accessory process and 2 setae near apex. Postantennal process slender, without papillae. Dentiform process of maxillule (Figure 22.3E) blunt, with numerous small points embossed on medial and apical surfaces. Corpus of maxilliped (Figure 22.3F) broad, with corrugated area on proximolateral margin, 3 large medial processes on myxal region, and 3 small proximal processes on anteromedial surface; subchela with relatively long, curved shaft. Legs 5 and 6 (Figure 22.3G) highly reduced, each represented by lobiform projection on genital complex; leg 5 with 1 small lateral seta and 2 apical pinnate setae; leg 6 larger than leg 5, with 2 apical pinnate setae.

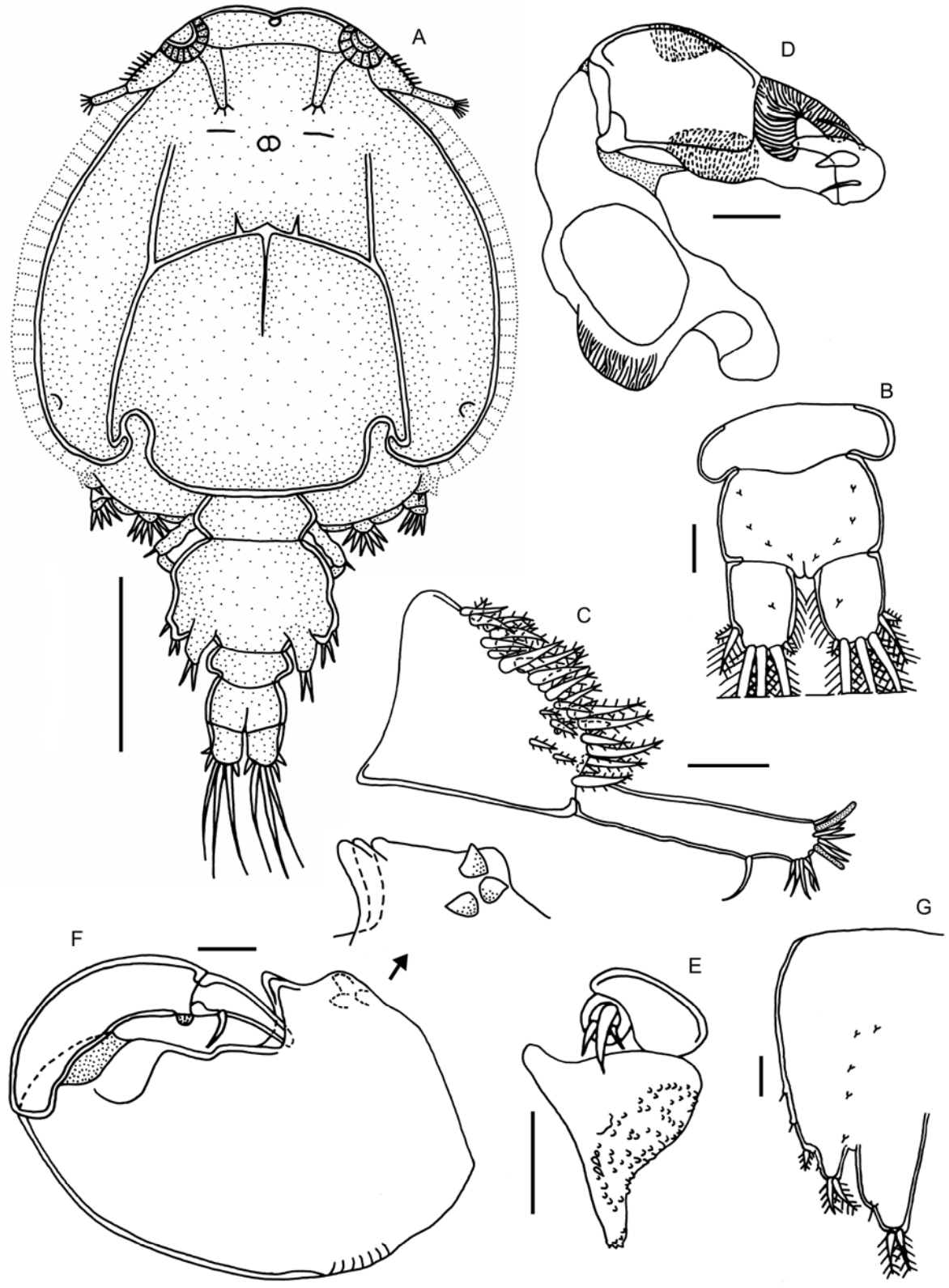

Figure 22.3. *Caligus nuenonnae* n. sp., adult male. (A) habitus, dorsal; (B) abdomen and caudal rami, dorsal; (C) antennule, ventral; (D) antenna, ventral; (E) maxillule; (F) maxilliped, with enlarged dorsal view of myxal region; (G) legs 5 and 6. Scale bars: A, 1.00 mm; B–D, F, H, 100 μm; E, I, 50 μm.

Etymology: The specific name refers to the south-eastern region of Tasmania, known as ‘nuenonne’ by indigenous Australians, where the new caligid species occurs.

Remarks. *Caligus nuenonnae* n. sp. shares an accessory process on apical spines two and three of the distal exopodal segment of leg 1, three well developed inner setae on the distal exopodal segment of leg 1, a 2-segmented leg 4 exopod with I-0; I, III armature and a 1-segmented abdomen in the female that is about a third the length of the genital complex in common with *C. acanthopagri* Lin, Ho & Chen, 1994, *C. asymmetricus* Kabata, 1965, *C. dieuzeidei* Brian, 1933, *C. latigenitalis* Shiino, 1954, *C. pomacentrus* Cressey, 1991, *C. serratus* Shiino, 1965, *C. willungae* Kabata, 1965 and *C. zei* Norman & T. Scott, 1906.

The following comparisons refer to female characteristics unless stated otherwise. *Caligus nuenonnae* n. sp. differs from *C. acanthopagri* and *C. latigenitalis* by having a spatulate rather than pointed proximolateral process on the first segment of the antenna, a proximal ridged protrusion on the maxilliped corpus, widely separated furcal tines, an outer flange on each apical spine of the terminal exopodal segment of leg 1, a relatively shorter accessory process on the middle and inner apical spines of the terminal exopodal segment of leg 1, a 1-segmented instead of 2-segmented leg 1 endopod, a relatively longer distolateral spine on the terminal exopodal segment of leg 2 and two small adhesion pads on the leg 3 protopod (see Ho & Lin 2004; Izawa & Choi 2000).

Caligus nuenonnae n. sp. can be distinguished from *C. asymmetricus* by the presence of a large rather than small proximolateral process on the first segment of the antenna, widely separated furcal tines, a relatively shorter proximolateral spine on the terminal exopodal segment of leg 2 and a lateral row of setules rather than large coarse teeth on the middle endopodal segment of leg 2. Further differences include the absence of a medial tooth-like protrusion and basal irregular outgrowth on the maxilliped corpus and spinules on the protopod of legs 1 and 3 in the new species (see Ho & Lin 2004).

Caligus dieuzeidei differs from the new taxon by having a slender rather than broad proximolateral process on the first segment of the antenna, an accessory basal process on the postantennal process, a secondary process on the maxillulary dentiform process and a relatively longer sternal box.

Caligus nuenonnae n. sp. can be distinguished from *C. pomacentrus* by having a nearly subequal instead of distinctly wider abdominal somite, a spatulate rather than pointed proximolateral process on the first segment of the antenna, relatively slimmer furcal tines, a flange rather than fine teeth on the outer margin of the middle and inner apical spines on the terminal exopodal segment of leg 1, a flange rather than denticles on the outer margin of the lateral spine on the middle exopodal segment of leg 2 and a considerably longer distolateral spine on the second exopodal segment of leg 4.

Caligus serratus differs from the new species by having furcal tines that are spaced closer together, relatively slimmer apical spines on the terminal exopodal segment of leg 1, a relatively slimmer lateral spine on the proximal exopodal segment of leg 2 and a relatively shorter distolateral spine on the terminal exopodal segment of legs 2 and 4. The former taxon also lacks a proximolateral process on the first segment of the antenna, a proximal ridged protrusion on the maxilliped corpus and an outer flange on each apical spine of the terminal exopodal segment of leg 1.

Caligus nuenonnae n. sp**.** can be distinguished from *C. willungae* by possessing a subcircular rather than pyriform cephalothoracic shield, a recurved postantennal process, a pointed maxillulary dentiform process, a proximal ridged protrusion on the maxilliped corpus,

widely separated furcal tines and a relatively shorter middle apical spine on the terminal exopodal segment of leg 4. The new species also lacks an accessory basal process on the postantennal process and an apical flange on the postantennal process, maxillulary dentiform process and each furcal tine.

Caligus zei differs from *C. nuenonnae* n. sp. by having a genital complex that is nearly as large as the cephalothoracic shield, a relatively shorter outer apical spine on the terminal exopodal segment of leg 1 and a relatively shorter inner apical spine on the distal exopodal segment of leg 4 (see Kabata 1979).

Caligus nuenonnae n. sp. can be further distinguished from those eight congeners by having a mid-lateral indentation and strongly concave distal margin on the female genital complex, a distinctly broader first abdominal somite relative to the second abdominal somite in the male, numerous small points embossed on the surface of the male maxillulary dentiform process and male legs 5 and 6 represented by distinct lobiform projections on the genital complex.

In summary, *C. nuenonnae* n. sp. is characterised by the following combination of features: 1) female genital complex with a mid-lateral indentation and highly concave posterior margin; 2) 1-segmented abdomen in the female that is about one-third the length of the genital complex; 3) distinctly broader first abdominal somite relative to the second abdominal somite in the male; 4) antenna with a spatulate process on the first segment; 5) recurved postantennal process lacking a basal process; 6) female maxilliped with a ridged protrusion on the posterior surface of the corpus; 7) sternal furca with widely separated, apically truncate tines; 8) distal exopodal segment of leg 1 with a lateral flange on each apical spine and an accessory process on apical spines two and three; 9) leg 3 with two adhesion pads on the protopod; 10) 2-segmented leg 4 exopod with I-0; I, III armature; 11) terminal exopodal segment of leg 4 with the outer apical spine being slightly shorter than the middle apical spine; 12) male maxillulary dentiform process with numerous small points embossed on the surface; and 13) relatively well developed, lobate male legs 5 and 6.

The presence of *C. nuenonnae* n. sp**.** in high numbers (40 parasites per fish) on cultured striped trumpeter has been associated with the development of small lesions on the host's body surface, which heal within several weeks once the parasites have been removed. It is uncertain whether *C. nuenonnae* n. sp. will have a negative effect on striped trumpeter health in a commercial setting. Further, it is unknown at this juncture whether *C. nuenonnae* n. sp. occurs on wild populations of striped trumpeter (and other marine fish) in Australia or elsewhere. To date, only four parasite species, i.e. two monogenean species: *Allomegalocotyla johnstoni* (Robinson, 1961) and *Pseudomegalocotyla latridis* (Robinson, 1961) and two unidentified nematode species each belonging to the genus *Anisakis* Dujardin, 1845 and *Cucullanellus* Törnquist, 1931, have been reported from wild striped trumpeter collected off the coast of New Zealand (Brunsden 1956; Robinson 1961). Clearly, a detailed parasite survey of wild striped trumpeter would be advantageous before intensive sea-cage farming of striped trumpeter commences in Australia.

Molecular analysis of the Caligidae is of particular interest, especially for the highly speciose genus *Caligus,* to delineate morphologically similar taxa and further understand the phylogenetic relationships amongst this diverse parasite group. For example, Øines & Heuch (2005) and Øines & Schram (2008) recently demonstrated the presence of sibling species for *Caligus elongatus* Nordmann, 1832 based on differences in morphology and mitochondrial

gene (CO1 and 16S) sequences. We have sequenced the mtCO1 gene of *C. nuenonnae* n. sp. and made it available on Genbank (accession number EF452642) for future investigators.

22.5 Acknowledgements

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23 THE FIRST CHONDRACANTHID (COPEPODA: CYCLOPOIDA) REPORTED FROM CULTURED FINFISH, WITH A REVISED KEY TO THE SPECIES OF *CHONDRACANTHUS*

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23.1 Abstract

A new species of the Chondracanthidae (Copepoda: Cyclopoida), *Chondracanthus goldsmidi*, is described based on material collected from the naso-branchial region of striped trumpeter (*Latris lineata* [Forster]) cultured at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories, Australia. This represents the first report of a chondracanthid copepod infecting cultured finfish and the first metazoan parasite from cultured striped trumpeter. *Chondracanthus goldsmidi* n. sp. can be distinguished from its female congeners by the absence of lateral processes on the head and presence of 3 pairs of lateral trunk outgrowths, 3 mid-dorsal body outgrowths (of which the first 2 are rounded), a small and subcylindrical antennule, and unornamented legs 1 and 2. A revised key to the 39 valid species of *Chondracanthus* is provided.

Latris lineata (Forster), known commonly in the southern hemisphere as striped trumpeter, is a demersal teleost species inhabiting the coasts of southeastern Australia, New Zealand, and South America (Kailola et al., 1993). This species, which grows to at least 1.2 m total length and 25 kg, has high quality flesh and is amenable to culture conditions; it is currently being investigated as a candidate for commercial aquaculture at the Tasmanian Aquaculture and Fisheries Institute (TAFI), Marine Research Laboratories, Australia (Kailola et al., 1993; Trotter et al., 2001; Morehead and Hart, 2003; Bransden et al., 2005; Brown et al., 2005; Battaglene and Cobcroft, in press).

To date, only one parasite, namely the myxozoan *Kudoa neurophila* (Grossel, Dykovà, Handlinger and Munday, 2003), is known to cause disease in cultured striped trumpeter. This myxozoan species specifically targets the tissues of the central nervous system of post-larval striped trumpeter, resulting in behavioral abnormalities such as loss of spatial control (Grossel et al., 2003). Recent routine examination of healthy and moribund striped trumpeter reared at the Tasmanian Aquaculture and Fisheries Institute resulted in the discovery of a new parasite belonging to the cyclopoid copepod genus, *Chondracanthus* Delaroche, 1811. The sessile parasites were commonly attached to tissue within the gill cavity, occasionally on the gills themselves, or in the nares. Host tissue appeared swollen at the attachment site and, in a severe infection of a population of 84 juveniles (250 g, 660-days-old), holes developed in the operculum. Furthermore, 36 of these 84 fish died or were euthanased when close to death over a 3-week period. This new species, the first within the

Chondracanthidae to be reported from cultured finfish, is herein described. Moreover, a revised key to the species of *Chondracanthus* is provided.

