Improvements in Yellowtail Kingfish Larval Survival and Juvenile Quality

Mike J. Thomson

Project No. 2009/749

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

This project was conducted by Clean Seas Tuna Ltd, in collaboration with SARDI, Flinders University, Institute for Marine and Antarctic Studies, and Northern Territory Government, Department of Primary Industries, Fisheries and Mines.

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Abbreviations

AB	Arno Bay hatchery
CIT	Challenger Institute of Technology, Fremantle, WA
CST	Clean Seas Tuna Ltd
DAC	Darwin Aquaculture Centre
DPH	days post-hatch
HPF	hours post-fertilization
PA	Port Augusta hatchery
SARDI	South Australian Research and Development Institute
TAFI/IMAS	Tasmanian Aquaculture and Fisheries Institute / Institute of Marine and Antarctic Studies
YTK	Yellowtail Kingfish

Definition of Key Terms

Fingerling:	A fish that has passed through the larval phase such that it has been fully weaned off live foods and is eating a formulated diet. Most organs are developed and the fish has more control over its position in the water column.
Hatchery run:	Broodstock YTK are conditioned and induced to spawn for a few days. Each spawning event can produce 20-100 million fertilized eggs. These numbers are sufficient to fill the capacity of the larval rearing and live food systems in the hatchery.
Hatchery season / Hatchery year:	The YTK hatcheries usually operate from August to October to undertake sufficient (usually 2 or 3) hatchery runs to produce the target number of quality fingerlings that can be stocked into seacages from September to December.
Hatchery:	A facility where fertilized eggs are reared through egg incubation, larval rearing and nursery/fingerling stages.
Larvae:	A newly hatched YTK fish that is ingesting live prey as food. Not all organs will have developed, and they usually have little control over their position in the water column.
Live feeds:	In the case of YTK this is a combination of microalgae, rotifers and *Artemia* nauplii, with the regime dependent upon the age and developments stage of the fish.
Malformation:	Developing larvae can be affected by endogenous and exogenous factors such that the spine, bones in the jaw and head, and/or swimbladder may not form properly. This can give rise to problems with the fish as it grows.

NON TECHNICAL SUMMARY

PROJECT 2009/749 Improvements in Yellowtail Kingfish larval survival and juvenile quality

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PROJECT OBJECTIVES:

1. To identify improvements to be made to commercial scale YTK larval and juvenile rearing systems and procedures resulting in higher survival (> 25% by end 2010), better growth, reduced levels (<5% by end 2010) and severity of malformations and more cost efficient juvenile production.

2. To assess the suitability of some novel larval and juvenile rearing techniques for YTK: recirculating intensive larval rearing system with semi-automatic feeding, artificial light, algal paste, small rotifer, high prey density and early weaning.

3. To test a range of key biotic and abiotic factors and rearing strategies on YTK larvae and juveniles in replicated tanks and identify optimal regimes for adoption in commercial scale hatcheries.

OUTCOMES ACHIEVED TO DATE

This project has confirmed that the low survival rates and high malformation rates that have previously been observed in two commercial Yellowtail Kingfish hatcheries (at Arno Bay and Port Augusta, South Australia) operated by Clean Seas Tuna Ltd were also experienced at two separate research hatchery facilities (SARDI and Darwin Aquaculture Centre) with YTK eggs supplied by Clean Seas and an independent research facility, the Challenger Institute of Technology. This strongly indicates that cause(s) of the problems are intrinsic to the species, and reconfirmed the need for commercial and research hatchery experts to work collaboratively to identify the cause(s) and ways to mitigate them.

The long term planned outcomes for this project were:

- Higher survival rates (>25% from fertilised eggs to 5g juveniles within 2 years), lower production costs and greater efficiencies in Australian Yellowtail Kingfish hatcheries

• Lower malformation rates (<5% commercial malformations up to 5g within 2 years) and
higher quality juvenile outputs in cultured Yellowtail Kingfish and therefore more cost-effective production of fingerlings

Specific short term outcomes included:

• Recommendations on how to improve larval and juvenile rearing systems and procedures
leading to better survival rates and reduced levels of malformation

• Rapid adoption of improvements in rearing techniques

Some progress was made meeting the short term outcomes of this project, but no major breakthroughs were made towards achieving the long term outcomes.

The work done at the Clean Seas hatcheries during this 2008-2009 project demonstrated shortcomings in both the infrastructure and company expertise to conduct the highest quality R&D within the organization. These findings provided some of the impetus to invest in a new R&D hatchery facility and the appointment or secondment of research trained staff, and to undertake substantial improvements in practices and procedures, training and quality control of hatchery staff. The collaborative nature of this project has increased the ability of staff to network with others to discuss ideas and issues.

OUTPUTS PRODUCED

To date (May 2012) three manuscripts have been prepared from the SARDI, Flinders University and IMAS components of the work (Appendix C) and their publication status is:

- Chapter 3: Effect of water temperature regimes on survival, growth and the incidence of jaw deformity during Yellowtail Kingfish, *Seriola lalandi*, larval rearing. Is being combined with some of Zhenhua Ma's PhD data into a more comprehensive manuscript.
- Chapter 4: Effect of live food feeding protocols on Yellowtail Kingfish *Seriola lalandi* larval performance: survival, growth and jaw malformation. Has been submitted to an international aquaculture journal and is being revised prior to acceptance.
- Chapter 5: Effect of age of Yellowtail Kingfish (*Seriola lalandi*) larvae on success of weaning from live food to a formulated diet. Manuscript in final stages of preparation.

In addition, two PhD theses will be submitted from Seafood CRC supported students through Flinders University.

A number of Clean Seas Tuna hatchery Standard Operating Procedure (SOP) documents have been written and/or updated as a result of the work carried out during this project.

These SOPs remain commercial-in-confidence as Intellectual Property of Cleans Seas Tuna Ltd.

NON TECHNICAL SUMMARY

This project was a collaborative approach to try to understand the cause(s) and ways to mitigate a number of production performance issues that have affected Yellowtail Kingfish (YTK) hatcheries for 10 years. The problems include low survival rates of larvae (commonly only 10-15% of hatched larvae survive to juvenile stage), and the high incidence of jaw malformations that can be severe enough to require 20-40% of juveniles to be culled. The cull is necessary because severe jaw malformations will affect how quickly the fish grow, and therefore how long it takes for them to reach a marketable size, and also even if they reach harvest size they need to be sold as fillets because the appearance of the fish would affect their marketability.

The research approaches used in this project were either (a) correlational, that is looking for relationships between key biotic and abiotic variables and larval survival or juvenile quality parameters using information recorded in the Clean Seas hatchery database, or (b) experimental, using replicated tank systems, several treatment groups and only changing one parameter at a time. The correlational approach did not reveal any key parameters that were either positively or negatively associated with survival or malformation rates, but this was almost certainly because of the variability and inconsistencies in the actual hatchery rearing conditions between each tank, hatchery run, hatchery site or spawning season. However, a review of this activity identified a disconnect between some of the senior hatchery management planned larval rearing strategies and the operational priorities of some of the hatchery workforce. Substantial changes have since been made to communications between the different levels of the organization to correct this.

The experimental approach proved extremely useful with clear results on the optimal water temperature regime for larval rearing, suitable rotifer and *Artemia* feeding densities, and optimal ages for weaning of larvae onto microparticulate diets. Whilst the survival rates of larvae did differ between treatment groups in these experiments, the growth rates, jaw and swimbladder malformation rates did not and remained higher than what is desirable. Thus, whilst these experiments provided some information that will help to increase larval survival, there is no immediate solution to the issue of poor juvenile quality.

An alternative larval rearing system using an intensive approach with constant addition of live feeds was trialed at the Darwin Aquaculture Centre. Only 2 batches of eggs were placed into the system, and although several thousand fish made it to day 20 or 26 post-hatch, the

overall survival rate and incidence of malformation were still very poor, probably as a consequence of the challenging egg and early larval life culture conditions. The fact that any larvae survived in this system is encouraging for further trials.

This project has proved that reducing the mortality and malformation rates of YTK larvae remains a substantial challenge, and that the cause(s) are not simple or straightforward. However, the collaboration shown in the execution of this project between all the teams has been a significant benefit to getting closer to the cause(s) of these problems.

KEYWORDS: Yellowtail Kingfish, larval rearing, live food, fingerling quality, jaw malformation, swimbladder malformation

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Dr John Carragher (Logifish Consulting) for reviewing, editing and finalising the draft report.

1. Background

In response to the increased demand for seafood and for recreational and customary fishing experiences Clean Seas Tuna Ltd. (CST) is a participant in the Australian Seafood Co-operative Research Centre (CRC). There are a suite of nine research projects addressing nine objectives set out in the overarching program of activity described in project 2007/707. The nine projects collectively endeavour to resolve the larval rearing, juvenile development and productivity constraints for propagated Southern Bluefin Tuna, *Thunnus maccoyii,* as well as continue to make improvements to the production of Yellowtail Kingfish, *Seriola lalandi* (YTK). The activities described in this report address objective 4 of that project, which aims to increase survival rates of YTK larvae to more than 25%, and reduce malformation rates to less than 10% before hatchery grading.

Current yields of high quality juvenile Yellowtail Kingfish are unacceptably low in Australian marine hatcheries. The survival rate is the number of juveniles that are transferred to sea divided by the total number of newly hatched larvae that were stocked into the larval rearing tanks in that hatchery run. Mortality values include both the number of fish that die in the tanks during the ~30 day hatchery phase, as well as the number of juvenile fish culled by hatchery staff (i.e. to eliminate malformed fish from transfer to at-sea growout cages) to meet industry quality standards. Thus, the percentage of juveniles (circa 5g bodyweight) transferred into sea cages for CST commercial YTK production runs between 2004 and 2007 was between 3 and 19% of newly hatched larvae (M. Deichmann, pers. comm. and CST production records).

At CST, larval mortality and juvenile deformity levels can be high and show substantial variability between rearing tanks, hatchery runs, hatchery sites and years. Common malformations include problems with the jaw and mouth, operculum, spinal column and swimbladder inflation (Cobcroft et al., 2004). These particular problems, and the high rates of mortality/culls, are by no means unusual for hatcheries rearing *Seriola* (the genus to which the YTK species belongs; Benetti et al., 2005; Kolkovski and Sakakura, 2007), or indeed most marine finfish hatcheries in early years of production. However, as most marine aquaculture industries have matured, the normal pattern has been that the mortality and cull rates have decreased as hatchery practices and procedures improve. At the time this project was initiated (2008), this was not the CST experience despite significant R&D on causes of mortality and malformations, and the potential for these to be minimised through alternative approaches and practices in YTK larval rearing. Some of these efforts are described below:

Seafood CRC project 2007/718 "Identify timing and nature of jaw deformities in YTK and scope the likely causes of this condition" was led by the University of Tasmania through the

Tasmanian Aquaculture and Fisheries Institute (TAFI; now IMAS) and provided good developmental and diagnostic information, and some useful results on the effects of various biotic and abiotic factors on the frequency and extent of jaw deformities (Battaglene and Cobcroft, 2008). Some alterations to commercial YTK rearing conditions instigated from that project have shown initial promise but a thorough understanding of the major underlying causes has not yet been achieved and practical steps to reduce deformities on a lasting basis have not yet been identified.

In this regard, further progress was likely to be made in the concurrent Australian Research Council (ARC-Linkage LP0882042) project “Reducing skeletal malformations in cultured marine fish using gene expression, improved nutrition and advanced system operation” led by TAFI/IMAS with CST, Western Kingfish and the Darwin Aquaculture Centre as industry partners in the three year study which started in April 2008.

The aquaculture team at Flinders University and SARDI focused considerable effort on the capability of YTK larvae to take exogenous feed by describing the structure and functional development of the digestive system. These studies characterized the ontogeny of the digestive system including the enzyme circadian rhythms, response to starvation and also the response to dietary changes including early weaning (Chen et al., 2006; 2007). This work will be extremely useful to YTK hatchery staff as they try to match the food type, density and nutritional value to the development stage of the larvae in order to avoid mortality through inappropriate feeding regimes and practices.