23.2 Materials and Methods

Parasites were collected from striped trumpeter *L. lineata* juveniles and adults that were either cultured or wild-caught animals held in captivity at the TAFI, Marine Research Laboratories in Tasmania. Fish were held in 25,000-L tanks supplied with unfiltered seawater at ambient temperature (annual range 9-19 ^{o}C). Fish were routinely removed from the tanks, anaesthetized (0.02% 2-phenoxyethanol, Sigma-Aldrich, St. Louis Missouri) and examined for chondracanthid copepods on 9 separate occasions (Table 23.1). Twenty-four transformed adult female copepods (13 each with 1 attached male; 1 with 2 attached males; 2 without attached males) were removed from striped trumpeter, preserved in either 10% formalin or 70% ethanol, and later soaked in lactic acid for at least 24 hr prior to examination using an Olympus BX50 compound microscope. Three female and 4 male specimens were measured using an ocular micrometer. Three specimens from each sex were dissected and examined according to the wooden slide procedure of Humes and Gooding (1964). All drawings were made with the aid of a camera lucida. Anatomical terminology follows Boxshall and Halsey (2004).

Table 23.1 Infection perameters of *Chondracanthus goldsmidi* paratizing *Latris lineaa* cultured at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories, Australia

Date	No. hosts examined	Mean† host length (cm)	Mean† host weight (kg)	Host age (days)	Prevalence (%)	Parasite counts (range)
4 June 2003	38	41.6 ± 4.5	1.08 ± 0.27	1,453	92	0 to >25
11 March 2005	48	24.3 ± 2.1	0.21 ± 0.05	675	54	0 to >8
21 April 2005	22	47.5 ± 2.8	1.76 ± 0.34	2,120	100	1 to >10
15 December 2005	5	28.9 ± 3.1	0.36 ± 0.35	954	80	0 to 8
20 December 2005	6	63.5 ± 5.2	4.03 ± 0.95	9–17 years‡	33	0 to 13
22 March 2006	154	28.0 ± 4.5	0.36 ± 0.25	683	42	0 to 4
30 March 2006	19	49.6 ± 5.1	2.08 ± 0.89	3,285	100	1 to 25
9 May 2006	58	41.2 ± 4.8	0.95 ± 0.54	1,385	100	5 to >60
23 May 2006	18	49.6 ± 4.5	2.26 ± 0.68	3,338	94	0 to 15

* Parasites were removed manually and fish treated with Neguvon® following each date given.
† Data are mean ± SD.
‡ Estimated age of wild-caught fish held in captivity for several years as brood stock.

Description

Chondracanthus goldsmidi n. sp.

(Figure 23.1 to Figure 23.4)
Adult female: Body (Figure 23.1A, B) divided into head, short neck, and stout trunk. Total length (from anterior margin of head to distal end of posterior processes on trunk) 4.92 ± 0.35 mm; trunk width 2.57 ± 0.43 mm; head length 1.23 ± 0.03 mm and head width 1.53 ± 0.20 mm. Head composed of cephalosome only, broader posteriorly, lacking processes. Neck region composed of first pediger only, with a rounded outgrowth on mid-dorsal surface. Pedigerous somites 2, 3, and 4 fused to form a large trunk, bearing 3 pairs of lateral outgrowths (middle pair largest of three pairs), 1 pair of posterior processes, and 2 outgrowths along mid-dorsal line; posterior, mid-dorsal outgrowth larger than anterior, mid-dorsal outgrowth, and protrudes either partially or completely over the genitoabdomen in dorsal view. Genitoabdomen (Figure 23.1C) divisible as 2 tagmata by transverse constriction; anterior tagma bearing a minute seta near opening of each genital aperture and a

sensilla on each posterolateral surface; posterior tagma suboval, narrower than genital somite, with a dorsal pair of sensillae and anteroventral pair of caudal rami. Caudal ramus (Figure 23.1D) spiniform, armed with 2 ventral setae, 1 dorsal seta, and a medial knob.

Antennule (Figure 23.1E) small, subcylindrical, with an armature of 1-1-1-3-2-8. Antenna (Figure 23.2A) 2-segmented, composed of coxobasis and 1-segmented endopod; coxobasis short, unarmed; endopod forming uncinate claw, with transverse striations near apex. Labrum (Figure 23.2B) with a small protrusion on each lateral margin and patches of minute spinules along posterior margin. Mandible (Figure 23.2C) 1-segmented, bearing apical falcate blade armed with 40–51 teeth on convex margin and 29–38 teeth on concave margin (counts based on each pair of mandibles from 3 specimens). Paragnath (Figure 23.2D) trilobate, with spinules on small, outer lobe and large, medial lobe. Maxillule (Figure 23.2E) lobate, bearing a large basal protrusion, a subapical patch of spinules, and 2 terminal, unequal elements. Maxilla (Figure 23.2F) 2-segmented, comprised of syncoxa and basis; syncoxa robust, unarmed; basis forming a claw-like process, armed with 2 unequal basal setae and 11–13 marginal teeth (counts based on each pair of maxillae from 3 specimens). Maxilliped (Figure 23.2G) 3-segmented, composed of syncoxa, basis and terminal claw (formed from fused endopod and claw); syncoxa naked, longer than last 2 segments combined; basis stout, with 2 large patches of minute spinules along inner margin; claw short and robust, bearing 1 accessory tooth. Leg 1 (Figure 23.2H) fleshy and bilobate, armed with an outer protopodal seta; both rami subequal and naked. Leg 2 (Figure 23.3A) similar to leg 1, except larger in size.

Adult male: Body (Figure 23.3B) 465 ± 30 µm long and 337 ± 12 µm wide; body segmentation indistinct; cephalothorax globose, comprising more than half total body length; urosome flexed ventrally. Genital somite completely fused with abdomen (Figure 23.3C), bearing paired apertures ventrally; opercula unarmed. Caudal rami (Figure 23.3C) spiniform, each bearing three basal setae, a small medial knob, and minute spinules apically.

Antennule (Figure 23.3D) filiform, with an armature of 1-1-2-3-8. Antenna (Figure 23.3E) short and stout, with 1 seta on the coxobasis and a medial seta on the claw. Labrum (Figure 23.3F) as in female, except with median knob. Mandibular blade (Figure 23.4A) bearing 13–23 teeth on convex side and 9–14 teeth on concave side (counts based on each pair of mandibles from 3 specimens). Maxillule (Figure 23.4B) with 2 subequal, terminal elements, a small patch of spinules, and a medial lobe bearing an acuminate tip. Maxillary basis (Figure 23.4C) lacking teeth; large basal seta unilaterally spinulated. Maxilliped (Figure 23.4D) as in female, except with smaller patches of spinules on basis. Leg 1 (Figure 23.4E) with inner basal protrusion, a long subapical seta (representing the protopodal seta), 2–3 apical setae (representing the exopodal elements), and an inner subtriangular process (representing the endopod). Leg 2 (Figure 23.4F) similar to leg 1, except smaller in size and bearing 1–2 apical setae. One dissected specimen lacking endopod on leg 2 (Figure 23.4G).

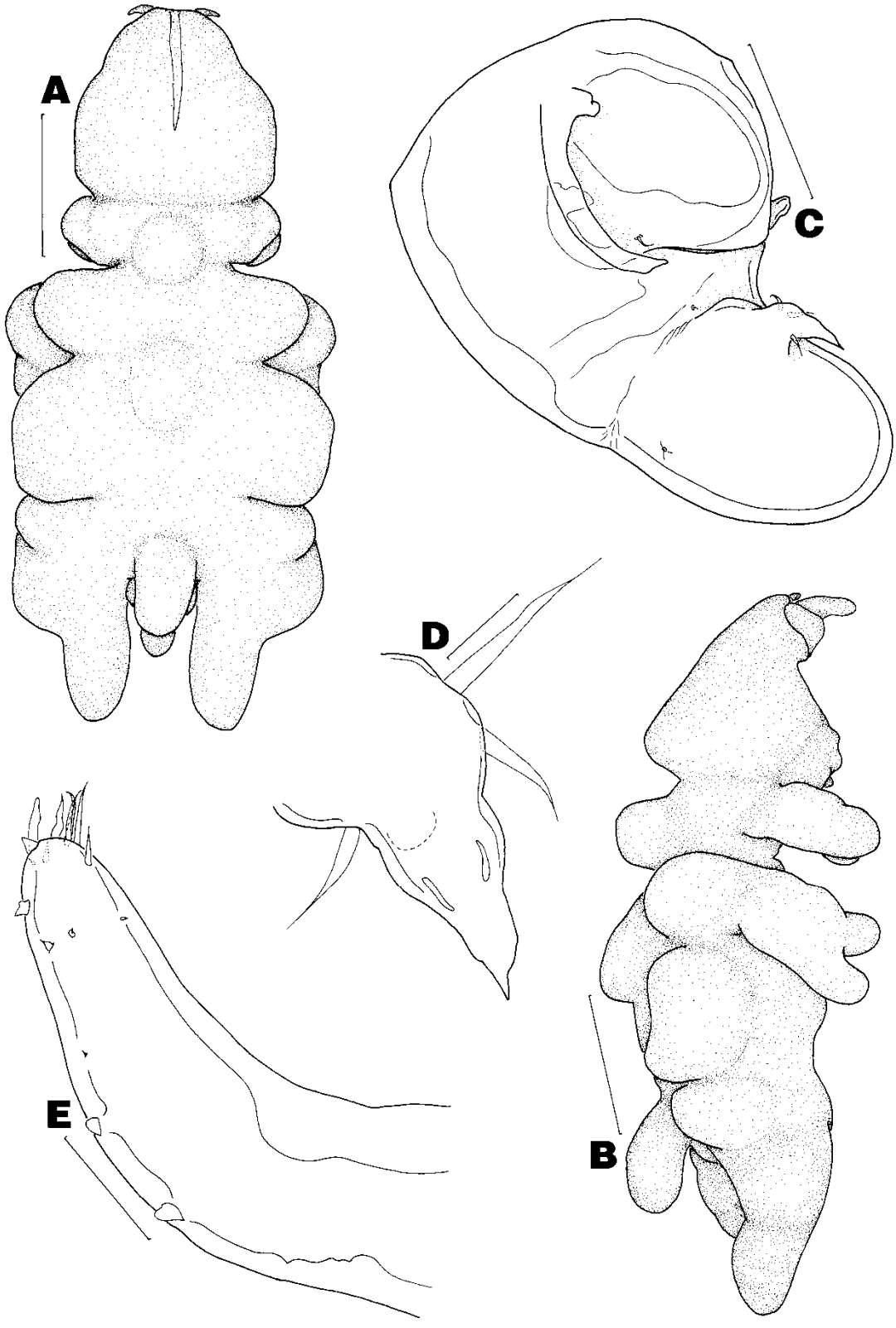

Figure 23.1 *Chondracanthus goldsmidi* n. sp., adult female. (A) habitus, dorsal; (B) same, lateral; (C) genitoabdomen, lateral; (D) caudal ramus; (E) antennule. Scale bars: A, B = 1.00 mm; C = 200 µm; D = 25 µm; E = 50µm.

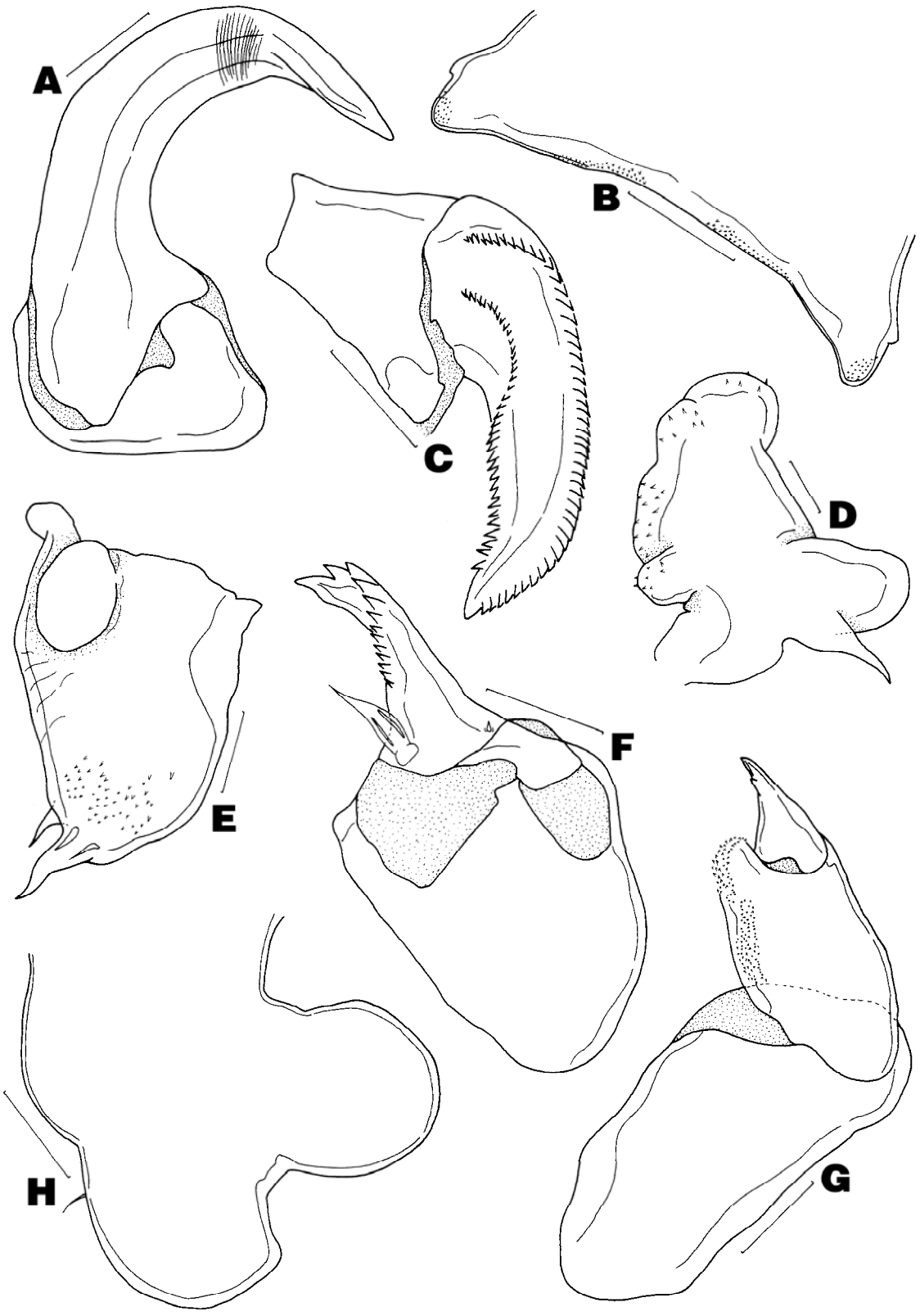

Figure 23.2 *Chondracanthus goldsmidi* n. sp., adult female. (A) antenna; (B) labrum; (C) mandible; (D) paragnath; (E) maxillule; (F) maxilla; (G) maxilliped; (H) leg 1. Scale bars: A, B = 100 µm; C, F, G = 50 µm; D = 12.5 µm; E = 25 µm; H = 200 µm.

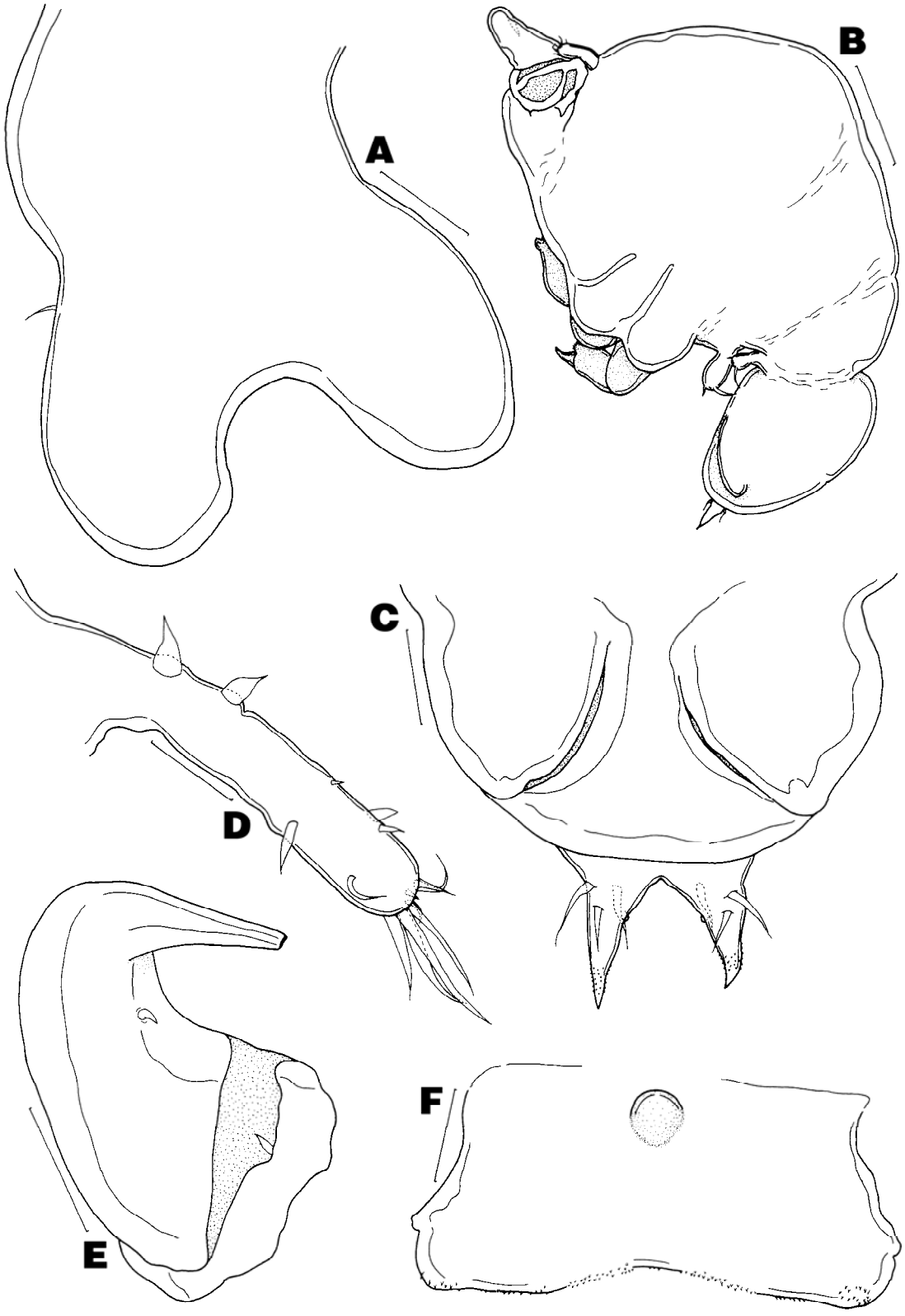

Figure 23.3 *Chondracanthus goldsmidi* n. sp., adult female (A) and adult male (B-F). (A) leg 2; (B) habitus, lateral; (C) genitoabdomen, ventral; (D) antennule; (E) antenna; (F) labrum. Scale bars: A = 200 µm; B = 100 µm; C, E, F = 25 µm; D = 12.5 µm.

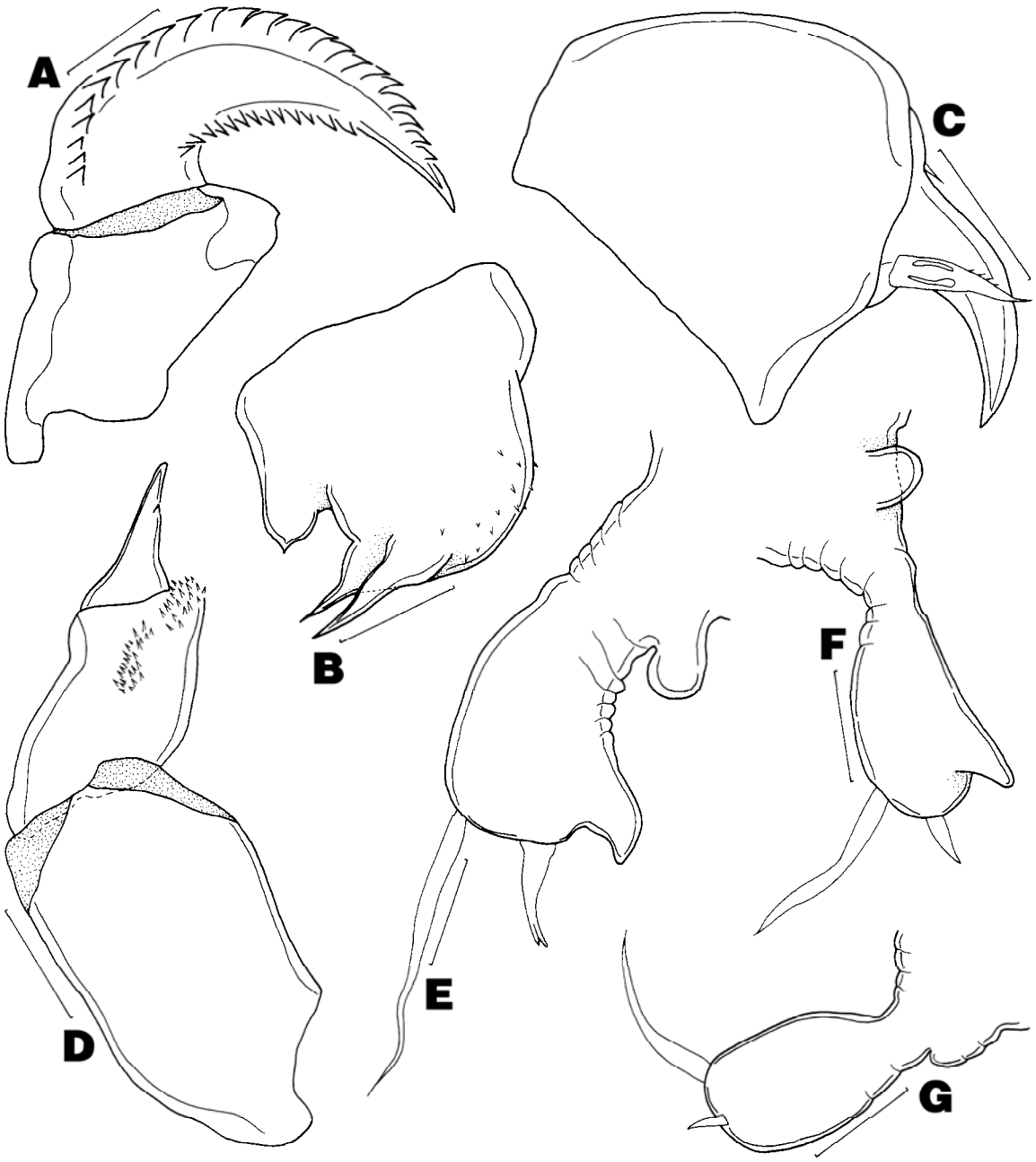

Figure 23.4 *Chondracanthus goldmidi* n. sp., adult male. (A) mandible; (B) maxillule; (C) maxilla; (D) maxilliped; (E) leg 1; (F) leg 2; (G) abnormal leg 2. Scale bars: A, B, E, F, G = 12.5 µm; C, D = 25 µm.

23.3 Taxonomic summary

Type host: Latris lineata (Forster, 1801) (Perciformes: Latridae).
Infection site: Branchial cavity wall, operculum, gills, nares.
Type locality: Crayfish Point, Taroona, Tasmania, Australia (43°35′S, 147°35′E).
Prevalence and parasite counts: see Table 23.1.

Type material: The holotype female (AM P.73329), allotype (AM P.73330) and paratypes (3 females, each with attached male; AM P.73328) are deposited in the Australian Museum, Sydney, Australia.
Etymology: The specific name is in honor of Ross Goldsmid for his continuing work and commitment to broodstock husbandry and the culturing of striped trumpeter juveniles, including parasite monitoring and management.

23.4 Remarks

With the absence of an atrophied tip on the antenna and possession of a cephalosomic head region, outgrowths on the trunk region, and 2 pairs of modified legs in the transformed adult female, the new species is unequivocally a member of *Chondracanthus* Delaroche, 1811. *Chondracanthus goldsmidi* n. sp. closely resembles *Chondracanthus irregularis* Fraser, 1920, in lacking lateral processes on the head and having 3 pairs of lateral outgrowths on the trunk and 3 mid-dorsal outgrowths on the body. However, *Ch. goldsmidi* can be distinguished from *Ch. irregularis* by differences in the shape of the first two mid-dorsal body outgrowths (rounded in *Ch. goldsmidi*; digitiform in *Ch. irregularis*), complexity of the mid-lateral trunk outgrowth (lacks a ventrolateral, digitiform outgrowth in *Ch. goldsmidi*; with ventrolateral, digitiform outgrowth in *Ch. irregularis*), antennular structure (small and subcylindrical in *Ch. goldsmidi*; large and fleshy in *Ch. irregularis*), and leg ornamentation (naked in *Ch. goldsmidi*; covered with fine denticles in *Ch. irregularis*) of the female, as well as in the shape of the antennule (slender in *Ch. goldsmidi*; inflated in *Ch. irregularis*) and structural details of the legs (with an inner basal protrusion in *Ch. goldsmidi*; lacking inner basal protrusion in *Ch. irregularis*) of the male.

All specimens identified to date have been from fish held in land-based tank systems at one site and the effects of the parasite in sea cages and at alternative sites are unknown. However, this parasite has the potential to affect striped trumpeter in a sea-cage industry via mortality in the event of heavy infection as has occurred in land-based systems, or possibly through reduced growth rates of infected fish, treatment costs, or reduced quality of harvested fish. Research to culture and describe the developmental stages of the parasite, examine the histopathology of infection and to test potential control and treatment methods for *Ch. goldsmidi* are currently underway and will be dealt with in detail elsewhere. Furthermore, wild striped trumpeter populations from Tasmanian waters will be sampled and examined to determine whether *Ch. goldsmidi* is a natural parasite of this finfish species.

23.5 Key to the species of *Chondracanthus*

In his preliminary review of *Chondracanthus*, Ho (1991a), recognized 37 valid species. Subsequently, Ho and Kim (1995) transferred *Acanthochondria solida* Gusev, 1951 to *Chondracanthus*, and Tang and Ho (2005) transferred *Chondracanthus quadratus* (Heegaard, 1945) to *Acanthocanthopsis* Heegaard, 1945. Although Ho et al. (2005) established 2 new *Chondracanthus* species, *Ch. parvus* and *Ch. yabei*, we consider the former species to be synonymous with *Chondracanthus solidus* (Gusev, 1951), as there are no marked differences between the descriptions and drawings of *Ch. solidus* and *Ch. parvus* given in Ho and Kim (1995) and Ho et al. (2005), respectively. Thus, with the establishment of *Ch. goldsmidi*, a total of 39 *Chondracanthus* species are currently considered valid.

The following key, which utilizes features of the transformed adult female only, is adapted from Ho (1991a). Additionally, the following errors from Ho (1991a) have been amended in this revised key: (1) *Chondracanthus neali* Leigh-Sharpe, 1930, which was omitted

previously, was added; (2) the first option in step 15 should state “trunk region with outgrowths on mid-dorsal surface” rather than “trunk region with an outgrowth on mid-ventral surface” as previously noted; and (3) the reference given for *Chondracanthus colligens* Barnard, 1955, should be Ho (1972b) rather than Ho (1972a), as previously noted. Following Ho (1991a), the species identified with this revised key should be confirmed by checking with the reference given in brackets following the species name.