Thus, this project, by undertaking a number of trials with expert researchers from SARDI, Flinders University, TAFI/IMAS and the Darwin Aquaculture Centre, as well as at both CST commercial hatcheries, should make considerable progress toward reducing mortalities and culls due to poor larval growth, inappropriate larval behaviour, skeletal malformations, poor swim bladder inflation and poor weaning success.

The variety of biotic and abiotic factors being tested across the different components of this project include:

- Egg quality factors and egg transportation practices
- Light conditions: Artificial versus Natural
- Water supply: recirculation versus flow through
- Rearing temperature and salinity
- Tank setup and hydrodynamics
- Tank colour and pattern
- Microbial characterisation and microbial management strategies in rearing and live food systems

- Nutrition: e.g. INVE Culture Selco®, yeast and live algae versus algal pastes, enrichment strategies
- Larval stocking rates and live food (prey) density interactions
- Weaning strategies: products and timing

Whilst genetics has not been identified as a major factor in YTK deformities, the implementation of a broodstock development program by CST may bring significant improvements in larval survival through domestication selection. Scoping of genetic options going forward is the subject of a further CRC project: 2008/723 "The development of a genetic management and improvement strategy for temperate marine finfish (SBT, YTK & Mulloway)".

1.1 Need

YTK aquaculture is a rapidly expanding industry in Australia, particularly in rural South Australia. CST is the largest producer of YTK in Australia having produced over 1.25 million juveniles in both 2007 and 2008 and over 800,000 in 2009. The company operates two hatcheries at Arno Bay and Port Augusta to achieve these numbers. The production of quality larvae from both hatcheries underpins the commercial growout of YTK for harvest at age 2 years and 3.5-4.5kg bodyweight, and thus low larval survival and high levels of culls significantly decreases the number of juveniles available for stocking into sea cages as well as substantially increases their cost.

Survival of Yellowtail Kingfish juveniles in Australian marine hatcheries is very low in comparison to many other marine species such as sea bass and bream produced in larger more mature industries, for example in Europe. In addition, several skeletal malformations have been reported in marine hatcheries in Australia and New Zealand (e.g. Trotter et al., 2001; Cobcroft et al., 2004; Cobcroft and Battaglene, 2009). Overall mortality rates, from to the summation of deaths occurring through the larval rearing stage and the malformed juveniles that are culled during laborious hand-sorting, are increasing the workload of hatchery staff and putting extra strain on facility infrastructure, and broodstock productivity in order to meet a particular target quota of fingerlings for growout. These factors are also likely to be at least partly responsible for the high variability in hatchery survival rates and the rate and severity of deformities between different production runs and commercial hatcheries (Kolkovski and Sakakura, 2007; Battaglene and Cobcroft; 2008). Both of these issues need to be overcome in order to reduce the costs of producing juvenile YTK from hatcheries to be stocked at sea for growout.

1.2 Objectives

The objectives of this project, and the progress made in addressing each of them is described below:

1. To identify improvements to be made to commercial scale YTK larval and juvenile rearing systems and procedures resulting in higher survival (> 25% by end 2010), better growth, reduced levels (<5% by end 2010) and severity of malformations and more cost efficient juvenile production.

This objective was not achieved during this project, though some progress was made towards identifying the main causal factors.

2. To assess the suitability of some novel larval and juvenile rearing techniques for YTK: recirculating intensive larval rearing system with semi-automatic feeding, artificial light, algal paste, small rotifer, high prey density and early weaning.

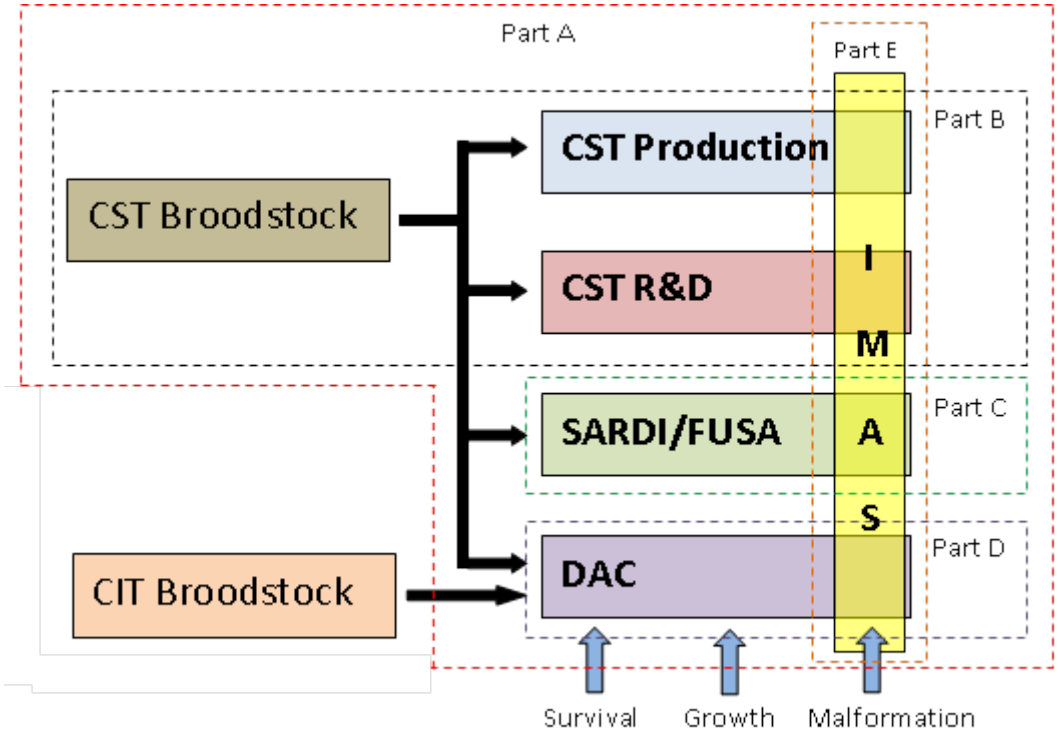
This objective was partly achieved during this project, with 2 batches of YTK eggs being reared in the intensive larval rearing system at the Darwin Aquaculture Centre. Artificial lighting, algal paste and early weaning studies were conducted the CST hatchery at Arno Bay and at the SARDI facility in Adelaide. Problems with egg quality after transport to Darwin and the water parameters at the DAC hatchery prevented a proper evaluation of the suitability of these approaches.

3. To test a range of key biotic and abiotic factors and rearing strategies on YTK larvae and juveniles in replicated tanks and identify optimal regimes for adoption in commercial scale hatcheries.

This objective was partly achieved during this project, with replicated experiments at the SARDI facility in Adelaide assessing the best water temperatures, identifying the best rotifer and Artemia feed densities and the earliest age for effective weaning for maximising larval survival and reducing malformation rates. Although these studies provided robust data, particularly on the temperate and weaning questions, the overall levels of larval survival and malformation were still too high.

1.3 Design and Structure of this Final Report

The multi-component nature of this project, with each of the four contributing groups being responsible for different aspects of the research (e.g. CST supplied batches of eggs for DAC and SARDI/Flinders experimentation, as well as its own trials), or analysing samples produced at several facilities (e.g. the IMAS team assessed larval deformities from both CST hatcheries, and the DAC and SARDI runs), and the way in which these different activities were contracted to each collaborating organisation by Seafood CRC, have all affected how this final report is constructed (see diagram).



Diagrammatic representation of how the different subprojects were arranged with respect to the source(s) of YTK eggs and their common overlaps with subproject E. Abbreviations: CST, Clean Seas Tuna Ltd.; SARDI, South Australian Research and Development Institute; FUSA, Flinders University of South Australia; DAC, Darwin Aquaculture Centre; IMAS, Institute of Marine and Antarctic Studies; CIT, Challenger Institute of Technology.

The contributions from the four collaborating parties have each been retained as complete documents, included as Appendices B, C, D and E. They have been edited and formatted to make them appear to be generally consistent with each other, but also, and more importantly, so as not to affect the authenticity of the researcher contributions, and potentially misconstrue the expert scientific interpretation of the data, by 'cutting and pasting' all of the supplied material into single document.

The initial section of the report (Part A) has taken a 'whole of project' approach and has been put together by the Principal Investigator. This section of the report cross-references to the appropriate data in the various Appendices, as and when required, in order to bring together the key findings and outcomes of the project. This report structure will reflect and emphasise the inter-organisational linkages that were intended when the detailed planning phase of the project occurred. In this way, the benefits of the collaborative research approach taken in this project are clearly evident.

Note:

The work described in this report was carried out in the 2008 and 2009 YTK spawning seasons, therefore the procedures described herein reflect the YTK larval rearing practices at that time. As a result of this project, and the others carried out by our CRC collaborators, many of these practices and procedures will since have been modified as necessary. In this way, the hatchery manuals and SOPs developed by CST are very much 'living documents'.

2. Key Findings

The approach of this section will be to consider the process of producing juvenile YTK for at sea stocking from the beginning, broodstock and egg quality, through egg incubation (including the transport of fertilized eggs), egg hatching and larval rearing. It will consider the biotic and abiotic conditions that have been found to either affect survival and/or malformation rates. The critical issues of live feed, jaw malformation and swimbladder inflation will be dealt with in specific sections. Finally, there will be some comments about improving hatchery practices and procedures, the value of SOPs and rigorous adherence to planned strategies.

2.1 Egg Quality

With the exception of 1 batch of eggs which was obtained from the CIT, Fremantle, WA, all the other activities in this project were derived from commercial YTK broodstock at CST hatcheries at Arno Bay and Port Augusta. CST hatchery records for 2008-2009 (the duration of the project), show there were 279 spawning events by 4 different groups of broodstock across both hatcheries (CST component; Appendix B). Mean egg diameter (1.3-1.5mm), mean oil droplet diameter (0.27-0.35mm), mean fertilization success (>90%) and mean egg viability (>90%) were consistent from batch to batch, and hatchery to hatchery, and year to year. Occasionally, egg viability rate (%) would drop below 90%, usually in the first few spawning events after spawning was initiated for a particular group of broodstock. Of the 279 spawning events, eggs (0.7-2.5 million) were collected (usually from one, but on some occasions eggs from two broodstock tanks were combined) and incubated as a total of 46 batches that were taken through the two CST hatcheries.

Attempts were made to determine the biochemical quality of fertilized eggs during this project to see if there were inconsistencies in fatty acid and vitamin contents of YTK eggs and those of other species, and whether there was variation between broodstock groups and runs. Despite collecting multiple lots of samples from both CST hatcheries, the data generated are probably of limited value due to violations of the recommended storage protocols. Fatty acids, particularly the polyunsaturated omega-3 variety, are susceptible to degradation even at -18^{o}C. Similarly, the antioxidant vitamins E and A are also prone to degradation. Thus, the apparent fatty acid profiles and vitamin concentrations reported in the IMAS component of this project (IMAS component; Appendix E), are to be interpreted with caution. At best, some batches of eggs might have undergone little degradation; at worst, a batch could have lost the majority of the initial content of these substances. Unfortunately,

there is no way of identifying how much post-sampling degradation a batch of eggs has undergone.

If the fatty acid and vitamin content data are taken at face value, the levels in 2008 and 2009 were generally lower than comparable data from 2007 or reported values from YTK in New Zealand or published work on other species. High levels of variability between different batches of eggs collected in 2008 and 2009 would suggest that the lower values probably reflect degraded samples and consequently they will drag down the apparent average concentrations.

Since this project (2008-2009), the new R&D hatchery has been equipped with a -60^{o}C freezer, which should significantly reduce the risk of post-sampling degradation of samples.

2.2 Egg Incubation

Egg incubation proved to be a considerable issue and, if not properly attended to, had serious consequences for the viability of that batch of eggs/larvae. The critical issues were, firstly, that although the YTK eggs were positively buoyant immediately after fertilization, over time, as the embryo developed, the eggs would become negatively buoyant. Secondly, the eggs needed to be kept in suspension in the water column; eggs that settled out on the bottom of the tank, even for a short time, would die or have delayed development and potentially compromised larval quality. Thirdly, the consequences of too vigorous aeration or water circulation to keep the eggs in suspension, might impart mechanical disruption of the yolk or oil droplet, which could, in turn, affect larval development (see in particular the DAC component; Appendix D).

Every hatchery has egg incubation tanks that are different sizes, shapes and with different configurations for water inlet and outlet flows, and means of aeration. All of these factors would affect the hydrodynamics within the tank such that each tank configuration would need to be ‘tuned’ to the batch of eggs placed into it. Unfortunately, the changing buoyancy of the eggs during development means that the tanks would need to be re-tuned several times during the incubation phase to prevent problems. Fortunately, each hatchery will have relatively few egg hatching tanks that will be used for every batch of eggs, so investment of time and hardware to create suitable hydrodynamic conditions in these few tanks will be rewarded many times over. SOPs have been modified to ensure that levels of aeration in each incubation tank are assessed every 2 hours in order to keep the eggs in suspension.

Decontamination of eggs to reduce microbial contamination and control the buildup of a microbial load on the egg surface using ozonated water was instigated at the CST

hatcheries during this project on the advice of collaborators who have proven the approach extremely useful in their facilities with other species. There was, however, insufficient or inconclusive data from the CST facilities to show that the ozonation treatment had any measureable benefit. Microbial loads in control (unozonated) eggs and incubation water were both low due largely to the excellent system of filtration and UV pre-treatment of the hatchery seawater supply (CST and IMAS components; Appendices B and E). At the same time, there were no data to suggest that the ozonation treatment did any harm to the eggs. Under the circumstances, it is considered sensible to take a precautionary approach and ozonate the eggs to minimise the risk of introduction of a microbial agent either from the broodstock or from the seawater supply if the filtration and UV system were compromised. An SOP for ozonation of eggs has been introduced to the hatchery for treatment of all batches of YTK eggs, not just those being sent to other hatcheries.

The mean water temperature for YTK egg incubation for the 46 batches of eggs at CST was between 21.0 and 22.5^{o}C, at SARDI it was 21.0^{o}C in one trial and 22-24^{o}C in two others (SARDI component; Appendix C), and at DAC it was 21.5^{o}C in one run and over 24^{o}C in two others (DAC component; Appendix D). The optimal egg incubation temperature has not been determined, and thus there would appear to be some benefit in a replicated trial with batches of eggs being incubated at water temperatures at (say), 18, 20, 22 and 24^{o}C, with the larvae being reared at the usual 24-25^{o}C (avoiding any changes in temperature of >1^{o}C per day following hatching) and being cultured using the same feed regime to 25 DPH to determine the effect (if any) on survival and malformation rates. Hatchery staff are now aware of the need to maintain a more consistent water temperature during the egg incubation stage, though the mechanisms for closely controlling water temperature are limited.

2.3 Transport of Fertilized Eggs

The data gathered in this project on best (and worst) practices for transporting fertilized eggs were essentially incidental to the main goal which was to see how YTK larvae would perform in the intensive larval rearing culture system at DAC. This system has been very reliable for the production on in excess of 1 million p.a. high quality barramundi fingerlings for commercial and research purposes (Curnow et al., 2005; Bosmans et al., 2006). Eggs were packaged in SA or WA and airfreighted to Darwin with total travel durations of between 12 and 24 h. Water quality was definitely an issue for the longer trips, but whether this was primarily due to time, or the packaging method used (e.g. egg density, temperature control) is difficult to say (DAC component; Appendix D).

Differences in dissolved oxygen levels of the transport water from SA and WA were pronounced, and given that one of the theories as to why egg settling might be so detrimental is that the restricted water movement over the surface of the eggs and a high metabolic demand from the embryo would create localised low DO concentrations that could retard or kill the embryos, it would seem sensible to do a study to determine the metabolic rate of YTK eggs during development. If the experiment includes a range of incubation temperatures, it would also help address the important question of the best water temperature to use during extended transport of eggs. It is common to think that there are no deleterious consequences of providing an excess of things that are essential to life (e.g. oxygen, vitamins, minerals etc), and that 'more is better'. However, this is incorrect, and not only could excess levels of these parameters be harmful to the individual, it usually adds significant and unnecessary cost to the process.

The CST SOP for preparing YTK eggs for transportation has been modified to reflect some of the findings from this project, but more work is required.

2.4 Egg Hatching

During this project egg hatching rates were not accurately estimated at CST facilities due to a poor sampling methodology that could introduce error in the estimate of total egg numbers for each batch/tank of eggs. Thus, reported apparent hatching rates varied from 32 to 160%. Unfortunately, because the methodological error was not consistent between tanks or batches, it is not possible to apply an average correction factor to the 2008-2009 data. This error has now been eliminated from the hatchery SOP for enumerating eggs (CST component; Appendix B).

There were some suggestions that YTK egg hatching appears less synchronised than observed for other species. Whether this is due to fertilization effects (e.g. there may be more than one spawning event in a tank each day such that some eggs are several hours older than others), or due to incubation conditions (e.g. egg settling) that retard development, is not known. Artificial fertilization of a batch of stripped eggs would be one way to see how much developmental variability there is post-fertilization. If most of the variation is shown to be related to the occurrence of multiple spawning events over the night then more frequent egg collector harvests could be made (with the associated higher costs of providing the additional labour or infrastructure to do that, e.g. by automated opening and closing of two overflow outlets to separate pre- from post-midnight spawned eggs), or the expectations of the staff can be modified to accept that this is a feature of the YTK hatchery process and that it is something that needs to be accepted and managed.

2.5 Larval Rearing

Despite the huge amounts of data recorded for each tank used in rearing the 46 batches of eggs/larvae at the CST hatcheries in 2008 - 2009, it is difficult to relate the production performance outcomes (survival, malformation rate and growth rate) to the abiotic and biotic culture conditions, because more than one parameter differed between tanks within a run, and also changed between runs (CST component; Appendix B). Thus, it's not possible to separate the effects of any parameter in isolation. This will be discussed further in the section on improving hatchery practices.

2.5.1 Abiotic factors

Water temperature. The replicated experiment carried out by the SARDI/Flinders team was hugely beneficial to this project (SARDI component; Appendix C). It clearly showed that a constant water temperature of 25ºC from 3 DPH was very deleterious to larvae (only 0.36% survival at 25 DPH); whereas a constant 21ºC and 23ºC resulted in better outcomes (12.8% and 8.8% survival, respectively). Growth rates of larvae reared at less than 25ºC until at least 18 DPH were similar to each other, and jaw deformity rates were lower in larvae that started at 21ºC and stepped up to 23ºC and 25ºC over time. The highest rate of jaw deformity was observed in the constant 25ºC treatment group (Figure 1). These results suggest that water temperature is a very important factor in determining the performance outcomes of a batch of YTK eggs, and that the larvae cope better with a lower starting temperature (e.g. 21ºC), but after 13 DPH or so, the temperature can be increased to 23 or even 25ºC (the latter in two steps). These results emphasise the benefits of conducting a similar study on water temperature during egg incubation that was suggested above. The importance of reducing the variability in water temperatures in larval tanks due to day/night ambient conditions, and between different hatchery runs, is now emphasised with hatchery staff. However, limitations of the hatchery building and water supply/recirculation infrastructure to maintain a narrow range of water temperatures at this time of year and when the facility is being fully utilised, is sometimes challenging, and temperatures can fall outside of the target range.

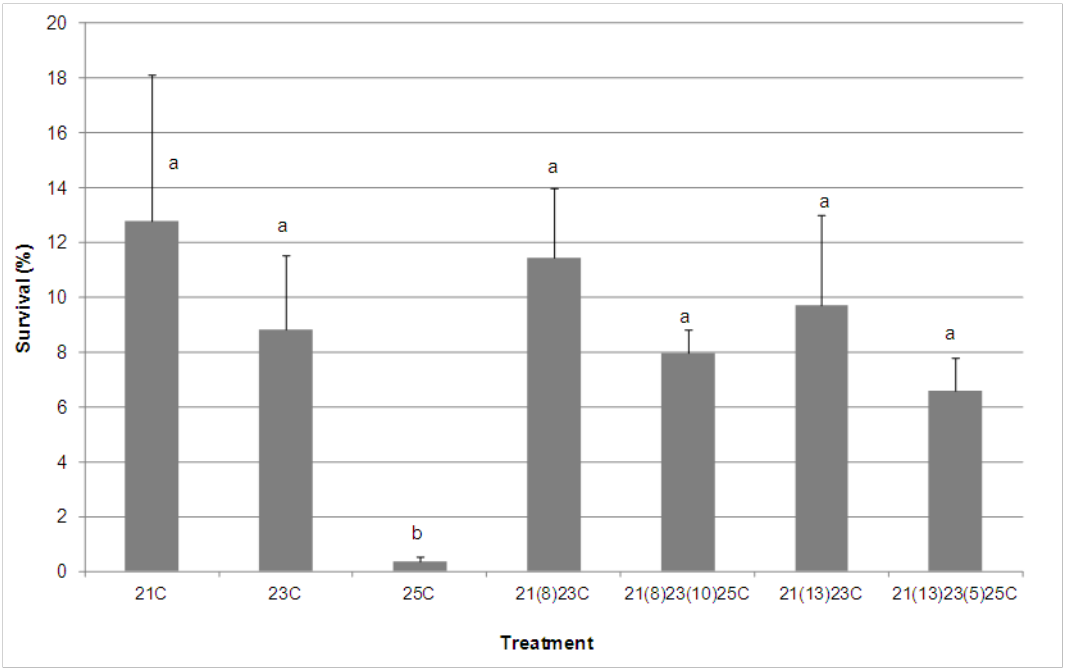


Figure 1: Mean final survival rate (%) of Yellowtail Kingfish larvae cultured from 3 DPH until 28 DPH using different water temperature regimes. The temperature regimes were: 21C, 23C and 25C (21ºC, 23ºC and 25ºC constant); and 21(8)23C (21ºC for 8 days, then 23ºC), 21(8)23(10)25C (21ºC for 8 days, then 23ºC for 10 days, then 25ºC), 21(13)23C (21ºC for 13 days, then 23ºC) and 21(13)23(5)25C (21ºC for 13 days, then 23ºC for 5 days, then 25ºC). Values are means ± SD (n = 4 replicates per treatment). Treatments that share a common letter are not significantly different (P > 0.05).

Tank wall colour. The significant progress that a reduction in walling behaviour by changing tank wall colour had on the survival and rates of jaw deformity in Striped Trumpeter larvae have not been translated to YTK performance. Until it is possible to reduce the between tank variability of a single batch of eggs to test only single factors (such as wall colour), then any conclusions about the best wall colour are largely moot. Walling behaviour will be affected by several factors including tank hydrodynamics, prey density and location, the location of 'structure' (e.g. airlines) in the tank, as well as tank wall colour/contrast.

Lighting. As described above, there is no definitive answer to the question of what sort of lighting - natural or artificial – is best for YTK larval performance. What level of light intensity is too high, or too low? What is the best photoperiod? Is it important to minimise extraneous light at night so as not to confuse the larvae or their live feeds? Until we can definitively test each of these questions, we should try to work with a single 'typical' regime used in a number of different hatchery runs.

Salinity. It is unlikely that salinity, within the ranges experienced at Arno Bay or Port Augusta, are going to affect the larval rearing performance of the hatchery YTK. Wild YTK

broodstock are known to spawn in the Port Augusta area, and with such little freshwater inflow to the whole Spencer Gulf system, it is unlikely that low salinity would be needed for successful larval rearing. If anything, the higher salinities found at the top of the gulf might keep the developing YTK larvae more buoyant, reducing the level of aeration vigor required to keep them in suspension.

Water Quality. It is probably not necessary to deviate from levels of water quality parameters (e.g. DO, pH, ammonia, total dissolved gases etc.) that have been used successfully when rearing other marine finfish larvae. Obviously, as with any hatchery, it is critical to ensure that the hatchery water source is also free of any potential toxins (hydrocarbons, pesticides, organic and inorganic heavy metals etc). None of these appear to be the case, and besides, the problems observed at CST hatcheries in terms of low survival and high malformation rates for YTK larvae, have also been experienced at the SARDI, NT, WA and New Zealand hatcheries.