1. Legs 1 and 2 unilobate……..*angustatus* (Raibaut et al. [1971]: 190–193)
   Legs 1 and 2 trilobate..... 2
   Legs 1 and 2 bilobate 5
2. Trunk region with outgrowths in the form of knobs, processes or protrusions on dorsal surface 3
   Trunk region without outgrowths on dorsal surface 4
3. Head without processes; trunk region with more than a dozen processes.....
   .....*zei* (Ho [1991a]: 50–54)
   Head with a pair of posterolateral processes; trunk region with less than a dozen processes .....*horridus* (Heller [1865]: 232–233)
   Head with lateral expansions; trunk region with less than a dozen processes.....
   ..... o*rnatus* (Kabata [1979]: 122–123)
4. Antennule extremely small; maxilliped claw with only one hooklet; caudal ramus with usual long terminal process.....*nodosus* (Ho [1971]: 27–31)
   Antennule large and fleshy; maxilliped claw bearing numerous hooklets; caudal ramus lacking long terminal process..... *wilsoni* (Ho ([1971]: 31–33)
5. Trunk region with only one pair of lateral outgrowths (knobs, protrusions or processes)..... 6
   Trunk region with more than one pair of lateral outgrowths (knobs, protrusions or processes)..... 13
6. Head with one or two pairs of small knobs on lateral surface 7
   Head with a pair of prominent lateral processes..... 8
   Head without knobs or processes..... 9
7. Head with one pair of small knobs; second pedigerous somite with pair of lateral processes .....*solidus* (Ho & Kim [1995]: 31–34)
   Head with two pairs of small knobs; second pedigerous somite without pair of lateral processes……*deltoideus* (Kabata [1984]: 1710–1713)
8. Posterodorsal portion of head protruded into a large crestlike outgrowth; abdomen greatly elongated .....*janebennettae* (Ho [1971]: 20–23)
   Head without crestlike outgrowth; abdomen smaller than genital area.....…….
   .....*merluccii* (Ho [1971]: 23–27)
9. Rami of leg 2 short and stout 10
   Rami of leg 2 long and slender 11
10. Trunk with a knoblike outgrowth on dorsal surface; posterior processes long .....
    ..... *gracilis* (Kabata [1968]: 332–335)
    Trunk without knoblike outgrowth on dorsal surface; posterior processes short ..
    .....*lepidionis* (Kabata [1970]: 180–182)
11. Anterior end of head distinctly narrower than its posterior end; posterior end of trunk with a ventral swelling..... 12
    Anterior end of head as wide as or slightly wider than its posterior end; posterior end of trunk without ventral swelling .....*psetti* (Ho [1977]: 164-165)
12. Leg 2 long, reaching or passing the posterior end of trunk.....
    ..... *palpifer* (Ho [1991b]: 2–4)
    Leg 2 short, barely reaching the posterior end of the third pedigerous somite.....

..... *australis* (Ho [1991b]: 5–8)

13. Trunk region with two pairs of lateral outgrowths (knobs, protrusions or processes).....14
    Trunk region with more than two pairs of lateral outgrowths (knobs, protrusions or processes) 26
14. Posterior processes well developed; head with or without lateral processes.....15
    Posterior processes poorly developed, appearing as a corner knob; head without lateral processes..... *narium* (Kabata [1969]: 3044–3047)
15. Trunk region with outgrowths on mid-dorsal surface 16
    Trunk region without outgrowths on mid-dorsal surface 21
16. Head with one pair of posterolateral processes or lateral expansions 17
    Head without processes or lateral expansions 20
17. First pedigerous somite with an outgrowth on dorsal surface 18
    First pedigerous somite without an outgrowth on dorsal surface 19
18. Lateral outgrowths on head and trunk short and stubby.....
    .....*multituberculatus* (Markevich [1956]: 161–162)
    Lateral outgrowths on head and trunk long and attenuate.....
    ..... *pinguis* (Kabata [1968]: 329–332)
    Lateral outgrowths on head and trunk medium size and bluntly pointed.....
    ..... *polymixiae* (Shiino [1955]: 77–79)
19. Trunk with two cylindrical dorsal processes; head with bilobate lateral protrusion.....*heterostichi* (Ho [1972a]: 527–529)
    Trunk with three rounded dorsal processes; head with lateral expansions.....
    .....*yabei* (Ho et al. [2005]:416–418)
20. All outgrowths on body large and massive; protopods of legs 1 and 2 with bulging outer surface ..... *lotellae* (Ho [1975]: 308–311)
    All outgrowths on body small and minute; protopods of legs 1 and 2 without bulging outer surface .....*pusillus* (Kabata [1968]: 335–339)
21. Head with one pair of posterolateral processes 22
    Head with lateral expansions, but not in form of process.....
    .....*yanezi* (Ho [1982]: 451–455)
    Head with lateral expansions and a knob in the anterior corners .....
    ..... *cottunculi* (Ho [1971]: 17–20)
    Head without expansions, knobs or processes.....24
22. Legs 1 and 2 long and slender 23
    Legs 1 and 2 short and stubby .....*theragrae* (Shiino [1955]: 74–77)
23. Exopods of legs 1 and 2 nearly twice as long as their respective protopods.....
    ..... *lepophidi* (Ho (1974): 870–873]
    Exopods of legs 1 and 2 as long as their respective protopods .....
    ..... *triventricosus* [Sekerak [1970]: 1944–1950)
24. Oral region separated from antennal area to form the first part of neck region; exopod of leg 1 twice as long as endopod.....
    .....*brotulae* (Ho & Rokicki [1987]: 1031–1033)
    Oral region set far behind antennal area but not separated into a neck region; exopod of leg 1 as long as endopod.....25
25. Legs 1 and 2 short and blunt; each protopod with prominent outer swelling.....
    ..... *genypteri* (Ho [1975]: 306–308)
    Legs 1 and 2 long and attenuate; protopods without such swelling .....
    .....*colligens* (Ho [1972b]: 151–152)
26. Head with one pair of lateral processes 27
    Head with two pairs of lateral processes 29

Head without lateral processes..... 30
27. Head large, its length about ¾ of trunk length.....
.....*tuberculatus* (Ho [1972b]: 155–158)
Head small, its length shorter than ¼ of trunk length..... 28
28. Posterior end of trunk with three processes (including paired posterior processes)
..... *lophii* (Kabata [1979]: 118–119)
Posterior end of trunk with five processes (including paired posterior processes)
.....*barnardi* (Ho [1972b]: 149–152)
29. Trunk with dorsal outgrowths; abdomen extended posteriorly .....
.....*neali* (Ho [1972b]: 152–155)
Trunk lacking dorsal outgrowths; abdomen not extended posteriorly.....
.....*distortus* (Shiino [1955]: 71–74)
30. Trunk with three pairs of lateral outgrowths..... 31
Trunk with six pairs of lateral outgrowths.....*shiinoi* (Shiino [1955]: 79–83)
31. Mid-lateral trunk outgrowth with ventrolateral, digitiform outgrowth; antennule large and fleshy ..... *irregularis* (Kabata [1968]: 323–328)
Mid-lateral trunk outgrowth without ventrolateral, digitiform outgrowth; antennule small and subcylindrical.....*goldsmidi* (this report)

23.6 Acknowledgements

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24 HOST RESPONSE TO THE CHONDRACANTHID COPEPOD *CHONDRACANTHUS GOLDSMIDI*, A GILL PARASITE OF THE STRIPED TRUMPETER, *LATRIS LINEATA* (FORSTER), IN TASMANIA

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24.1 Abstract

The chondracanthid copepod, *Chondracanthus goldsmidi* is an ectoparasite of gills, inner opercula and nasal cavities of cultured striped trumpeter, *Latris lineata* (Forster). Whilst often present in high numbers (up to 60 parasites per host), little is known about its effect on striped trumpeter. In this study *C. goldsmidi* was associated with extensive epithelial hyperplasia and necrosis. Pathological changes were most pronounced near the parasite's attachment site, with papilloma-like growths surrounding the entire parasite resulting in deformation of the filament. The number of mucous cells increased near the parasite attachment sites on both the opercula and gills. Mast cells were absent in healthy gills; in contrast numerous mast cells were identified in the papilloma-like growths. Immunostaining identified piscidin-positive mast cells in the papilloma-like growths, presenting the first evidence of piscidin in the family Latridae.

24.2 Introduction

Parasitic copepods naturally occur on wild fish, usually with little or no impact on the host, but for many species this relationship has changed with the increase of marine fish farming (Boxshall & Bravo 2000; Bravo 2003; Johnson, Treasurer, Bravo, Nagasawa & Kabata 2004). The actions of these parasites may result in mortality, decreased flesh quality and a need for increased drug treatment, adding as much as 6% to production costs (Rae 2002; Heuch, Bjørn, Finstad, Holst, Asplin & Nilsen 2005; Costello 2009). This has resulted in various studies being conducted on the effects of the feeding and attachment behaviour of parasitic copepods on the host (Jónsdóttir, Bron, Wootten & Turnbull 1992; Nolan, Ruane, van der Heijden, Quabius, Costelloe & Wendelaar Bonga 2000).

Changes in mucous cells and mast cells have been described as a response to the attachment of parasitic copepods in both marine and freshwater environments. An example is Ergasilus *sieboldi*, a gill parasite of many freshwater fish species in the United Kingdom (Alston &

Lewis 1994). Its attachment and feeding activity can result in extensive inflammation and granulocytosis of the gills of tench, *Tinca tinca* (L.), eventually leading to changes in the blood chemistry (Einszporn-Orecka 1973). Parasitism of bream, *Abramis brama* (L.), by *E. sieboldi* has been linked with epithelial hyperplasia, mucous cell proliferation and an increase in mast cells (Dezfuli, Giari, Konecny, Jaeger & Manera 2003b).

Mast cells have long been linked to a role in fish immune responses. They have been identified in organs that are often in contact with pathogens, such as the alimentary canal, skin and gills (Reite 1997; Silphaduang & Noga 2001), and are often associated with parasitic infections of these organs (Reite 1998; Dezfuli, Simoni, Rossi & Manera 2000). For example, mast cell inflammation was observed in *Eurasian minnows, Phoxinus phoxinus* (L.), parasitized by the nematode *Raphidascaris acus* (see Dezfuli et al. 2000). However, mast cells have not been reported in host responses to gill-parasitic copepods. Antimicrobial peptides (AMP), some of which are produced by mast cells, have been identified as important components of non-specific immune defence in many vertebrates (Jia, Patrzykat, Devlin, Ackerman, Iwama & Hancock 2000; Silphaduang & Noga 2001; Mulero, Noga, Meseguer, García-Ayala & Mulero 2008). Another cell type linked to localized irritants such as ectoparasites is the mucous cell. For example, Rogers (1969) reported mucous cell hyperplasia in cyprinids parasitized by *Ergasilus cyprinaceus*. However, the types of mucous cells (i.e. containing acidic, acidic-neutral, or neutral mucins) in copepod-parasitized gills has not been determined in any cases.

The striped trumpeter, *Latris lineata* (Forster), is a potential aquaculture candidate (Battaglene & Cobcroft 2007). The myxozoan *Kudoa neurophila* was previously the only parasite identified as a problem in this species (Grossel, Handlinger, Battaglene & Munday 2005). Recently, a new species of chondracanthid copepod, *Chondracanthus goldsmidi* Tang, Andrews & Cobcroft, 2007; was found in the buccal cavity and nares of the striped trumpeter, representing the first chondracanthid reported from a farmed fish species (Tang et al. 2007). The pathogenesis of *C. goldsmidi* is unknown, but heavy infestations are associated with decreased feeding, increased swimming speed and 'head shaking' (M. Andrews, unpublished data); infestations have also been associated with the development of ulcers on the operculum (Tang et al. 2007). The aim of our study was to determine the pathological changes resulting from *C. goldsmidi* infestation, as well as to examine the possible involvement of mast cells and mucous cells in this response.

24.3 Materials and Methods

24.3.1 *Sampling regime*

Striped trumpeter used in this study were held at the Tasmanian Aquaculture and Fisheries Institute (TAFI) Marine Research Laboratories in Taroona, Tasmania. Fish were held in 20 000 L tanks at densities of either ~2.6 kg m−3 (for <15-month-old fish) or ~1.9 kg m−3 (for >40-month-old fish); all fish were fed a commercial, pelleted diet. The tanks were supplied with sand-filtered sea water at ambient temperature (ranging from 10 °C in winter to 18 °C in summer) and photoperiod (ranging from 9 h day−1 in winter to 15 h day−1 in summer). All tanks were cleaned weekly and fish were observed for behavioural changes (e.g. anorexia, head shaking) which were diagnostic for *C. goldsmidi* infestation. When these changes were observed, the following health survey was conducted: all fish in the affected tank were examined for parasites and the numbers on each fish were recorded; all grossly visible parasites were then manually removed using forceps to lower the parasite burden. In instances, when the parasite load was deemed too high and affecting fish health, the tank was

treated with trichlorfon (300 ppm for 1 h) (M. Andrews, unpublished data). Parasitised striped trumpeter sampled during the current study were from a tank that had been undisturbed (received no drug treatment or parasite removal) for 12 months. Control fish were taken from a 3000 L tank provided with ozonated and sand-filtered sea water (ensuring no exposure to *C. goldsmidi*), and with a history of no parasitism.

Fifteen striped trumpeter (450–1307 days old, 203–1354 g) were sampled, including 10 infested fish and five control fish. During sampling, each fish was initially examined to grade the severity of parasitosis; the fish was then killed with 0.02% 2-phenoxyethanol, which does not detach the parasites (M. Andrews, unpublished data). Samples were rinsed in sea water to remove excess blood, fixed in seawater Davidson's for 24 h, and then transferred to 70% ethanol until processing.

The entire gills were removed from all 10 parasitised and five control fish, whilst both opercula were removed from five fish which had parasites present on the operculum; grossly normal tissue on the same opercula was used as a control.

24.3.2 *Histopathology*

A single gill arch bearing an attached parasite was selected from each parasitised fish, whilst one random gill arch was selected from each control fish. Samples were dehydrated, embedded in paraffin and sectioned at 5 μm. One section from each fish was stained with haematoxylin and eosin (H&E) for routine histopathology. For assessing mucous cells, one section each from five parasitised and five control fish was stained using the combined periodic acid-Schiff (PAS)/Alcian Blue (AB) technique at pH 2.5. Due to the large size of the parasite it attaches to either the gill arch or proximal region of the gill filament. This was taken into consideration when conducting mucous cell counts. In the PAS/AB sections, three areas were examined on the gill filament (proximal, middle and distal). For parasitized fish, this was in relation to the oral attachment site (considered proximal). In control fish, this was in relation to the distance from the gill arch. Sections were examined using a Leitz compound microscope with a Leitz Diaplan digital camera at 200× magnification (field of view of 900 × 700 μm). The three fields of view (proximal, middle and distal) were used to determine the density of each mucous cell type in each gill filament.

The operculum samples from the five parasitised fish were processed as for gill tissue, with sections from each fish stained with H&E and PAS/AB. Sections were examined and imaged as for gills, with three fields of view (900 X 700 μm) examined in close proximity to the parasite, whilst three other fields of view were examined from an area of grossly normal operculum not in contact with the parasite. From this, it was possible to determine the density of each mucous cell type in parasitised and healthy areas of operculum.

Mast cells were observed in papilloma-like growths of sections stained with H&E. Mast cells were defined as pleiomorphic cells with numerous eosinophilic granules. Measurements from 20 mast cells were taken from the longest point of the cell, and recorded as mean ± SE, to determine their mean size. Measurements were made using a Leitz compound microscope with a Leitz Diaplan digital camera at 400× magnification.