One aspect of abiotic conditions (water quality and potentially lighting) is the ability of the larvae to take a gulp of air at the water surface for the initial filling of the swimbladder. Four different factors could be affecting the ability of YTK larvae to undertake this step. Three involve physical aspects of the water surface: oil films, ripples and hydrodynamic barriers; the fourth is that the cues that larvae in the wild use to take this step are either not present, or if present they are not strong enough, in the hatchery to trigger the need to take the gulp at the water surface. These will be discussed in the subsection on swimbladder malformation.

2.5.2 Biotic factors

Microbial load. Larval rearing systems serve as wonderful opportunities for aquatic microbes to exploit. Not all microbes are bad; indeed, in most culture situations very few bad microbes will be present, most will be benign, and some will be beneficial to the aquaculture system.

On the other hand, live feed (open tank algae, rotifers and *Artemia*) cultures are generally rife with microbial life as the bacteria thrive in the warm, nutrient-enriched, aerated water. It is no wonder then, that adding volumes of live feed to a larval culture gives the accompanying microbes the opportunity to dominate the whole system, sometimes to the detriment of the intended culture target. That is why significant effort is being made to reduce the numbers of potentially harmful microbes in live food cultures. Proprietary probiotics are commonly used in live food systems, and live feeds are sometimes surface disinfected by ozone or UV rinses that are sufficient exposure to restrict the microbes, but not affect the survival of the live food items.

Again, these approaches and precautions have been shown to be effective in a number of larval rearing situations, but to date, their effectiveness and value has yet to be convincingly demonstrated for YTK larval rearing. As with many of the biotic factor mentioned above, perhaps this is due to there being limited opportunity to test the effectiveness of these approaches in a controlled and replicated manner. Until then, it may be wise to take a precautionary approach and keep using the disinfection or probiont products, but if the costs of hatchery production are to be minimised, it should be a high priority experiment.

An ‘emergency’ attempt to reduce a very high microbial load in a larval culture tank by addition of diluted formalin was futile. Instead it would be more sensible to treat the smaller volumes of live feed with a disinfection agent, so reducing the numbers of viable inoculants added into the system. Indeed, the potential for error and overdosing of the larvae in the tank with the highly toxic formalin would not be worth the risk. This practice has not been demonstrated to be effective in reducing microbial load in the rearing tanks, and needs to be abandoned for less risky approaches.

Larval density. In the project period (2008-2009) the newly hatched larvae were generally stocked into the rearing tanks at densities around 100 per litre. This is consistent with stocking rates for other marine finfish larvae of this bodylength/age.

2.5.3 Live feeds

All live food regimes for larval rearing are considered to be ‘a work in progress’, they will change as new approaches and products are publicised and marketed. Four hatcheries (both CST facilities, and the SARDI and DAC hatcheries) were used during this project to try to improve upon the then ‘best-practice’ methodology. The prolonged requirements for both rotifers and *Artemia* were a significant drain on staffing, demand on infrastructure, and impost upon the budget in the live feed section of the hatchery.

One of the first changes was to buy in some microalgal paste concentrates to reduce reliance on the continued availability of large volumes of live algae for green water in the larval rearing tanks and for feeding the rotifer cultures. There was no side-by-side comparison carried out beforehand (or since) on which to assess equivalency. Nonetheless, the algal concentrates have proven to be both effective and useful, and are now routinely utilised within the hatchery.

Rotifer production is a substantial and risky undertaking in a commercial hatchery. Reducing either the target number of rotifers per mL in the rearing tanks, and/or the duration over which they are fed to a tank, would be warmly welcomed by all concerned. The time that the

larvae spend on rotifers is largely dependent on their mouth gape, which in turn, is dependent on growth rate, as they cannot be successfully weaned onto *Artemia* nauplii until they can ingest the larger prey item. Thus, there was an extended period of rotifer and *Artemia* co-feeding, this suggests some scope to reduce the duration for supplying rotifers.

Although more convenient to provide on demand than rotifers, the substantial cost of both *Artemia* cysts and nutritional enrichment products also attract the attention of the hatchery manager who is trying to work within a budget. As with rotifers, reducing both the number of *Artemia* per mL and the duration over which they are required, would go a long way to reducing the production expenses of the hatchery. The main issue with weaning larvae off *Artemia* nauplii and onto a microparticulate diet, has been that the microparticulate diets do not invoke a strong feeding response in the larvae. The lack of movement (coupled with the poorly developed visual system in the larvae) and the relatively limited dissolved feeding stimulants in the microparticulate diets are believed to be the main barriers in this regard. With new microdiet products coming onto the market, each purporting to address these barriers, and new co-feeding strategies showing substantial improvement with weaning success in other species, the opportunity to advance the 2008 *Artemia* feeding regime was available.

The work on alternative feeding regimes that was carried out at the CST Arno Bay hatchery, in particular, was going to determine if the way the feed schedule was derived would make a difference to larval survival, growth and malformation rates, and reduce the total requirement for live feeds. The existing feed protocol was focused on adding a specified total number of enriched rotifers or *Artemia* per tank per day, and it was to be trialled against a strategy to maintain a target rotifer density by ‘topping up’ any residual rotifers with an addition of a new batch of live feed. Unfortunately, there were 3 issues with this approach:

1. All the larval tanks for each batch of eggs were (notionally) allocated to the same treatment, so there were no ‘control’ tanks of larvae from the same batch of eggs that got fed according to the 2008 standard live feeds strategy. Thus, it was not possible to make comparisons within a run.

2. The feed strategies that were planned were actually not executed, and often local hatchery management kept feeding live feeds at higher rates and for longer than was intended.

3. Even when the planned feeding schedule was not followed, there was relatively little consistency between runs in the under- or over-feeding practices.

The feeding trials at DAC were very much preliminary observations on how YTK larvae would perform in an intensive rearing system that was regularly topped up with inputs of fresh algae (green water) and enriched live feeds to a target feed density. Due to all the egg quality, egg incubation difficulties and high rates of early larval mortality, relatively few larvae made it into the intensive rearing system, and those that did were probably significantly compromised by their prior handling. Given this, the fact that several thousand juveniles up to 26 DPH were produced from effectively 2 batches of eggs, can probably be interpreted as a positive outcome of the efforts. Once the major issues to do with egg transport and incubation at DAC could be sorted out, it would definitely be interesting to see how the larvae performed in the intensive rearing system.

The feeding trials at SARDI/Flinders were, again, hugely informative in the context of this project. One experiment looked closely at the optimal density to feed rotifers at, and compared constant feed rates with different increasing rotifer density strategies. A second experiment compared performance outcomes of larvae fed increasing densities of *Artemia* to different maxima over 9 days, both with and without co-feeding of a low density of rotifers. A third trial determined the ability of larvae of different ages to wean from *Artemia* onto a particulate diet.

Unlike the CST trials at Arno Bay, these experiments had all the treatments being trialled at the same time with a single batch of eggs, replicate tanks for each treatment and the staff keeping to the intended target feeding profiles. This meant that the performance outcomes of the treatments within a trial could be compared to each other, and more confidence could be put into the conclusions.

The survival outcomes of the rotifer density trial are shown in Figure 2. The results are very clear: a constant rotifer density of either 10 or 40 mL^{-1} was detrimental to larval survival (<12%); whereas a constant rotifer density of 20 and 30 mL^{-1} were better (e.g. 28% survival for 20 mL^{-1}). Increasing rotifer density profiles improved larval survival, with the 15-40 mL^{-1} treatment group also resulting in 28% survival. Note that these experiments were done with an initial larval stocking density of 60 L^{-1}, not ~100 L^{-1} as was most common at CST. It would be prudent to repeat the trial with the higher larval density to determine whether the optimal rotifer density would change as the relative predator pressure increases.

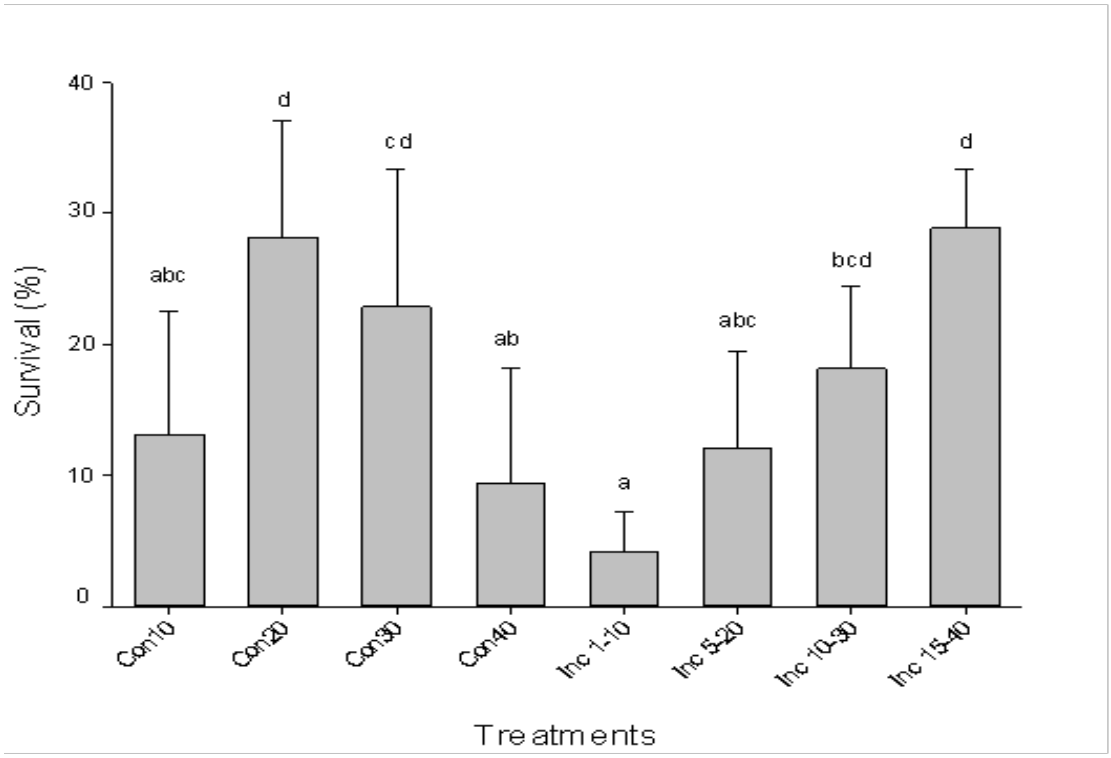


Figure 2. Mean survival rates (% ± SD, *n* = 4) at 13 DPH for Yellowtail Kingfish larvae fed with different rotifer density treatments from 2 DPH. Con10 = 10 rotifers mL^{-1}; Con20 = 20 rotifers mL^{-1}; Con30 = 30 rotifers mL^{-1}; Con40 = 40 rotifers mL^{-1}; Inc1-10 = density increased from 1 to 10 rotifers mL^{-1}; Inc5-20 = density increased from 5 to 20 rotifers mL^{-1}; Inc10-30 = density increased from 10 to 30 rotifers mL^{-1}; and Inc15-40 = density increased from 15 to 40 rotifers mL^{-1}. Treatments that share a common superscript are not significantly different ($P < 0.05$) in multiple comparisons.

The *Artemia* density trial provided some very encouraging survival rates that in some cases exceeded 50% (Figure 3). The rotifer only diet (a control) produced only 6% survival indicating that there is no value in trying to eliminate the transition to *Artemia* feeding. Feeding strategies that had peak *Artemia* densities of 15 to 35 mL^{-1} all produced >40% survival rates. Whereas a strategy that had Artemia peaking at 45 mL^{-1} was not as good as the protocol that peaked at 25 mL^{-1}. The larval stocking density in this trial was 40 L^{-1} at 10 DPH, so probably similar to commercial rearing conditions. Each of the *Artemia* densities was offered with and without co-feeding of 5 rotifers mL^{-1}, the results suggested there was no benefit of rotifer co-feeding.

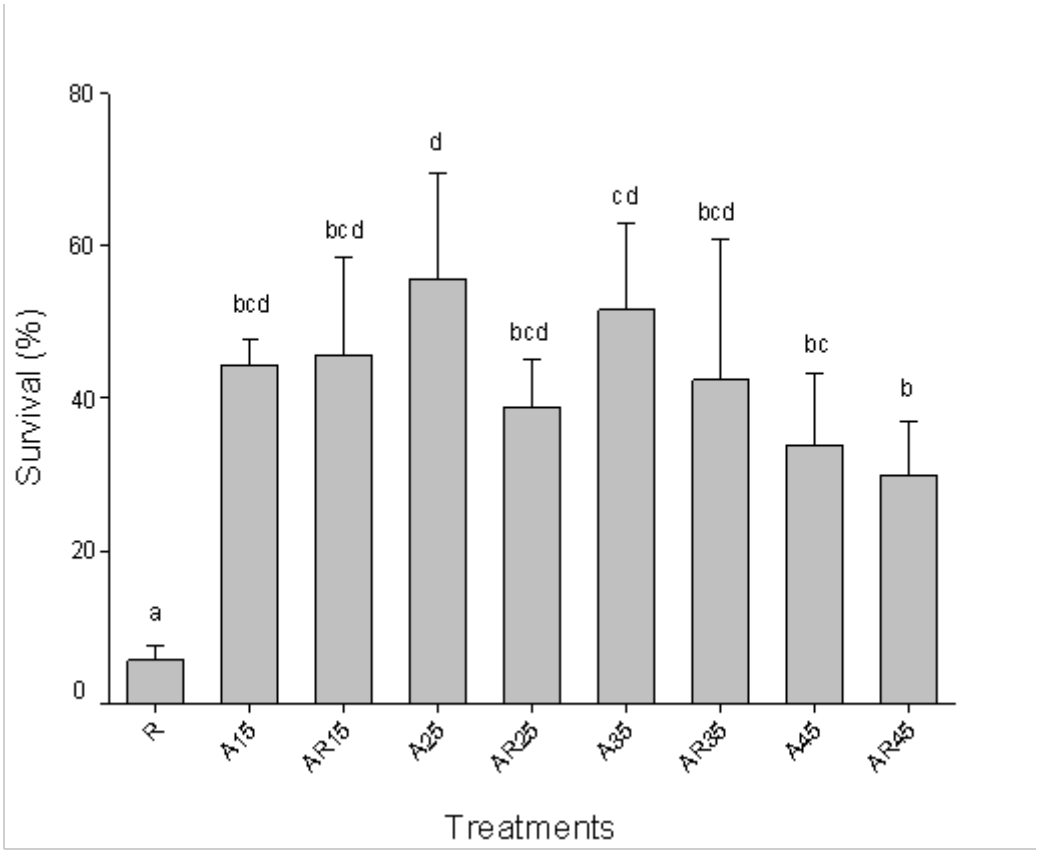


Figure 3. Mean survival rate (% ± SD, *n* = 4) at 21 DPH for Yellowtail Kingfish larvae fed different *Artemia* density treatments (number mL^{-1}) during the transition from rotifers to *Artemia* feeding between 12 and 21 DPH. (R = Rotifers only; A = *Artemia* only; AR = *Artemia* and rotifers until 21 DPH). Treatments that share a common superscript are not significantly different ($P < 0.05$) in multiple comparisons.

Weaning of larvae onto microparticulate diets started at 10 DPH with new treatment groups starting every 3 days, and introduction of microparticulate diets corresponded with a 5 day decreasing co-feed with *Artemia* nauplii. In comparison to the other SARDI/Flinders trials survival rates were very low (<5% at 33 DPH). Survival rates were higher in the groups where weaning was initiated later (e.g. weaning that started at 22 DPH was 3.2%). Weaning started at 10 DPH had 0.2% survival (Figure 4). Whilst the trend in the data was clear, the overall low survival rate suggests that the trial should be repeated.

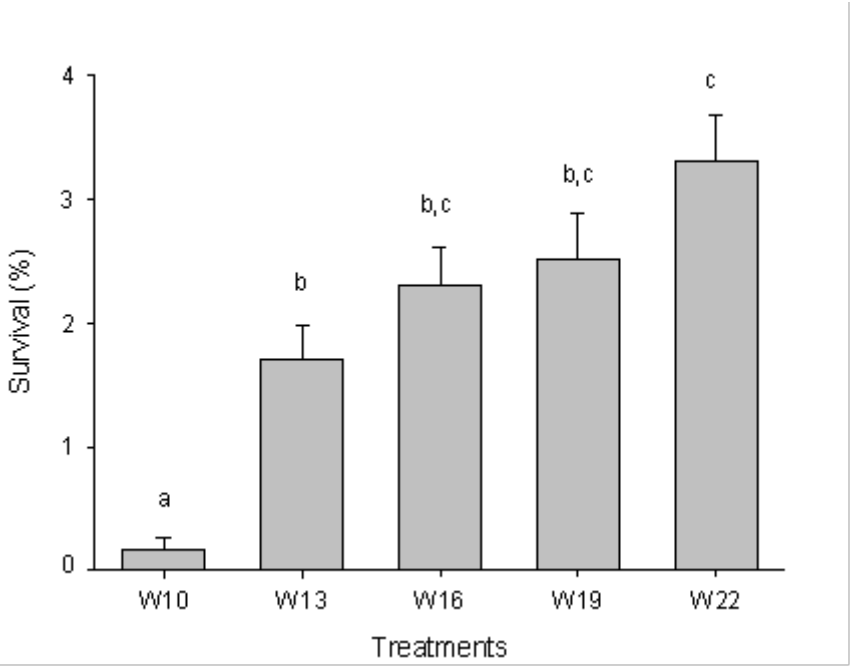


Figure 4. Survival rates (%; mean + SD, *n* = 5 replicates per treatment) until 33 DPH for Yellowtail Kingfish larvae weaned onto microparticulate diets starting at different ages (days 10, 13, 16, 19 and 22 post-hatch). All weaning protocols had a 5 day co-feeding with a decreasing density of *Artemia*). Treatments that share a common superscript are not significantly different ($P > 0.05$).

Growth rates of larvae did not show much difference between diet treatment groups (rotifers, *Artemia* or weaning studies), until the larvae were over 20 DPH. After this time the growth rates generally reflected the survival results, with relatively high survival treatments showing slightly better growth. Differences in jaw and swimbladder malformation in the 3 feeding trials will be presented and discussed in the relevant sections below.

Just considering the SARDI/Flinders feeding trial results, the best feeding strategy for YTK larvae would appear to be:

- Green water until weaning
- Rotifers – from 2 DPH, either constant at ~20-25 mL^{-1}, or starting at 15 and rising to 40 mL^{-1} by 10 DPH. The density should then probably be held at 40 mL^{-1} until 15 DPH when it should gradually decrease to zero by 20 DPH.
- Artemia – from 12 DPH, starting at 1 mL^{-1} and rising to 25 mL^{-1} by 20 DPH. Maintained at 25 mL^{-1} until 22 DPH, and then decrease to zero by 27 DPH.
- Microparticulate diet – start from 22 DPH with a 5 day co-feeding with decreasing numbers of Artemia.

Figure 5 shows how this compares to the 2008 feeding regime. The duration of green water is reduced by 7 days. The time on rotifers is similar, but the feeding rates are much more targeted and there is a 5 day decrease in rotifer density from 15 DPH. *Artemia* feeding can start 2 days later and finish 5 days earlier with a 4 day decreasing density, saving significant costs. Microparticulate diets can be introduced from 22 DPH. These findings are being incorporated into the CST hatchery live feed SOPs, but differences in the rate of growth and development of the larvae at different water temperatures during egg incubation and/or larval rearing phases in the larger commercial hatchery mean that these timings are somewhat aspirational, and the feeding regimes need to be modified according to the actual development stage of the larvae.

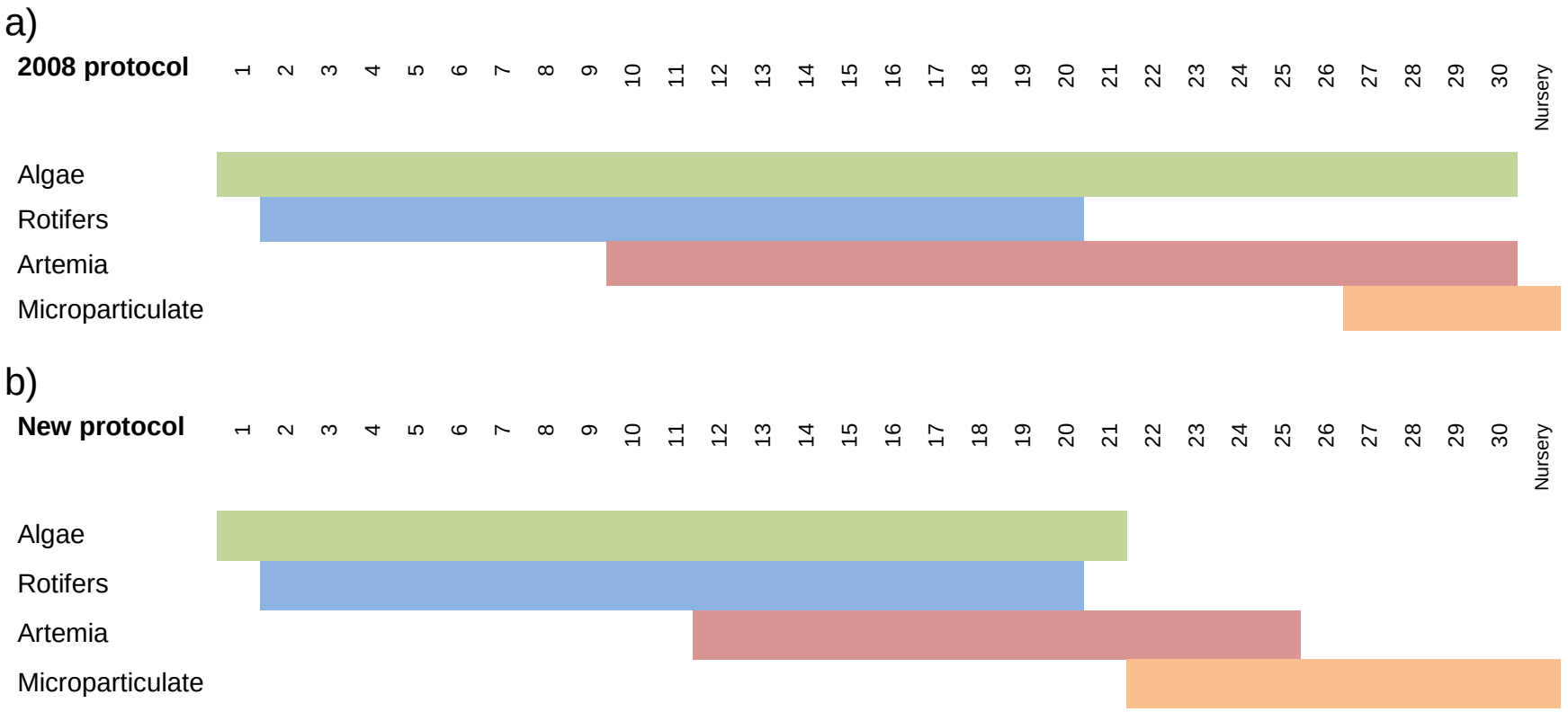


Figure 5. Schematic live food regime from Clean Seas Tuna Ltd hatcheries (a) as used in most commercial YTK hatchery runs prior to and in 2008, and (b) summarised from the results of this project (but not validated through a commercial hatchery run). The different shaded boxes indicate when that live feed item/microparticulate food was offered to the larvae.

2.5.4 Jaw Malformation

The work carried out by the IMAS team was key to characterising how the hatchery conditions and experimental protocols affected the incidence and severity of jaw deformities (IMAS component; Appendix E). The successful training of senior CST hatchery staff in the jaw malformation assessment and scoring was a significant improvement in the capability within the company. The jaw deformities characterised and quantified in this project were the same as were identified and described in earlier work (Cobcroft et al., 2004; Battaglene and Cobcroft, 2008).

In almost all assessments of jaw malformation the proportion of larvae showing score 2 and 3 (i.e. meeting the commercial cull threshold) at 12 DPH or later, was 20-40%, but rates up to 60% were recorded for groups where there was low survival rates (e.g. the constant 25°C group in the SARDI/Flinders larval rearing temperature experiment).