24.3.3 *Immunohistochemistry*

Mast cells were also probed using a specific antibody to piscidin 3, an antimicrobial peptide that was isolated from hybrid striped bass, Morone saxatilis × M. chrysops (see Silphaduang

& Noga 2001). Anti-piscidin 3 antibody (anti-HAGR) was produced by a commercial laboratory (Bethyl Laboratories) using the company's standard procedures. Briefly, 2 mg of a 12-mer peptide constituting the C-terminus of piscidin 3 (HAGRSIGRFLTG) was conjugated to keyhole limpet haemocyanin (KLH) using maleimide chemistry, which linked the peptide to KLH via a cysteine added to the N-terminal histidine. The conjugation via the terminal amino acid allows tertiary conformation of the peptide that may be expected to mimic that in the native peptide, thus eliciting anti-conformational antibodies important for recognizing the native peptide. Immunogen was mixed with complete Freund's adjuvant (1:1) and KLH-conjugated peptide was injected into two New Zealand white rabbits biweekly at five subcutaneous sites (0.2 mL−1 per site) using the following immunization schedule (100 μg/injection): days 0, 14, 28 and 42. Thirty mL−1 of antiserum was collected from each rabbit on days 35 and 45. The antisera were pooled and were then affinity-purified using the piscidin fragment conjugated to cyanogen bromide-activated agarose as an immunosorbent (10.5 mg of piscidin fragment was reacted with 15 g of agarose). One hundred and twenty mL−1 of antiserum (two 30 mL−1 bleeds from two rabbits) was loaded onto the affinity column (Uniflow 4; Sterogene). After washing, the affinity-purified antibody was eluted and concentrated.

This method produces greater than 0.1 mg of peptide-specific antibody mL−1 of antiserum, as determined by recovered affinity-purified antibody. Antibody was >95% IgG, as determined by immunoelectrophoresis using antibodies specific for rabbit IgG, IgM and serum proteins. The titre of the antibody was determined via ELISA, using the piscidin fragment as the antigen coated onto a microtitre plate. The 12-mer peptide (10 μg mL−1 in PBS, pH 7.2–7.5) was coated onto a microtitre plate at room temperature for 1 h. The plate was then washed and post-coated with 1% BSA in PBS for 30 min. The plate was washed and then dilutions of antibody in 1% BSA/PBS/0.01% Tween 20 were added, beginning at 1 μg antibody mL−1. After incubation for 1 h, the plate was washed, followed by addition of peroxidase-conjugated goat anti-rabbit IgG (h&l) in 1% BSA/PBS/0.01% Tween 20. After incubation for 1 h, the plate was washed and peroxidase substrate was added, incubated for 15 min, and then stopped with 1 N HCl (1:1). The absorbance was then read at 450 nm. The titre was read as the reciprocal of the antibody dilution (dilution of a 1 mg mL−1 solution) that produced a net optical density of 1.0, compared to a blank (non-coated well), which had an OD < 0.1. The titre of the antibody used in all assays was approximately 1:18 000.

The peptide-specific antibody had <1% cross-reactivity by ELISA, where 1% cross-reactivity is 100 times more antibody than is required to produce the same optical density with either free KLH, conjugated KLH, or free peptide that shares <3 amino acids in the sequence.

Piscidin 3-positive cells were detected in histological sections as described by Silphaduang, Colorni & Noga (2006) using a biotin based immunohistochemical (IHC) detection kit (Biogenex, cat# QD000-5 L−1) according to the manufacturer's instructions. Briefly, serial sections (5 μm) were deparaffinised in xylene, rehydrated, and blocked in 3% H2O2 (10 min). The sections were then rinsed in water, washed in phosphate-buffered saline (PBS, pH 7.2) and incubated with a 1:400 dilution of the primary antibody (anti-HAGR) for 30 min at room temperature. Following a wash in PBS, the sections were incubated with a biotinylated goat-rabbit serum (20 min), rinsed in PBS and incubated with streptavidin-conjugated horse radish peroxidase (20 min). The sections were then developed using 3,3'-diaminobenzidine (DAB), rinsed and counterstained with Meyer's haematoxylin. Non-immune rabbit serum and diluent only sections were used as negative controls. Positive control tissue was striped bass, M. saxatilis Walbaum, intestine (Silphaduang et al. 2006).

24.3.4 *Statistical analysis*

To determine if there were statistical differences in the mucous cell composition of parasitised vs. healthy gills, sections from five parasitised and five control fish were examined (one section/fish, n = 5). A Chi-square test was run for each mucous cell type (acidic mucin, neutral mucin, acidic and neutral mucin) to determine whether a statistical difference occurred in relation to cell density and proximity to the parasite. To determine whether there were statistical differences in the mucous cell composition of parasitised and grossly normal opercula, five sections from parasitised fish were examined (n = 5). A Chi-square test was run to identify any differences between the density of mucous cells in parasitised and grossly normal opercula. All statistics were conducted using the statistical program SPSS 15 for Windows (© SPSS Inc.) the results were considered to be statistically significant if $P < 0.05$.

24.4 Results

Our study examined the effects of a low grade (<5 adults per fish) infestation of *C. goldsmidi*. The observed pathological changes were related to the proximity of the parasite to host tissue. At its oral attachment site, there was necrosis (Figure 24.1a) and extensive mucous cell hyperplasia (Figure 24.1b,c) (Table 24.1). More proximal, near the cephalic region of the parasite, there was extensive epithelial cell hyperplasia interspersed with mast cells. Large, ~5 mm, papilloma-like lesions (PLL) surrounded the parasite with more pronounced regions observed where the legs of the parasite were in direct contact with gill tissue (Figure 24.1d). The PLL consisted of undifferentiated epithelial cells and large numbers of mucous cells interspersed throughout; no compression was observed in these PLLs, even when situated between the first and second legs of the parasite (Figure 24.1e). The middle region of the gill filament, which was in constant contact with the parasite's egg sacs, also had extensive lamellar fusion caused by extensive epithelial cell hyperplasia (Figure 24.1f). No cellular changes occurred in the distal region of the gill filament which had no contact with the parasite.

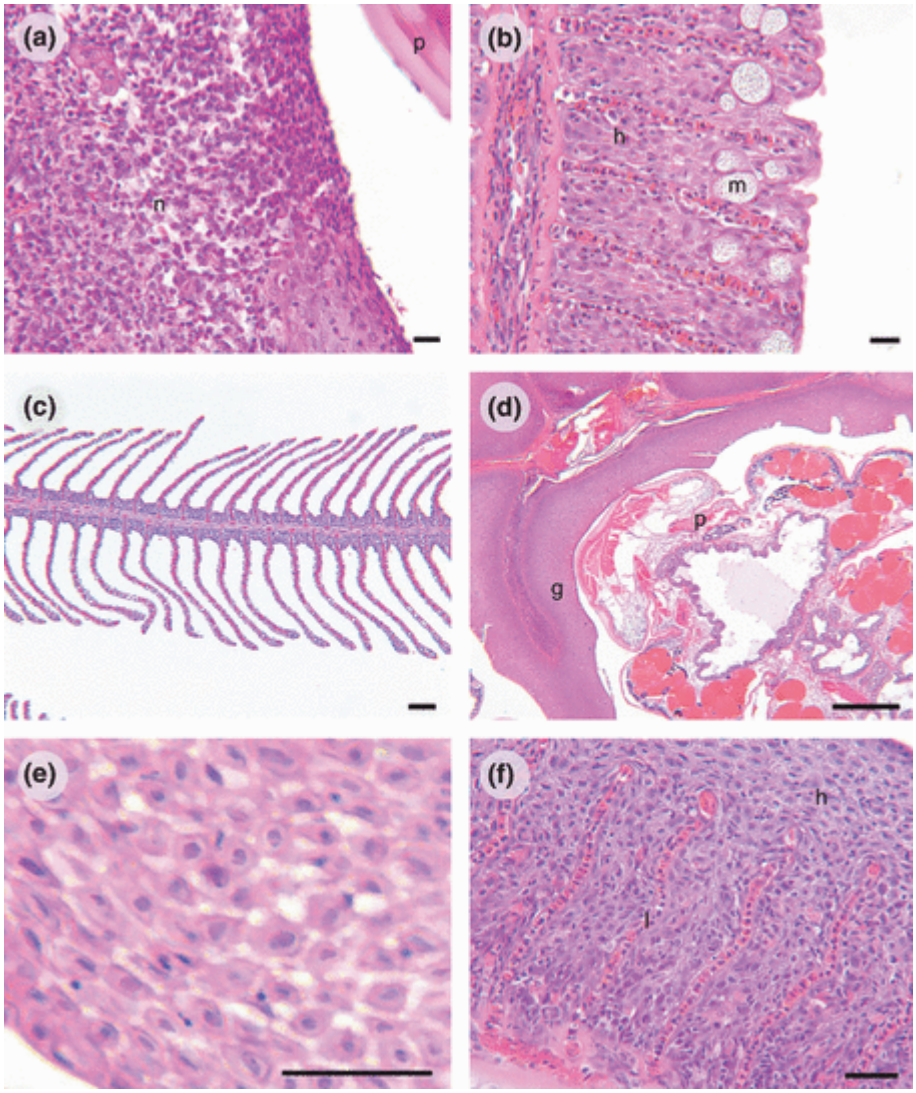

Figure 24.1 Adult *Chondracanthus goldsmidi* parasitising the gills of striped trumpeter. (a) Necrosis (n) occurring near the parasite (p). (b) Epithelial hyperplasia (h) with mucous cells present (m). (c) Healthy gill filament. (d) Papilloma-like growth (g) in contact with the parasite (p). (e) Magnified view of the papilloma-like growth. (f) Lamellar (l) fusion due to epithelial hyperplasia (h) (bars: A, B, F, E = 25 μm; C = 100 μm; D = 0.5 mm; A–F, H & E).

Table 24.1 Mean number (±SE) of mucous cells containing neutral mucin, acidic mucin, and a combination of acidic and neutral mucin from parasitised and healthy gills of striped trumpeter (n = 5)

Mucin type	Filament area	Parasitised gills	Healthy gills
Acidic	Proximal	7.5 ± 4.4	11.7 ± 2.4
	Middle	25.5 ± 17.3	31.1 ± 4.0
	Distal	14.8 ± 6.2	50.3 ± 9.7*
Acidic and neutral	Proximal	97.4 ± 36.7*	0 ± 0
	Middle	80.3 ± 10.6*	0.9 ± 0.4
	Distal	6.5 ± 2.0*	1.5 ± 0.7
Neutral	Proximal	0.5 ± 0.5	0.1 ± 0.1
	Middle	0.1 ± 0.1	1.4 ± 0.8*
	Distal	0 ± 0	1.3 ± 0.4*

*Indicates significant differences, $P < 0.05$.

The PAS/AB technique stains mucous cells having only acidic mucins as blue, those only with neutral mucins as pink, and those having a combination of acidic and neutral mucins as magenta (Bancroft & Cook 1994). The density of mucous cells containing acidic, acidic-neutral and neutral mucins changed with distance from the attachment site (Table 24.1). Significantly lower densities of mucous cells containing acidic mucin were observed in parasitised gill tissue throughout all three regions of the gill filament ($\chi 2 = 35.3$, d.f. = 2, $P < 0.001$). In contrast, mucous cells containing a combination of acidic and neutral mucins occurred in significantly greater densities in parasitised gills ($\chi 2 = 91.5$, d.f. = 2, $P < 0.001$), whilst mucous cells containing neutral mucins occurred at significantly lower densities in the middle and distal regions of the parasitised gill filaments ($\chi 2 = 10.3$, d.f. = 2, $P = 0.006$). The PLLs occurred in the proximal and middle region of the parasitised gill filament, coinciding with changes in the mucous cell distribution.

Mast cells could be presumptively identified in H&E stained sections of parasitised gill (Figure 24.2a), primarily within the PLLs, by their irregular, ovoid shape (mean length 4.93 ± 0.21 μm) and granular appearance. No such cells were evident in grossly normal tissue (Figure 24.2b). This was supported by immunochemical probing with piscidin 3 antibody, which showed that the mast cells were piscidin-positive within the PLL (Figure 24.2c,d).

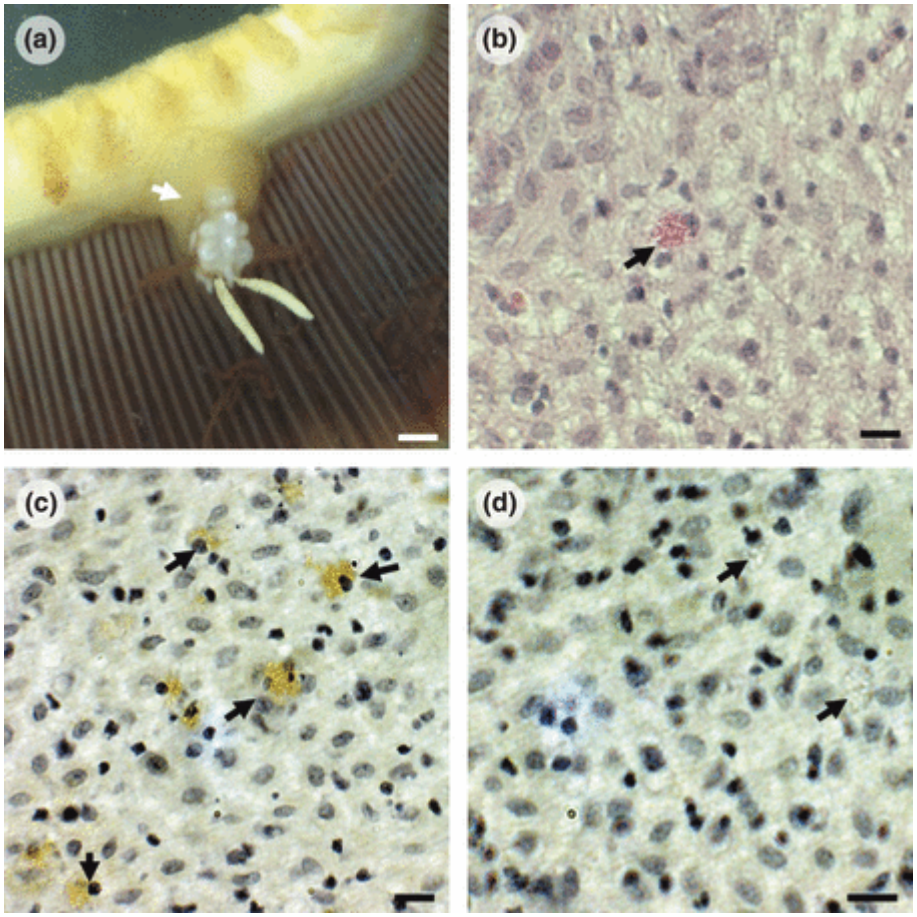

Figure 24.2 Adult *Chondracanthus goldsmidi* parasitising the gills of striped trumpeter. (a) Gross lesion associated with parasite attachment (arrow). (b) Putative mast cell (arrow) within the previously indicated lesion, note the presence of characteristic eosinophilic granules. (c) Positive immunostaining of putative mast cells (arrows) with anti-piscidin antibody from the corresponding serial section. (d) Negative control showing no immuno-reactivity (arrows) in corresponding tissue (bars: A = 2 mm; B–D = 20 μm; B–D, 3,3'-diaminobenzidine (DAB) counterstained with Meyer's haematoxylin).