The only time when low (<20%) score 2+3 jaw deformity rates were measured were in larvae that were <10 DPH (as it takes time for some of the severe categories to transition from lower scores as the fish ages/grows), or in nursery tanks of juveniles that had been graded by size and the larger, faster growing fish tended to have fewer, less severe jaw deformities. It is not known whether they grew faster because they had less severe deformities, or they had fewer deformities because they grew faster (i.e. the ‘cause or effect’ question).

Thus, during this project (2008-2009) there was no indication that any particular live feed regime, nutritional enrichment protocol, tank colour, tank hydrodynamic pattern, broodstock or egg quality parameter, water temperature or lighting condition, had any particular association with either a high or low incidence of severe jaw malformation. Clearly, with such a high incidence of the condition, further research is required to identify the cause(s) and mitigate them.

2.5.5 Swimbladder Inflation

In most hatchery runs up until the 2008 spawning season, poor swimbladder inflation only affected a relatively few percent of juvenile YTK. However, in 2009 things changed and swimbladder inflation problems were common in most batches of larvae at both CST hatcheries, and the hatchery runs that were carried out at SARDI and DAC. The nature of the swimbladder problem was either no inflation, or in some cases, hyper-inflation of the organ. Swimbladder inflation in most marine finfish species happens about the time when fins start to develop, and the larvae tries to exert some autonomy in its movement, rather than just being at the mercy of the circulation pattern in the tank. It usually happens around the time the skin develops some pigmentation, so it can be difficult to visualise once this process occurs.

The swimbladder organ develops as an offshoot of the gut, and in most species of fish, the first gas to fill the organ is via a gulp of air taken at the water surface. After that time, in most species, the connection between the swimbladder and the gut breaks down and they become independent organs. As mentioned above, there are a number of different ways in which swimbladder inflation can be less than optimal. The potential causes include:

- physical aspects of the water surface; oil films, ripples and hydrodynamic barriers, and
- the absence of sufficient cues to trigger the need for the larva to take a gulp at the water surface.

This report (and the detailed component included as Appendix B), have acknowledged that at times during this project, the attention paid to skimming of surface oil films was probably insufficient. The oil comes from two main sources, the nutritionally enriched (generally with high levels of polyunsaturated fatty acids) live feeds, and the oil droplets from deteriorating dead eggs and/or larvae. As the lipid is less dense than seawater, it rises to surface and spreads in a very thin layer over a large area. The oil film acts as a barrier between the surface of the water and the air above, and so a larva that makes it to the surface would not be able to gulp any air.

The hydrodynamic setup in the larval rearing tank may be too strong (because it's trying to stop the larvae from settling to the bottom of the tank) such that the larva, which has little control over its position in the water column, does not have enough time at the surface to take the gulp before it is swept off in the circulation. Constant air movement across the tank may cause ripples on the surface of the water and set up a condition called 'laminar flow' in a thin layer immediately under the surface of the water. This has a boundary with the turbulent flow condition that is in the rest of the tank, and that boundary is robust enough to stop larvae from attaining the water surface.

Finally, larval fish have been shown to use environmental cues to synchronise their movement to the water surface to take the gulp of air that will fill the swimbladder (e.g. Trotter et al., 2003). In the case of Striped Trumpeter, the larvae aggregated at the water surface during darkness, and swimbladder inflation was affected by photoperiod and light intensity (Trotter et al., 2003). Similar studies using appropriate culture conditions and techniques may help to determine what these cues might be for YTK larvae.

It is interesting that whatever the cause(s) of poor swimbladder inflation, the condition affected the larvae reared at 4 different hatcheries, from eggs spawned by CST and CIT broodstock, and appeared suddenly. This information should be further analysed to determine what might have changed between 2008 and 2009.

2.6 Improving Hatchery Practices and Procedures

It was stated previously, but will be restated here, that comments made about CST hatchery practices and processes will reflect the project period (2008-2009), and do not indicate the current (2011) circumstances and situation at these facilities.

Through this, and other projects, CST has had many specialist researchers visit the Arno Bay hatchery, with fewer visiting the Port Augusta site. All of these specialists will have identified practices and procedures that were less than ideal, either in methodology and/or execution. The collaborative nature of these projects ensures that hatchery staff build up a network of contacts who they can turn to for advice or opinion, without prejudice as all parties will have signed confidentiality agreements. New appointees with experience in other hatcheries have also helped to address some of the practices and procedures that needed attention. One of the key improvements to hatchery performance was the updating and re-emphasising of SOPs.

2.6.1 The Value of SOPs

Standard Operating Procedures (SOPs) are imperatives in any technical and quality-focussed activity. They should always be up to date, on display in the areas where that activity is carried out, and contain only the essential and relevant information to that task. New staff should be trained using the SOPs and some level of quality control needs to be conducted to ensure the SOPs are being utilised. SOPs should enhance productivity because they reduce the risk that unexpected outcomes will occur, and this means that staff won't feel as though they can't take time off, because they are irreplaceable, and that reduces the chances of mistakes being made through exhaustion. Since this project ended, all the hatchery practices and procedures have had new SOPs written, and they are now key reference points for all hatchery workers. These SOPs are commercial-in-confidence IP of CST and will not be disclosed.

2.6.2 Rigorous Adherence to Planned Strategies

Much of the experience from the CST component of this project reflect a significant disconnect between CSTs senior hatchery management, who set the overall goals and devised a suitable rearing strategy for each hatchery site in each year, and the people at the hatcheries who had to handle the workload associated with producing the live foods, eggs, larvae and juvenile fish 7 days a week during the spawning and larval rearing season. In contrast, the hatchery staff considered that their highest priority was to keep as many fish alive for as long as possible, and that the highest quantities/densities of live food the system could produce would help do that. So, as a consequence there was little or no implementation of the modified live feed strategy that the senior managers had devised for 2009. Thus, when the production outcomes were reported back to senior management, there was an assumption (now shown to be incorrect) that

the new feeding strategies were being implemented, and so they concluded that these feeding changes had made no improvement in survival and/or malformation rates.

As with the previous two sections, this serious shortcoming was recognised at the end of the 2009 hatchery season, and has since been rectified. The senior hatchery managers discuss feeding strategies, and especially any changes in those strategies, with the YTK production hatchery and R&D hatchery workers so that the intentions and reasons for the changes are understood and their importance is emphasised. The appointment of several key staff that have research backgrounds to the hatchery team will also help to ensure that R&D activities are given a high priority in a mainly commercial setting.

3. Benefits and Adoption

This project contributed to the realisation that CST had to make significant investments in hatchery infrastructure and staff capability in order to be better able to address the questions that could not be answered with the existing hatchery setup. The new R&D hatchery was purpose built so that replicated trials can be conducted, with appropriate controls, and that experimental protocols will be followed. So whilst this project did not provide answers to many of the questions, it has substantially improved the probability that those answers will be found soon.

The close collaboration and interdependence between commercial industry and specialist research scientists and facilities has been another major benefit of this project. Many of the barriers that existed (from both sides) have been discarded, and the mutually-beneficial interactions are continuing to 2011. The value of the role and function that specialist researchers can play in addressing apparently intractable industry problems has been acknowledged, and the importance of having a pool of that expertise available has been demonstrated. Many of the technologies (e.g. ozonation) that were previously in the R&D domain, have now become integral to the commercial facility.

The CST facilities at Arno Bay and Port Augusta have created many new, and updated other existing, SOPs for both commercial and R&D hatchery runs. The benefits of this should be much more consistent results between tanks, between batches, facilities and years. Anything that reduces the risk in hatchery output will contribute to predictability and confidence in the production chain. These SOPs are commercial-in-confidence and remain the IP of CST.

4. Further Development

The outcomes of the project were not realised, but they are still key goals of the YTK production platform at CST. The new R&D hatchery, additional skilled staff, and ongoing interactions between CST staff and research expertise described in this project all contribute to a sense of optimism that these problems can be addressed soon.

The key issues that will form the basis of future research are still:

1. improving egg incubation (the effects of water temperature, the effects of vigorous water circulation, potential benefits of food grade thickening agents in maintaining buoyancy, oxygen demands of eggs and larvae; biochemical analysis of eggs using appropriately stored samples, are concentrated algal pastes equivalent to live algae)
2. abiotic factors during larval rearing (lighting regimes for improving swimbladder inflation)
3. biotic factors during larval rearing (larval behaviour for initial swimbladder inflation, biochemical analysis of live feeds using appropriately stored samples, feeding times and the subsequent effects on larval buoyancy, further trials with the DAC intensive larval rearing system, are the probionts reducing microbial loads in live feed)

The performance outcomes of these trials need to include survival, growth and malformations (jaw and swimbladder).

5. Planned Outcomes

Public and Private Benefit Outcomes

The planned outcomes were:

> ***Higher survival rates, lower malformation rates, higher quality juveniles, lower production costs and greater efficiencies in YTK hatcheries.***

These outcomes have not been achieved. The mortality and malformation issues are almost certainly not simple, single-factor cause – effect situations. These problems will continue to affect the profitability and expansion of the YTK sector in Australian aquaculture.

> ***Recommendations on how to improve larval and juvenile rearing systems and procedures leading to better survival rates and reduced levels of malformation.***

Partly achieved. The project showed that the existing hatchery facility and staffing capability were not appropriate to address these issues. Significant investment in a new R&D hatchery, new and retrained staff, and a re-vamping of all hatchery SOPs were required to address to these production issues. These outcomes benefit both private and public aspects of the YTK sector.

Linkages with CRC Milestones

The increased profitability and industry value through efficient delivery of Australian seafood to the consumer is the definitive outcome of this project. This project has several Seafood CRC Milestones associated with it, including:

Milestone 1.3.1: New genetic tools and/or appropriate breeding strategies developed for genetic management and improvement of at least two aquaculture species

Milestone 1.3.3: Strategic disease management approaches and technologies developed for at least two aquaculture species.

Milestone 1.3.5: Production efficiency gains from genetic, health management and nutritional interventions quantified to inform long-term strategies and estimate commercial benefits

6. Conclusion

This project sought to try to understand the cause(s) and ways to mitigate a number of production performance issues; low survival rates of larvae (commonly only 10-15% of hatched larvae survive to juvenile stage), and the high incidence of jaw malformations that can be severe enough to require that 20-40% of juveniles are culled, that have affected Yellowtail Kingfish (YTK) hatcheries for 10 years. Furthermore, in 2009, during this project, there was a substantial increase in the incidence of swimbladder malformation in larvae and juveniles. This deformity leaves the fish with no, or a much reduced swimbladder, and these fish also need to be culled from the juveniles that are placed into sea cages for grow out.

This project used a collaborative approach and was carried out at four hatcheries – the Arno Bay and Port Augusta facilities run by Clean Seas Tuna Ltd, and the specialist research facilities at SARDI (with SARDI and Flinders University researchers), and the Darwin Aquaculture Centre. The experience and expertise of the researchers at TAFI (now Institute of Marine and Antarctic Studies) from the University of Tasmania, underpinned the identification and quantification of jaw and swimbladder malformation rates at all four hatcheries.

The research approaches used in this project were (a) correlational (using the Clean Seas hatchery database), and (b) experimental using replicated tank systems, several treatment groups and only changing one parameter at a time. The correlational approach did not reveal any key parameters that were either positively or negatively associated with survival or malformation rates, but this was almost certainly because of the inconsistencies and variability in the actual hatchery rearing conditions between each tank, batch, hatchery or year. This finding identified something of a disconnect between the senior hatchery management team that planned the larval rearing strategies, and the on-site hatchery workforce. Substantial changes have since been made to correct this.

The experimental approach proved extremely useful with clear results on the optimal water temperature regime for larval rearing, suitable rotifer and *Artemia* feeding densities, and optimal ages for weaning of larvae onto microparticulate diets. Whilst the survival rates of larvae were different between treatment groups in these experiments, growth rates and the jaw and swimbladder malformation rates were not. Thus, there is some information that will help to increase larval survival rate, there is no immediate solution to the issue of poor juvenile quality.

An alternative larval rearing system using an intensive approach with constant addition of live feeds was trialed at the Darwin Aquaculture Centre. Only 2 batches of eggs were placed into the system, and although several thousand fish made it to day 20 or 26 post-hatch, the overall survival rate and incidence of malformation were still poor, probably as a consequence of the

challenging egg and early larval life culture conditions. The fact that any larvae survived in this system is substantially encouraging for further trials.

This project has confirmed that the low survival rates and high malformation rates that have been observed in two commercial Yellowtail Kingfish hatcheries operated by Clean Seas Tuna Ltd were also experienced at two separate research hatchery facilities with eggs supplied by Clean Seas and an independent research facility. This strongly indicates that cause(s) of the problems are intrinsic to the species, and reconfirmed the need for commercial and research hatchery experts to work collaboratively to identify the cause(s) and ways to mitigate them.

This project has proven that YTK larval survival and malformation rates are a substantial challenge, and that the cause(s) are not simple or straightforward. The collaboration shown in the execution of this project between all the teams has been a significant benefit to getting closer to the cause(s) of the problems.

The results of this R&D project have been incorporated into new and modified SOPs that are used in the CST hatcheries at Arno Bay and Port Augusta for both commercial and R&D hatchery runs. The benefits of these SOPs should be more consistent results between tanks, batches, sites and years. Anything that reduces the risk in hatchery output will contribute to predictability and confidence in the production chain. These SOPs are commercial-in-confidence and remain the IP of CST.

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Appendix 1 – Intellectual property

There was some new intellectual property arising from this project. This IP has been identified and utilized in new and updated SOPs that are used in both CST hatcheries. These SOPs are commercial-in-confidence documents and remain the property of CST Ltd.

Appendix 2 – Staff

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Morten Deichmann

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Arno Bay Hatchery team

Port Augusta hatchery team

Collaborators

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SARDI – Wayne Hutchinson, Dr Ben Nan Chen, Steven Clarke

Flinders University – Assoc Prof Jian Qin

Darwin Aquaculture Centre – Jerome Bosmans and Glenn Schipp

Improvements in Yellowtail Kingfish Larval Survival and Juvenile Quality

Appendix B – Clean Seas Tuna Component

Alexander L. Czypionka

Project No. 2009/749

This project was conducted by Clean Seas Tuna

The Australian Seafood CRC is established and supported under the Australian Government's Cooperative Research Centres Program. Other investors in the CRC are the Fisheries Research and Development Corporation, Seafood CRC company members, and supporting participants.

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Part B - Table of Contents

Abbreviations

AB	Arno Bay hatchery
CST	Clean Seas Tuna Ltd
CT	contact time (for ozonation; calculated as exposure in ppm x time in mins)
DAC	Darwin Aquaculture Centre
DPH	days post-hatch
HPF	hours post-fertilization
PA	Port Augusta hatchery
ppm	parts per million
ppt	Parts per thousand
SARDI	South Australian Research and Development Institute
TAFI/IMAS	Tasmanian Aquaculture and Fisheries Institute / Institute of Marine and Atmospheric Sciences
YTK	Yellowtail Kingfish

Non-Technical Summary

PROJECT 2009/749 Improvements in Yellowtail Kingfish larval survival and juvenile quality (Clean Seas Tuna component)

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NON-TECHNICAL SUMMARY:

The two Clean Seas Tuna Ltd hatcheries at Arno Bay (AB) and Port Augusta (PA) produced 4 and 2 runs of juvenile Yellowtail Kingfish (YTK), respectively, in each of the 2008 and 2009 production seasons. The production performance criteria of larval survival, juvenile deformity, swimbladder inflation and larval growth were estimated and were discussed in light of various biotic and abiotic parameters that affected each of those runs. These biotic and abiotic factors included: tank wall colour, larval density, live feed density and enrichment products and protocols, water temperature, broodstock and egg quality factors.

In addition, the AB hatchery was used to carry out several experiments to try to determine the effects of factors such as lighting (natural vs artificial), different methods for creating flow in the tanks (air vs water) to keep YTK larvae and live feeds in suspension and the effects of ozone disinfection on the microbial loading of YTK eggs. The same production performance criteria were used in most of these experiments.

The project showed that:

- The data routinely collected by the hatchery staff on each tank, on each day, of each hatchery run carried out each year, can be a valuable R&D resource.
- To be used effectively, however, the data going into that database must be standardized, interrogated and evaluated objectively so that any deviations from the intended rearing protocol can be rectified as soon as possible.
- Proper training of staff in all sampling and assessment practices and processes is critical to the quality of the data.
- Ongoing interactions with specialist hatchery R&D collaborators will help to improve on site R&D practices.
- Biotic and abiotic conditions both between hatchery runs, and between tanks within a single hatchery run, do affect larval survival and deformity rates in the

commercial YTK hatchery.

- R&D must continue, both on-farm and in specialist facilities, to resolve the current situation whereby larval survival and juvenile quality are too low.

Acknowledgements

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Dr John Carragher (Logifish Consulting) for reviewing, editing and finalising the draft report.

1. Background

This report describes the work done in the CST hatcheries at Arno Bay and Port Augusta. Separate reports are provided by TAFI/IMAS, SARDI and DAC for their respective components of the project and will be cross-referenced as appropriate. The work described in this report was carried out in the 2008 and 2009 YTK spawning seasons, therefore the procedures described herein reflect the YTK larval rearing practices at that time. As a result of this project, and the others carried out by our CRC collaborators, many of these practices and procedures will since have been modified as necessary. In this way, the hatchery manuals and SOPs developed by CST are very much 'living documents'.

Survival rates of YTK from hatched larvae to weaned juveniles is very low (~5-10%) in comparison to many other marine species produced in larger more mature industries, for example the sea bass and seabream sectors in Europe. Mortality in the hatchery stage can be classified into a number of categories, the most common of which are deaths in the tank and culls due to deformities. Of particular note, several skeletal malformations particularly of the spine, jaws and opercula, have been reported in Australian and New Zealand marine hatcheries, although few are well documented (Cobcroft *et al*., 2004).

A number of biotic and abiotic factors have been shown to be associated with higher levels of mortalities and deformities at the hatchery stage in other species, and it seems likely that some of these factors are also likely to be important for YTK larval rearing success. Whilst trials in specialist R&D facilities are extremely useful in trying to understand the effect of these factors, either alone or in combination, there is also great value in looking at the production performance data collected by commercial hatchery facilities to see if there are some clues to indicate which factors might be more, or less, important. This is the aim of the current project.

The variety of biotic and abiotic factors being examined in this project include:

- Egg quality factors
- Light conditions: Artificial versus Natural
- Rearing temperature and salinity
- Tank setup and hydrodynamics
- Tank colour and pattern
- Microbial characterisation and microbial management strategies in rearing and live food systems

- Nutrition: e.g. INVE Culture Selco®, yeast and live algae versus algal pastes, enrichment strategies
- Larval stocking rates and live food (prey) density interactions

Some of these factors were assessed via additional sampling of normal commercial production runs of YTK eggs, larvae and juveniles by hatchery R&D staff, and using the inherent variability in culture conditions to correlate with larval survival and deformity rates. The effect of other factors were assessed via replicated (where possible) tank experiments, where one or more factors were deliberately changed or manipulated by the hatchery R&D staff.

2. Need

The costs of producing juvenile YTK which are fit (i.e. no or low level of deformity) to be stocked at sea for growout need to be reduced. Currently, the low survival rates and high deformity rates of YTK larvae and juveniles is constraining the growth and profitability of this industry in price-sensitive markets. This project aims to determine the factors that contribute most to the low survival and high deformity rates, and to seek industry-relevant ways in which to address them.

3. Methods

3.1 General

CST employs both R&D and commercial hatchery staff (managers and technicians in the broodstock, larval rearing and live feeds sections) to complete all the necessary tasks required for producing the target number of YTK juveniles to go to sea each year. Hatchery staff record a large amount of information that can be specific to each department or relevant to general production during each run. To assist this process the hatchery R&D staff follow a sampling schedule (Appendix 3a) which requires a variety of samples to be collected according to the standard sampling protocols (Appendix 3b), as well as recording all the standard information gathered during each production run.

The vast amount of information collected over the production periods at both hatcheries is entered into a comprehensive Excel database. Standard output reports from the database include: broodstock and egg production data, incubation data, tank setup data, parameter data, feed data, larval data, nursery data and sea transfer data. This database contains all the R&D and production information collected by hatchery staff throughout the 5 years of operations and it is an excellent management tool. Continual development and interrogation of the hatchery database will deliver ongoing benefits to improve the overall efficiency of the process of producing large numbers of healthy juvenile YTK for stocking at-sea.

The production process can divided up into several stages that are described in detail below.

3.2 Broodstock and egg production

The production process begins when selected broodstock fish are triggered to spawn. The broodstock staff are responsible for the production, collection, enumeration, disinfection and incubation of the eggs to hatch. The date of every spawning event for each respective tank of broodstock, along with the number of eggs in each spawn was recorded. Initially egg enumeration was done by removing the eggs from the egg collectors, settling them in a graduated container and recording the volume (in litres) of buoyant eggs, with the number of eggs estimated by multiplying the volume of eggs (L) by 350,000. Unfortunately, during the course of this project this method was found to be too inaccurate and has since been changed.

Now the total number of eggs in a spawning event is estimated by gently suspending them in 150L of sterile sea water, then several replicate samples are taken using a pipette and the number of eggs in each sub sample is counted to determine the total amount of eggs in a known volume of water, and then extrapolating this number to 150L.

Broodstock staff record egg quality data on each batch of YTK eggs used for production and/or research purposes. The egg quality parameters were assessed using a dissecting microscope and transmitted light and include measurement of egg and oil globule diameters using an eyepiece graticule, as well as the viability rate as determined by the counting the proportions of eggs at advanced stages of cell division as shown in the reference photographs (Appendix 3c). Fertilisation rates were also estimated in 2008 but were discontinued due to the difficulties in predicting spawning events and because the sampling needed to happen late in the evening after spawning events occurred. At various times during the project samples of egg were frozen and sent to TAFI for biochemical analysis that included fatty acid profiles and vitamin profiles (these data are reported in the TAFI/IMAS document).

In 2008, following a recommendation by our TAFI collaborators, the hatchery R&D staff conducted trials on the effects of ozonation on YTK eggs. The results (shown later in this report), showed no negative impact on the eggs and as such the practice was adopted by CST in 2009. Now, therefore, before any eggs are placed into incubators or packaged for transport, they are ozonated at an ozone contact time (CT; see later section for calculation) of 0.5.

At various times during this project the CST hatcheries supplied the collaborating institutions with fertilized YTK eggs in order that they could conduct their own trials. The egg transport procedure has been developed over the years as the demand for egg shipments has increased. After ozonation, the fertilized eggs are bagged in 5L of highly oxygenated sterile sea water with 10L oxygen gas above, and packed into 20L foam cool boxes before being transported by bus or air. Only if the shipment is of long duration, or is at a particularly hot time of year, is any ice included in the esky.

3.3 Egg incubation

The initial egg stocking density into each incubator was recorded, together with daily water quality parameters such as salinity, temperature, dissolved oxygen and pH using calibrated probes. Water exchange rate was also measured daily by recording

the time it took to fill a 10L container from the incubator outlet pipe. This allowed the percentage of tank volume exchange per day to be calculated. The broodstock staff monitored the eggs and made any necessary adjustments to the system during the 3 day incubation period.

After hatching the YTK larvae were harvested, concentrated into a known volume of water and enumerated by taking several replicate samples using a wide bore graduated pipette and counting the number of larvae in each subsample. The average count per 10mL was then extrapolated to estimate the total number of larvae in the harvester. The hatch rate was determined by dividing the total number of larvae in the harvester by the total number of eggs placed into the incubator.

The larvae were then handed over to the larval rearing staff and distributed to each larval rearing tank by gently using a hand jug to measure the required volume of water with the appropriate number of larvae to achieve the desired socking densities. The day the larvae were stocked into the larval rearing tanks was recorded as 1 day post hatch (DPH).

3.4 Larval and Nursery Tank Setup

There were some common features at both Arno Bay (AB) and Port Augusta (PA) hatcheries: both hatcheries operated under natural light conditions, using flow through systems with UV sterilised micro-filtered seawater. However, there were some fundamental differences too.

AB used seven 8m^3 tanks, five of these tanks had outlet standpipes located on the side wall and two tanks had central standpipes. In 2008, the walls were marble coloured but in 2009 the tanks were green.