When attached to the operculum, *C. goldsmidi* is in constant contact with the gill, resulting in epithelial hyperplasia and necrosis of the gill (similar to effects with gill attachment, Figure 24.1a,b). Mucous cells were present in both grossly normal and parasitised opercular epithelium (Figure 24.3a), but there was a significant increase in the density of the mucous cell types in parasitised opercula ($\chi 2 = 8.36$, d.f. = 2, P = 0.015). There were significantly higher densities of all three mucous cell types in the parasitised operculum (Table 24.2), with cells containing acidic and neutral mucins at the greatest density; mucous cells containing acidic mucins were absent from healthy operculum (Figure 24.3b). The feeding activity of the parasite resulted in regions of compression as well as the formation of large necrotic areas (Figure 24.3c,d) which showed signs of inflammation (Figure 24.3d).

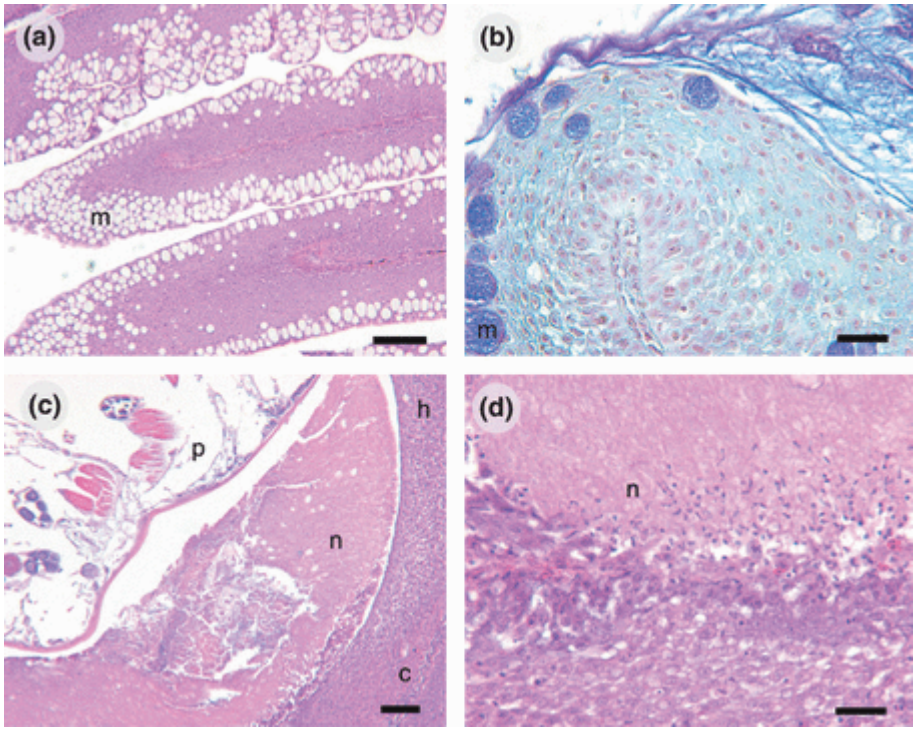

Figure 24.3 Adult *Chondracanthus goldsmidi* parasitising the operculum of the striped trumpeter. (a) Normal operculum showing numerous mucous cells (m). (b) PAS/Alcian Blue section of tissue and mucous cells containing acidic mucin (m) near the attachment site. (c) Necrotic tissue (n), compressed tissue (c) and hyperplasia (h) near the parasite's (p) cephalic region. (d) Necrotic region (n) near the attachment site infected with the adult copepod (bars: A, F = 25 μm; B–D = 100 μm; E = 200 μm; A, C, D, H & E; B PAS/Alcian blue, pH 2.5).

Table 24.2 Mean number (±SE) of mucous cells containing neutral mucin, acidic mucin, and a combination of acidic and neutral mucin from parasitised and grossly normal opercular tissue from striped trumpeter (n = 5)

Mucin type	Parasitised operculum	Grossly normal operculum
Acidic	6.3 ± 3.5*	0 ± 0
Acidic and neutral	177.3 ± 6.5*	122.7 ± 22.8
Neutral	49.0 ± 20.6*	18.3 ± 5.4

*Indicates significant differences, $P < 0.05$.

24.5 Discussion

Chondracanthus goldsmidi attaches to both the gills and opercula of the host. As would be expected, attachment to the gills resulted in more severe damage, as evidenced by severe epithelial cell hyperplasia (including induction of PLL), mast cell inflammation, mucous cell hyperplasia, and necrosis.

Adult *C. goldsmidi* are sessile and remain attached to a single site, resulting in chronic tissue changes that are restricted to the immediate area surrounding the parasite. The thorax and appendages of attached females flex and contract, which might ensure water movement over the egg sacs to prevent settling of mucus and debris (M. Andrews, unpublished data). This continuous movement may induce formation of the PLL. To our knowledge, PLLs have not been previously associated with gill parasitism. However, Avenant-Oldewage (1994) observed a similar response to the branchiuran Dolops ranarum on the skin of the African catfish, Clarias gariepinus (Burchell). The PLLs were observed near the parasite's swimming legs (Avenant-Oldewage 1994), and since D. ranarum is mobile, this resulted in a more widespread tissue response compared to that of *C. goldsmidi*.

Epithelial hyperplasia, resulting in the formation of PLLs near the cephalic region, could be advantageous to the parasite by providing a more secure attachment to the host and more tissue on which to feed. A grossly similar response has also been observed with the lernaeid copepod, *Lamproglena clariae*, whose cephalic region is often enveloped in hypertrophic connective tissue when attached to the gill filaments of *C. gariepinus* (Avenant-Oldewage 1994; Tsotetsi, Avenant-Oldewage & Mashego 2005). Similar hyperplasia resulting in the formation of nodules was observed in the gills of the leopard coralgrouper, *Plectropomus leopardus* (Lacépède), parasitised by the dissonid copepod *Dissonus manteri* (Bennett & Bennett 2001). However, in both these cases, the nodules were formed by connective tissue proliferation rather than the epithelial cell hyperplasia, as observed in this study.

Where the egg strings of *C. goldsmidi* contacted gill tissue, the PLLs were absent, but epithelial hyperplasia was still present, resulting in the fusion of secondary lamellae. The distal region of the parasitised gill filament, which was not in contact with the parasite, showed no obvious cellular changes. This differs from the lernanthropid copepod, *Lernanthropus atrox*, that parasitises the surf bream, *Acanthopagrus australis* (Günther), where there is extensive haemorrhage and the collapse of the efferent filament artery (Roubal 1989). Necrosis was observed in close proximity to the mouthparts of *C. goldsmidi*. This, in combination with the action of the attachment organs, may restrict blood flow, as was observed in bream parasitised by *E. sieboldi* (Dezfuli et al. 2003b). However, necrosis may be caused by release of digestive enzymes during feeding, as has been reported during ergasilid feeding (Paperna & Zwerner 1982). Although the feeding mechanism of *Chondracanthus* is unknown, it may be similar to that of other sessile, parasitic copepods, such as *L. clariae*. Tsotetsi et al. (2005) described the maxillae and maxillipeds of *L. clariae* as having a 'scraping and rolling movement' that brings gill tissue to the buccal cavity. This may also occur with *C. goldsmidi*, which has strong and well-developed mouthparts (Tang et al. 2007). This movement may also release digestive enzymes, causing necrosis.

The PAS/AB mucous cell staining highlighted the changes caused by *C. goldsmidi*. The PLL had greater densities of mucous cells containing acidic and neutral mucins, with densities decreasing in areas not in contact with the parasite. Healthy, unparasitised gills had very low mucous cell densities when compared to parasitised gills, with densities decreasing distally along the filament. Parasitic and bacterial infections both can increase mucous secretion in gill and intestinal epithelia (Adel-Meguid, Esch & Eure 1995; Lodemel, Mayhew, Myklebust, Olsen, Espelid & Ringø 2001). An increase in mucous secretion was linked with an increase in mucous cell density, as in sea trout, *Salmo trutta L.*, infected with the acanthocephalan *Pomphorhynchus laevis* (Bosi, Arrighi, Di Giancamillo & Domeneghini 2005). As in the case of *C. goldsmidi*, mucous cells containing mixed and acid mucin were dominant in the presence of *P. laevis*. *Acidic mucin* has been linked with a defensive role in the intestine and may contribute to the removal of attached parasites (Domeneghini, Straini

Pannelli & Veggetti 1998). The findings of this study support the possible defensive role of mucous cells containing acidic mucin, which may be the culmination of the host attempting to exclude the parasite.

The appearance of mast cells in the PLL that were immunoreactive with the antibody to piscidin 3 represents the first evidence of piscidins in the family Latridae. No piscidin-positive mast cells were present in grossly normal tissue. Piscidins have been localized in the gill and other portals of pathogen entry in several economically important fish families, including the Belontidae, Cichlidae, Moronidae, Sciaenidae, Serranidae and Siganidae (Silphaduang et al. 2006). The previous studies used a probe to the highly conserved N-terminus of the piscidin family (anti-FFHH antibody). The anti-piscidin 3 antibody that we used to probe striped trumpeter tissues is raised against the more variable C-terminus (Mulero et al. 2008).

High numbers of mast cells have been reported in tissues damaged from the actions of parasites and pathogens (Murray, Leggiadro & Douglas 2007). The high number of piscidin-positive mast cells in the presence of *C. goldsmidi* compared to their lack in grossly normal gill tissue may indicate that the mast cells migrate to the affected area. Such migration was reported in the gills of rainbow trout, *Oncorhynchus mykiss* (Walbaum), affected by amoebic gill disease (see Powell, Wright & Burka 1990). *Ergasilus sieboldi* infestation of bream gills resulted in mucous cell infiltration in damaged tissue (Dezfuli et al. 2003b). Similarly, attachment of *P. laevis* to the alimentary canal of the chub, *Leuciscus cephalus* (L.), causes mast cell inflammation (Dezfuli, Giari, Simoni, Bosi & Manera 2003a). Piscidins are often expressed by mast cells with piscidin 2 in particular linked with an inhibitory effect on pathogens (Colorni, Ullal, Heinisch & Noga 2008).

The presence of AMPs in the PLL surrounding *C. goldsmidi* may be a combined immune response resulting from both the parasite's actions and the threat of pathogens entering through disruption of the epithelial surface. The inflammatory response resulting from the infestation may have resulted in the accumulation of numerous mast cells increasing the up-regulation of AMPs. Similar cellular responses were observed in chub parasitised by *P. laevis* (Dezfuli et al. 2003a). Given the lack of mast cells in healthy tissue, their abundance in damaged tissue (such as the PLL), and expression of piscidin in the mucous cells, strongly suggests that these cells are an important component of the host defence against this parasite in striped trumpeter. The staining methods used identified the expression of piscidin-positive mucous cells in the affected area with none identified in unaffected areas. To find unstained mucous cells in unaffected areas is unexpected as these cells would not be responding to a stimulus (e.g. disruption caused by an attached parasite).

In this study, pathology is described in relation to parasites attached to striped trumpeter gill filaments. In contrast, Covello, Bird, Morrison, Battaglene, Secombes & Nowak (2009), whilst primarily examining the expression of the pro-inflammatory cytokines, also described the effects of *C. goldsmidi* attached to the gill arch. Covello et al. (2009) found large areas of epithelial and mucosal hyperplasia in close proximity to the parasite; with inflammatory cells in the area adjacent to the attachment site. The present study has identified the extensive development of PLLs and the expression of piscidin 3. As these studies were conducted on naturally infected fish, the attachment site (varying from the gill arch to midway along the filament) and infection duration could not be controlled, which may account for the different observations.

Heavy copepod infestations can reduce gill function, resulting in decreased growth rate due to impaired respiration, as well as mortality (Wang, Li, Yao & Nie 2002; Molnár & Székely 2004; Tsotetsi et al. 2005). Multiple generations, as can occur in *C. goldsmidi* infestations, exacerbate the effects on the host. We have identified numerous pathological changes associated with these parasites including presence of mast cells, PLL, necrosis, and epithelial cell hyperplasia and mucous cell hyperplasia, with lesions most severe in closest proximity to the parasite. We identified piscidin-positive mast cells in this antiparasitic response and other piscidins are known to have potent antiparasitic activity (Colorni et al. 2008). This host response also includes up-regulation of cytokine genes (TNF-α, IL-1β and IL-8) in *C. goldsmidi*-parasitised fish (Covello et al. 2009). It is evident that *C. goldsmidi* has a negative impact on the striped trumpeter even at low infection intensity. This highlights a need for future controlled studies to more precisely determine host–parasite interactions and the factors responsible for the resulting cellular changes and immune response.

24.6 Acknowledgements

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25 PERFORMANCE OF HATCHERY REARED STRIPED TRUMPETER (*LATRIS LINEATA*) ON-GROWN IN SEA CAGES

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25.1 Abstract

Four groups of high quality hatchery produced striped trumpeter juveniles were successfully transported and stocked into sea cages in the Huon River region between October 2006 and October 2007. A total of 2,483 fish were stocked from three hatchery cohorts ranging in mean size from 64 to 193 g. The fish were successfully on-grown for 427 to 793 days. The fish were fed a selection of formulated diets through automated Aquasmart feeders in three 20 m sea cages within separate predator exclusion nets. The trials provided the first hatchery reared striped trumpeter grown to market size in sea cages. Overall survival was 66, 93 and 94% for the three cohorts. Males from all three cohorts were precociously spermiating during spring 2008. Females remained sexually immature. The water salinity was often below full salinity during the winter. Sea temperature at the cage sites throughout the period at sea ranged from 9.4^{o}C to 18.9^{o}C and averaged 13.9^{o}C. Three copepod parasites *Chondracanthus goldsmidi, Caligus nuenonnae* and *Ceratothoa imbricata* were detected on farmed fish but none proved problematic and fish were not treated.

The data collected were used for growth model predictions. The results suggest that southern Tasmanian conditions especially cage culture in a site dominated by freshwater inflow are not optimal for growth. The model predicts that striped trumpeter stocked at 100 g would attain a weight of 1.6 kg within three years at sea. The model suggests that the variation of growth with temperature was not significant. Future trials would be best carried out at higher temperatures in a marine dominated site. The average weight of harvested fish was 1430±9g. HOG (head-on, gilled, gutted) yield was excellent at 94%, while fillets were 61% total recovery for a basic trim and 55% for a full trim. The product can be hot and cold-smoked, with hot-smoked product testing most favourably. A basic economic analysis of striped trumpeter farming suggested it would take 14 years to reach 5000 t production at which point cumulative profit could eclipse cumulative spend.

25.2 Introduction

The Tasmanian salmon industry is Australia's largest and most valued fish producer with ~26,000 t of Atlantic salmon worth >$290 million produced in 2007/8 (ABARE 2009; Battaglene et al., 2008). There is concern that ocean warming associated with climate change could impact the industry (Battaglene et al., 2008). One of the long-term adaptation strategies is the farming of alternative native species. Striped trumpeter is highly prized as

one of the best eating fishes in Australia and was chosen as the top candidate for diversifying sea cage culture in Tasmania in the late 1980's. The current study reports on the first sea cage trials of cultured striped trumpeter and identifies the performance of fish grown in sea cages in southern Tasmania between December 2006 and April 2009.

The capacity to produce high quality striped trumpeter juvenile fish at the Marine Research Laboratories allowed the first ever trials of cultured fish. This was a very significant achievement and marked a turning point in the development of striped trumpeter as a new species for diversifying aquaculture in Tasmania. Before trials commenced detailed negotiations were conducted with the Huon Aquaculture Group (HAG), the second largest producer of salmon in Tasmania. The grow-out trials were conducted under a signed agreement between the company and UTAS. The major considerations of the agreement were:

1. Trials were carried out with the knowledge, necessary permits and approval of the Department of Primary Industries and Water. Huon Aquaculture Group was responsible for obtaining all permits and approvals.
2. UTAS through TAFI made the fish available to Huon Aquaculture Group at no cost, for the purpose of conducting grow-out trials in sea cages. All costs incurred in rearing the striped trumpeter in sea cages, including but not limited to transporting, holding, feeding, treating and harvesting these fish, were the responsibility of HAG.
3. Grow-out trials were carried out by Huon Aquaculture Group at their own risk and neither UTAS nor the Aquafin CRC was responsible for any direct or indirect losses incurred as a result, including the loss of the striped trumpeter, impediments to the production of salmon or any other cause.
4. Striped trumpeter are susceptible to a range of bacterial, viral and parasitic diseases. UTAS through TAFI in good faith explained the health issues to Huon Aquaculture Group representatives and undertook to inform HAGof any disease outbreaks prior to transfer. Huon Aquaculture Group undertook its own risk assessment in relation to all health issues including but not restricted to cohabitation with salmon. Neither UTAS nor the Aquafin CRC was responsible for any loss or damage resulting from any such disease, whether to striped trumpeter or to any other fish.
5. Disposal of the striped trumpeter was at the discretion of HAG which marketed these fish and kept all revenue generated by the sale.
6. Huon Aquaculture Group, with assistance form TAFI staff, carried out regular sampling of the striped trumpeter in cages to measure their growth and condition (and mortalities) and provided the information to UTAS through TAFI.

25.3 Methods

25.3.1 *Stocking*

Four transfers of high quality hatchery-produced striped trumpeter from three hatchery cohorts (ST0601, ST0701 and ST0702), were successfully transported and stocked into sea cages at a Huon Aquaculture Group farm in the Huon River region (Figure 25.1). A total of 2,483 were stocked: 404 fish in December 2006, 1556 fish in March 2007 and 523 fish in October 2007. The details of the fish, when they were stocked and harvested are provided in Table 25.1.

Figure 25.1 Transfer of juveniles to sea-cages

Figure 25.2 Weight checking fish

Table 25.1 Stocking date, last weight check date and age, number of fish and weight of fish.

Cohort	Received	Age (dph)	# Fish	Mean weight (g)	Weight check	Age (dph)	# Fish	Mean weight (g)	Min weight (g)	Max weight (g)
ST0601	10/10/2006	493	404	129	11/12/2008	1286	324	1290	657	1911
ST0701	30/03/2007	337	1556	64	9/12/2008	954	1445	785	238	1426
ST0702	11/10/2007	373	523	105	11/12/2008	800	493	557	256	915

25.3.2 *Husbandry*

The fish were fed on a range of diets from three suppliers, Skretting, Ridley and Alitec (Table 25.2). Dietary information is being held commercial-in-confidence by Huon Aquaculture Group along with temperature and husbandry records. Diets were fed via automated Aquasmart (AQ1) feeders in three 20 m sea cages, contained within separate predator exclusion nets. Problems with feeders and small pellets and the consumption of food by wild fish made Food Conversion Ratio (FCR) data unreliable. Weight checks were

conducted on individual fish on 14 occasions and for pooled fish (bulk weights) on six occasions (Figure 25.2).

Table 25.2 Stocking date, weight of fish at weight checks and feed type throughout the on-growing period.

	ST0601		ST0701		ST0702	
Start	Weight (g)	Type	Weight (g)	Type	Weight (g)	Type
Dec 2006	193	Skretting Nova				
Jan 2007		Skretting Nova				
Feb 2007		Skretting Nova				
Mar 2007		Skretting Nova	64	Skretting Nova		
Apr 2007	413	Skretting Nova		Skretting Nova		
May 2007	481	Skretting Nova	121	Skretting Nova		
Jun 2007		Skretting Nova		Skretting Nova		
Jul 2007		Skretting Nova		Skretting Nova		
Aug 2007		Skretting Nova		Skretting Nova		
Sep 2007		Skretting Nova		Skretting Nova		
Oct 2007	599	Skretting Nova	183	Skretting Nova	105	Skretting Nova
Nov 2007	713	Skretting Nova		Alitec		Skretting Nova
Dec 2007		Skretting Nova		Alitec	153	Skretting Nova
Jan 2008		Skretting Nova		Ridley		Skretting Gemma
Feb 2008		Skretting Nova		Ridley		Skretting Gemma
Mar 2008		Skretting Nova		Ridley		Skretting Gemma
Apr 2008		Skretting Nova		Alitec		Skretting Gemma
May 2008		Skretting Nova	561	Ridley		Skretting Gemma
Jun 2008	1035	Skretting Nova		Ridley	420	Ridley
Jul 2008		Skretting Nova		Ridley		Ridley
Aug 2008		Ridley		Ridley		Ridley
Sep 2008		Ridley	654	Ridley		Ridley
Oct 2008	1189	Ridley		Ridley	473	Ridley
Nov 2008		Ridley		Ridley		Ridley
Dec 2008	1290	Ridley	785	Ridley	557	Ridley

25.3.3 *Health monitoring*

Fish were checked regularly for parasites and disease, and mortalities were removed and recorded. In general, the fish did not experience any major health issues or mortalities except for a small number of fish becoming entangled during the changing of nets.

25.3.4 *Growth models*

Growth was modelled as a function of body mass and sea surface temperature. This mass/temperature model was used to identify periods of under- or over-performance and help identify the optimal temperature for growth. There were suggestions initially that there was underperformance during the summer in the populations compared to the mass/temperature model and these prompted management (net maintenance and diet formulation) changes prior to the summer of 2008/2009.

The data from the summer of 2008/2009 were added to the dataset and this prompted a revision of the model. It was apparent that, despite the management changes, temperature and mass had little impact on growth, the best predictor of growth was age, this is unusual and is indicative of some overriding limit on “normal” growth. A model projecting growth as a function of days from hatch was produced using proprietary software kept in-confidence (D. Whyte personal communication).

25.3.5 *Recovery rates and taste tests*

Thirty fish were harvested from ST0601 on 16th December 2008. These fish were dip-netted, weighed to target fish within 1300-1500g, killed by a blow to the head, bleed bled via a cut to the gills and packed on ice before transferring to the processing facility at Port Huon. All fish were weighed again and groups of fish were weighed at various points during the gutting and filleting process including the final full-trim. Ten fish (20 fillets) were sent to Hideaway Bay for fresh taste testing and 20 fish were sent to Mt Barker in Adelaide for cold-smoking (20 fillets) and hot-smoking (1h cold-smoke = 10 fillets and 12h cold-smoke = 10 fillets). Tasting sheets were completed for all products tested.

25.4 Results

After 793, 617 and 427 days at sea the striped trumpeter from cohorts ST0601, ST0701 and ST0702 had reached 1290, 785 and 557 g average weight, respectively. All cohorts maintained normally distributed frequencies at each weight check and had Coefficients of Variation (CV's) of 16.3, 24.6 and 17.6 for ST0601, ST0701 and ST0702, respectively at their last weight check in December 2008 (Figure 25.3). The maximum fish weight within each cohort was on average 65% heavier than the average for the population (Table 25.1). Overall survival was 66, 93 and 94% for ST0601, ST0701 and ST0702, respectively and included fish that were culled at weight checks (i.e., survival was better than estimates). Conditions for growing an oceanic deepwater fish were not ideal at the lease site. The water salinity was often below full salinity during the winter. Sea temperature at the cage sites throughout the period at sea ranged from 9.4^{o}C to 18.9^{o}C and averaged 13.9^{o}C.

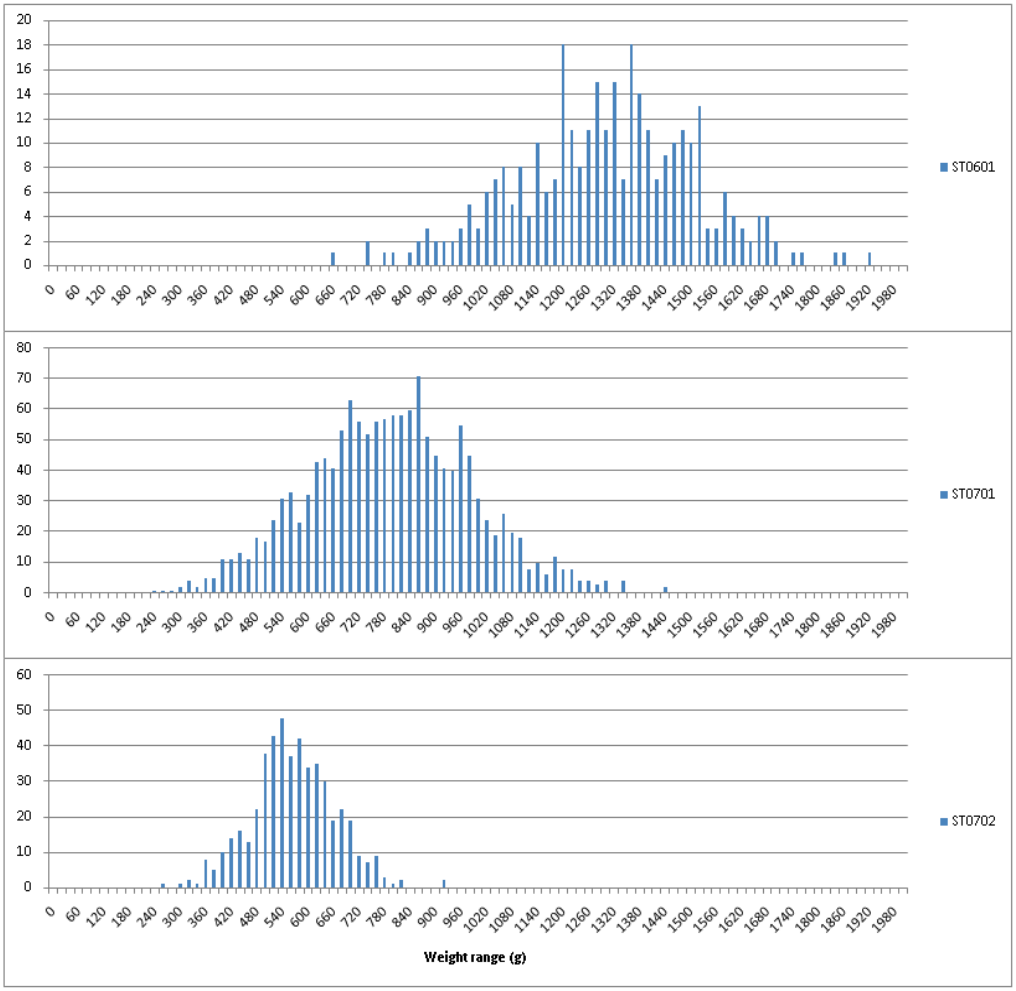

Figure 25.3. Frequency distribution of wet weight (g) of striped trumpeter from cohorts ST0601, ST0701 and ST0702 at 1286, 954 and 800 days post-hatch, respectively.

Almost all the fish sampled were in excellent condition. Some of the stocked fish had minor lesions on the first weight check but these had generally cleared by the second weight check. It appears the lesions may be due to acclimation to the sea cage environment and fouling on the nets. Three copepod parasites *Chondracanthus goldsmidi, Caligus nuenonnae* and *Ceratothoa imbricata* were detected on farmed fish. *Chondracanthus goldsmidi, and C. nuenonnae* were uncommon. The highest prevalence of *C. nuenonnae* was 2.5% in one sea cage, while the highest prevalence of *C. goldsmidi* was 3.3% (Andrews, 2010).The isopod *C. imbricata* was recorded in increasing prevalence in two cohorts during 2008, ranging in prevalence from 9.8% to 17.5% in one cohort and from 27.7% to 67.2% in the second (Andrews, 2010). Increasing isopod numbers may be a result of transference from wild fish species such as mackerel that were more common in cages with high parasite loads. No treatment of fish was undertaken during the trials.

Fish from ST0601 performed to the mass/temperature model on four out of six occasions, but under-performed in both summer periods. ST0701 and ST0702 showed a similar lack of performance against the model during the summer (Figure 25.4). This prompted a re-evaluation of the model. Generally speaking fish growth is impacted by three factors; body mass, temperature and sexual maturation. For the second successive year the populations did not respond to the increasing summer temperatures with increasing growth rates. This was unexpected and remodeling the growth data showed a strong correlation with age, and temperature was not a factor in weight gain (Figure 25.5). Males from all three cohorts were precociously spermiating during spring 2008. Females remained sexually immature.

The average weight of harvested fish was 1430±9g. Harvested fish had a cumulative HOG and fillet recovery of 94.4 and 58.1%, respectively, which is not dissimilar to Atlantic salmon with 88.5 and 62.4%, respectively (Table 25.3).

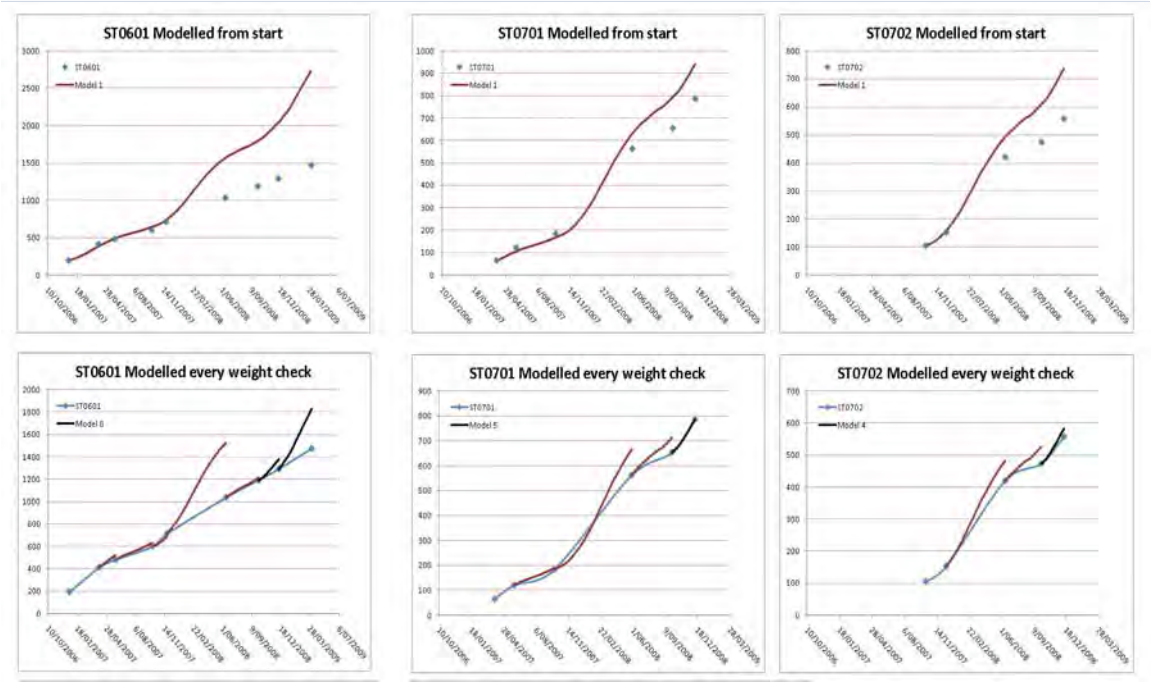

Figure 25.4 Model data from start and for each weight check compared with actual growth data for ST0601, ST0701 and ST0702.

Table 25.3 Comparison of yield data for striped trumpeter and Atlantic salmon.

	Striped trumpeter			Atlantic salmon		
	Recovery (%)	Cum recovery (%)	Cum loss (%)	Recovery (%)	Cum recovery (%)	Cum loss (%)
Bled	100.0			100.0		
HOG	94.4	94.4	5.6	88.5	88.5	11.5
Fillets	61.6	58.1	41.9	70.5	62.4	37.6

Fresh product was inadvertently frozen for two days (placed in freezer on ice rather than in chiller on ice). Upon thawing, fillets were cooked and the response for taste was positive. The flesh had a clean, white colour, pulled apart in large flakes and was considered to have a very good taste.

The cold and hot-smoked product was sampled at Mt Barker and Hideaway Bay (Table 25.4). Water phase salt is critical in cold smoked product (>3.5%), but not in hot smoked product due to the heat treatment of hot smoking. The results for the cold smoked product varied from 'unusual', 'very salty' and 'chewy' to 'perfect', 'delicious' and 'beautiful', highlighting the differences in preference between samplers. In contrast to the cold-smoked, both hot-smoked products (1hr CS and 12 hr CS) were well received across the board with generally favourable comments. Over 2 t of fresh striped trumpeter was sold to local retail outlets just before Easter 2009. The consumer feed back was highly positive and demand was high with retailers requesting more product.

Table 25.4. Product results from Mt Barker for cold-smoked and hot-smoked after 1 or 12 hrs cold-smoking.

	Cold smoked	**Hot smoked (1hr CS)**	**Hot smoked (12 hr CS)**
Taste	Very salty, smokey & chewy. Unusual flavour and texture.	Very moist, very salty, but good flavour.	Less moist but very smokey.
Salt (%)	4.23	67.45	5.9
Moisture (%)	3.34	56.57	5.58
Water phase salt (%)	3.03	52.93	5.41

25.5 Discussion

The mass/temperature model suggests that fish from ST0701 and ST0702 under-performed within the size range 183-654 g and 153-473 g, respectively. However, fish from ST0601 performed to model within the approximate size range (193-599 g), suggesting under-performance of the younger cohorts the following season rather than failure of the model.

Although fish were fed different diets, all fish under-performed to model between Dec 2007 and May 2008. Temperature was similar both seasons (13.1-18.5^{o}C first season when ST0601 performed to model and 12.0-18.9^{o}C second season; with means of 15.18 and 15.48^{o}C, respectively), suggesting temperature was not the cause of the under-performance. Heavy fouling of nets during the second season was noted during the second summer and may have been a causative factor for under-performance across all three cohorts. Net hygiene was improved for the third season and there was no growth increase in response to the higher ambient temperatures. It would therefore appear that there was no temperature mediated growth response in striped trumpeter or that there was another factor that prevented a temperature mediated response from being expressed. The suppression of "normal" response has been seen in other species, for example Atlantic salmon at low water temperatures will adopt a growth rate that is independent of mass (D. Whyte personal

observation). This suggests that the temperature range experienced by the fish during this trial was not optimal and further work will be needed to determine causes of poor growth.

Using ambient sea temperatures from the farm the mass/temperature model suggests that fish put to sea at 104 g mean weight at 365 days post-hatch (i.e., same as for ST0702) would reach an average weight of 1.62 kg within two years (three years from hatching; Figure 25.5). As striped trumpeter are caught throughout Tasmanian waters year-round, but predominantly in the NE of the state off St Helens in summer, it is likely that they have a higher temperature optima than Atlantic salmon and a preference for waters warmer than experienced within the Huon River. When sea temperature within the model was raised by one and two degrees the average weight attained for fish within two years increased to 2.15 and 2.77 kg, respectively.

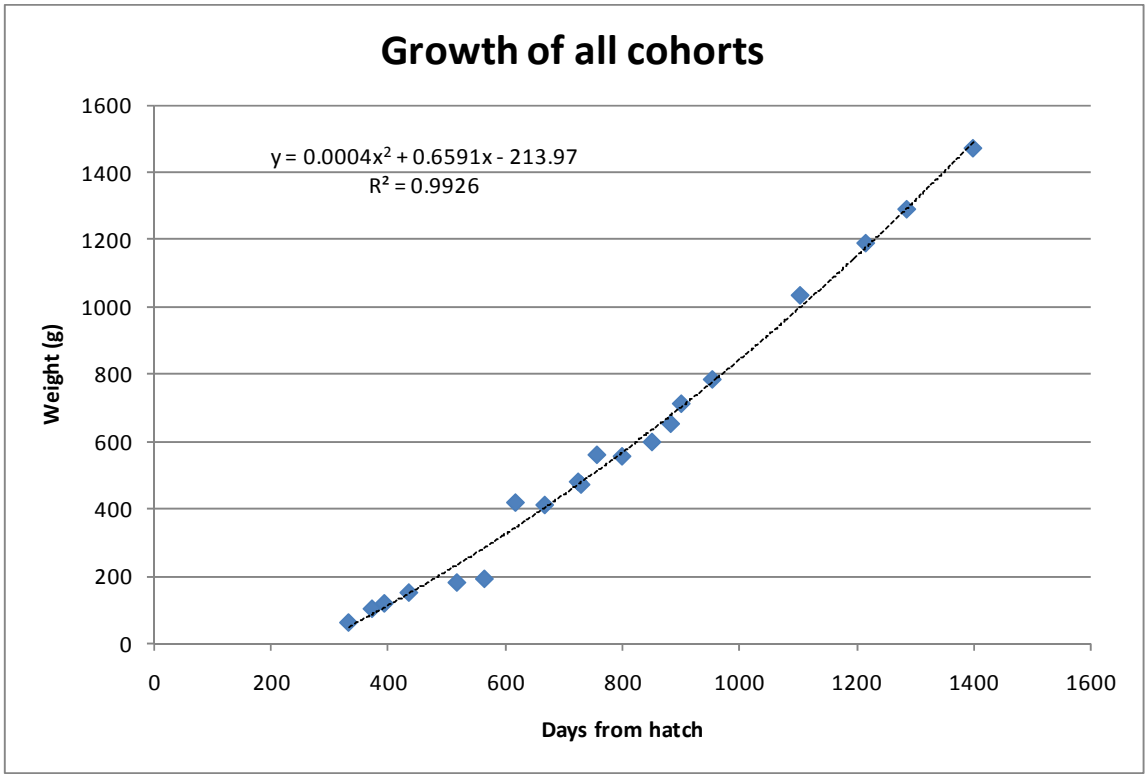

Figure 25.5 Individual weight checks plotted against age for all cohorts.

The mass/temperature model suggests that juveniles put to sea three months earlier (i.e.,100 g at 270 days) and experiencing 2^{o}C warmer water than in the Huon River would reach 3.2 kg after just over two years at sea (three years from hatching) (Figure 25.6). It is anticipated that better quality juveniles, with fewer deformities and uncompromised growth in the hatchery, would perform better at sea and that improved farm management including higher stocking densities and tailored feed regimes would result in further growth improvements. The size range in the existing populations supports the capacity for improved growth, with the largest fish within ST0701 being 82% heavier than the average weight for the population.

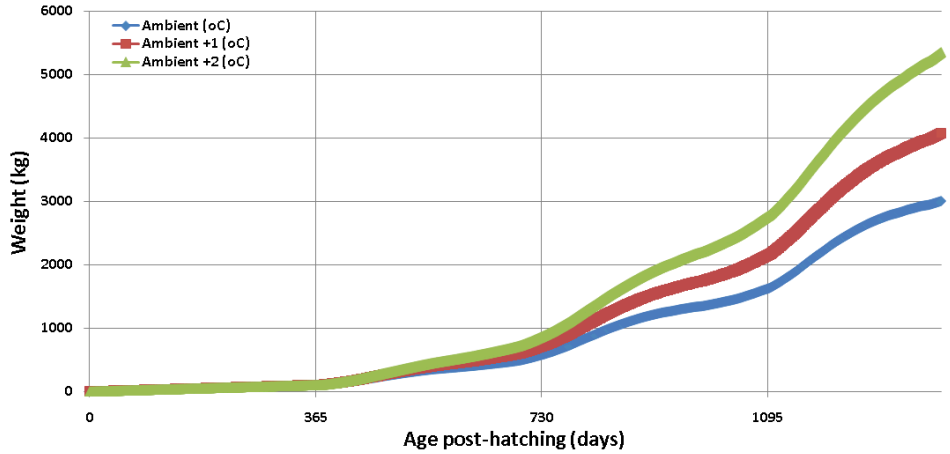

Figure 25.6 Modelled striped trumpeter growth data under ambient sea water temperature, based on the last three years data, and at simulated increased temperatures of 1 and 2^{o}C.

Yield from the striped trumpeter was excellent for HOG (94.4%) and, while not as good as for Atlantic salmon, quite good for fillets (basic trim = 60.5%; full trim = 54.9%). Value-adding of product appeared possible with good feed-back for cold-smoked and very good feed-back for hot-smoked.

An assessment of the economics of farming striped trumpeter, based on Atlantic salmon operating costs and a profit margin of 15% (post feed, labour, packaging, transport and marketing) suggested cumulative profit would eclipse cumulative spend after around 14 years of farming (Table 25.5). This coincided with harvests reaching 5,000 tonnes per annum (based on a 148% increase in fish harvested each year).

Table 25.5 Economic assessment of striped trumpeter farming based on growth per annum of 148% and profits of 15% after feed, labour, packaging, transport and marketing costs.

Stocked	Number of Pens	Pen purchases	Cost per pen ($)	Total ($)	Cumulative spend ($)	Harvested	Tonnes[1]	Sales Price per Kg	Profit[2] ($)	Cumulative Profit ($)
31/12/2012										0
31/12/2013	6	4	100,000	400,000	400,000					0
31/12/2014	7	1	100,000	100,000	500,000					0
31/12/2015	10	3	100,000	300,000	800,000	31/12/2015	100	$12	180,000	180,000
31/12/2016	15	5	200,000	1,000,000	1,800,000	31/12/2016	148	$12	266,176	446,176
31/12/2017	21	6	200,000	1,200,000	3,000,000	31/12/2017	219	$12	393,610	839,787
31/12/2018	31	10	200,000	2,000,000	5,000,000	31/12/2018	323	$12	582,054	1,421,841
31/12/2019	46	15	200,000	3,000,000	8,000,000	31/12/2019	478	$12	860,717	2,282,558
31/12/2020	68	22	200,000	4,400,000	12,400,000	31/12/2020	707	$12	1,272,792	3,555,350
31/12/2021	100	32	200,000	6,400,000	18,800,000	31/12/2021	1046	$12	1,882,151	5,437,502
31/12/2022	100	0	200,000	-	18,800,000	31/12/2022	1546	$12	2,783,245	8,220,747
31/12/2023	100	0	200,000	-	18,800,000	31/12/2023	2287	$12	4,115,745	12,336,493
31/12/2024	100	4	200,000	800,000	19,600,000	31/12/2024	3381	$12	6,086,190	18,422,683
31/12/2025	100	1	200,000	200,000	19,800,000	31/12/2025	5000	$12	9,000,000	27,422,682

25.6 Acknowledgements

Andrew Bourke, Robbie Churchill and Colin Johnson maintained fish at sea and provided assistance with all weight checks and sampling. Dave Mitchell, Dale Russell and Mark Garland assisted with nets, feeds and management of farm operations. Staff from Skretting and Ridley supplied feed. Staff from TAFI assisted with all weight checks and sampling, Ross Goldsmid is thanked for coordinating TAFI staff.

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