PA had seven 5m^3 tanks and one 10m^3 tank. Each tank had a central outlet standpipe, all had marble coloured walls and all used air to maintain the hydrodynamic environment of the tanks. This was the case for both 2008 and 2009.

At different times during this project a number of the tanks at each site were modified to change one or both of the lighting and hydrodynamic conditions to determine their effect on larval survival and deformity rates. These circumstances will be described in later sections.

3.5 Live Feeds

YTK, like most finfish larvae, require a series of different sized food items (prey) presented to them as they grow, in order to match the gape size and ability of the larvae to see, lunge and engulf the item. To be successful YTK larval rearing requires operators to use the 'green water culture' approach. This has traditionally been achieved by adding large volumes of live algae (grown in a separate area of the hatchery) to the larval rearing tanks with the density of live algae monitored by a visual assessment (until tank bottom could not be seen).

As part of this project, the effectiveness of using a commercial algal paste concentrate to create the green water was tested. In 2008, the PA hatchery used the traditional live algae (*Isochrysis* and *Nannochloropsis*) in the tanks, whereas the AB hatchery used the microalgal paste. However, in 2009 both hatcheries used the algal paste. The amount of algal paste used in each tank was adjusted daily and monitored by visual assessment.

The YTK feeding regime is to start external feeding with rotifers at 2DPH, and then at about 10DPH progress to *Artemia* (brine shrimp) nauplii, at about 25DPH the larvae can be weaned onto a microparticulate formulated diet at which time the green water can be abandoned.

Providing the large volumes and numbers of each of the live food items to maintain an optimal prey density in the nursery tank over several days is a major limitation to many, if not all, hatcheries. Not only do prey numbers need to be maintained, but the nutritional value of that prey needs to be boosted by pre-conditioning them in commercial enrichment supplements. Proper feeding of rotifers is absolutely critical to their replication, the health of the culture and their usefulness as a prey item. At CST the following approaches were used during the project (2008-2009):

1. Maintenance feeding and multiplication of cultures:
    - Recirculation systems used INVE S.parkle, INVE Culture Selco, yeast
    - Batch systems used live algae (*Nannochloropsis*), INVE S.parkle, INVE Culture Selco
2. Enrichment of harvested rotifers prior to them being fed to YTK larvae was acheived with INVE S.parkle, INVE S.presso, INVE roti Selco
3. Bacteria control using the probiotic INVE Sanolife MIC-F

Feeding regimes in different hatcheries will vary according to past experience, the preferences of the hatchery manager and/or the amount of live food available on the day. The latter factor may mean that whilst the manager would prefer a particular feed regime to be used, on some runs or days the amount of feed distributed to the nursery tanks may be more, or less, than the target amount. Even in a R&D hatchery, where adherence to a specific feeding regime is more critical, the staff sometimes find it difficult to throw excess live feed away (overfeed) or always achieve the numbers of prey that are needed (underfeed).

Any discussion about ways to manage live feeds in a hatchery is complicated by the different numerical approaches that different operators use when they describe their feeding regime. This report has three ways of presenting the feed data.

1. Total feed: the total amount of live feed added to a tank in a day. This is the sum of live feed added to the tank at each of several feeds and is generally expressed in millions of rotifers/*Artemia* per tank. This figure does not take into account the volume of a tank, and so a bigger tank will require more feed that a smaller tank.
2. Total feed density: This is simply the total feed given to a tank in a day divided by the volume of the tank. In this report, total feed density values (*Artemia*/mL) are only used to make comparisons where tank sizes differ.
3. Target feed density: This figure takes into account the counts of residual prey items that are carried out before the next feed. In this report, target feed density is only used to compare the rotifer feeds (rotifers/mL).

The following formula is used to calculate target feed density:

$$\frac{[(\text{residual1} + \text{feed1}) + (\text{residual2} + \text{feed2}) + (\text{residual3} + \text{feed3}) + \ldots]}{\text{Number of feeds}}$$

Example: This calculation shows how the target density would be maintained at 25 rotifers/mL by topping up the rotifers at each feed if the residual counts before each feed were 0, 3 and 8 rotifers/mL respectively:

$$\frac{[(0 + 25) + (3 + 22) + (8 + 17)]}{3}$$

$$= \frac{[25 + 25 + 25]}{3} = \frac{75}{3} = 25 \text{ rotifers/mL}$$

During this project at the CST hatchery live feed technicians produced and enriched the live feeds using different batch and recirculation systems according to hatchery SOPs. Each day the amount of rotifers and/or *Artemia* fed at each feed to every tank

was recorded along with residual prey counts. Daily records of total live feed production are maintained on the database.

As was the case with YTK eggs in this project, samples of both rotifers and *Artemia* were taken at regular intervals and frozen for biochemical analysis and bacteriology at TAFI. Finally, the potential for introducing large numbers of bacteria, including some that could be harmful to the larvae, with the live feed is a substantial risk in nurseries. A commercial probiotic formulation was added to live food cultures to mitigate this risk.

3.6 Larval rearing

Larvae is the name given to the stage of development after hatch through until the individual has most, if not all, of the internal, external and behavioural characteristics of the adult, and then it can be called a juvenile or fingerling. In most marine finfish the larval phase includes a number of critical stages, including the transition from endogenous (yolk sac) to exogenous (live prey) feeding, significant physical and functional development of a number of organs including the eyes, gills, gut, musculature, fins, scales and the first filling of the swimbladder with a gulp of atmospheric air. The transition to a juvenile is usually signalled by weaning (the ability to take a formulated diet) and a change in the behaviour of the fish as it is able to move at least partly independently of the water currents. The larval rearing staff need to be aware of when each of these different stages occur in order to anticipate and, to the best of their abilities, provide for the changing needs of the larvae.

From the long list of critical physical, functional and behavioural changes described above, it is not difficult to see why larval rearing is most critical potential bottleneck in hatchery production. If there is poor survival or a high incidence of deformities in the larval rearing phase this naturally translates to low numbers and/or high levels of deformities in the juvenile phase. For this reason, the hatchery staff need to be vigilant, taking samples accordance to the sampling schedule (Appendix 3a) so that, even if (when) things do go wrong, there are samples in storage that can be studied to see if the cause(s) can be determined and prevented in future runs. Whilst destructive sampling of larvae is essential, consideration must be given to minimise the number of larvae removed from each tank and to balance that with the need for the sampled larvae to be representative of the tank. Care must also be taken to ensure that sampling does not otherwise disturb or contaminate the larvae remaining in the tanks.

The sampling procedures used in this project were standardised by the protocols agreed with SARDI and TAFI (Appendix 3b). The range of samples that were taken included: histological (10% formalin), biochemical (flash frozen), genetic (70% ethanol) and dry weight samples. All samples taken were recorded on the sample record sheet before being stored or transported. Biochemical samples were stored in a -60˚C freezer before being sent to TAFI/IMAS for analysis. Histological samples used for deformity checks were also sent to TAFI/IMAS at the end of the production season for assessment. A number of other histological sampling events were for health purposes, and the remaining samples were retained to create an archive for further analysis if required. Detailed presentations of the different types and extents of deformities, and the biochemical data, will be included in the TAFI/IMAS report.

Training in the deformity assessments occurred on site at CST by TAFI/IMAS staff in both 2008 and 2009. This was done to better enable CST hatchery staff to conduct their own deformity assessments in the future. The deformity rate used by CST in the reporting of its overall production results is calculated by dividing the number of deformity culls by the initial number of juveniles that entered the nursery in that batch.

Larval mortalities in each hatchery were estimated after the production staff had vacuumed the bottoms of each tank in the morning. The mortalities from each tank were transferred to a 10L bucket and 3 sub samples were removed from the stirred bucket using a modified pipette. The numbers of dead larvae in each sub sample were counted and averaged. The average was then extrapolated to 10L volume of water in the bucket to provide an estimate of the daily number of mortalities from the tank.

Several factors with regards to fish ontogeny were measured and monitored throughout the larval rearing phase. These measurements included standard length, total length, yolk sac and oil globule dimensions, swimbladder inflation percentages and gut fullness scores. A compound or dissecting microscope was used to complete all these measurements.

When the juveniles were transferred to the nursery a wet weight measurement was used to estimate the number of juveniles produced. A bucket containing seawater was placed onto a balance and zeroed. A minimum of 300 randomly selected juveniles were taken from the tank and counted into the bucket. The count of juvenile fish and their mass were recorded in order to calculate an average mass per juvenile.

The remaining fish in the tank were then anaesthetised, netted out and placed into buckets of seawater that had been zeroed on the scale. The mass of juvenile fish in each bucket was recorded before the bucket was taken to the nursery for grading. After all the fish had been removed from the tank the total mass of the tank was calculated and divided by the average mass of a juvenile to estimate the number of fish transferred from each tank. This value only provided management with an estimate of what entered the nursery from each run.

3.7 Nursery

Each morning the nursery the tanks were siphoned and the mortalities were counted. If the fish were less than a 3mm grade (that is, they would pass through a grill that had parallel bars 3mm apart), they were considered to still be in the weaning phase. If the juveniles were larger than the 3mm grade they stopped receiving *Artemia* and were completely weaned onto artificial diets. Weaning mortality could be significant, but after the 3mm grade natural mortalities in the nursery decreased substantially with the majority of mortalities being due to culling as a result of structural deformities around the jaws, head, spine, or swimbladder.

Deformities were identified and culled by hand sorting the juveniles. This was a labour intensive process usually requiring all staff on site as well as some casual labour. The hatchery R&D staff trained all hand sorters in the subjective aspects of the different types/varieties of deformities and the cull threshold standards. Every fish in the nursery passes over the hand sorting table and, once a tank has been hand sorted, deformity checks are carried out to assess the quality of hand sorting. More often than not the standard is high and tanks are very rarely hand sorted twice.

Nursery sampling in 2009 was extended because there were significant issues with low rates of swimbladder inflation. Fifty random juveniles from each tank were dissected and if fewer than 47 (95%) of the fish had properly inflated swimbladders then the whole tank of fish was ‘floated’ to separate the non inflated fish (which were discarded) from the inflated fish (which were retained). The floating procedure is described in the hatchery SOPs.

Prior to transfer to the ocean cages, the health team from the growout section would sample 20 random fish from each tank and remove the second gill arch and tissue from the left side of the fish. These samples would be sent to an external commercial

pathology service for processing and the information passed onto growout managers. After the juvenile fish leave the hatchery they are no longer property of the hatchery.

3.8 Bacteriology

As mentioned previously, at regular intervals or at the request of management, water samples were taken from larval rearing, rotifer culture, *Artemia* and broodstock tanks and plated onto various agar plates. The culture media included thiosulphate citrate bile sucrose (TCBS; a *Vibrio* selective agar), marine agar (MA; to indicate the general abundance of bacteria) and sheep blood agar (SBA; to indicate the presence of haemolytic bacteria). The plates were incubated at 30^{o}C for 24 h and the number of colony forming units were counted and categorised according to colour. Some colonies of each colour types that grew on each type of agar were sent away to the Gribbles pathology laboratory to be identified to genus or species, where possible.

Commercial probiotic formulations were used at both hatcheries being added to algal cultures, live feed cultures and larval rearing tanks to assist with microbial management. In 2009, 100ppm of formalin was added on 3, 6 and 9 DPH to attempt to reduce the bacterial load in the tanks. Unfortunately no control tanks could be set up to gauge the effect (if any) of the formalin. This will be discussed later.

3.9 R&D case studies and trials

Some substantive trials were conducted at the CST hatcheries to provide some insight into improving the efficiency and effectiveness of operations. These included:

- A comparison of two types of upwelling systems
- The time course of swimbladder inflation in larvae
- The effectiveness of ozonation for disinfection of eggs
- A comparison of natural and artificial lighting of larval rearing tanks

4. Results and Discussion

4.1 General

The data collected from the two CST hatcheries during the production season are extensive and so, consequently, are the analyses. This report uses information collected during hatchery runs in 2008 and 2009 under the CRC project to illustrate and describe how the main performance indicators, namely larval survival, deformity rates and growth, are affected between and within the hatcheries. Careful consideration of the data may help to determine the factors that have the greatest impact on those performance indicators, and indicate the culture conditions under which mortality and deformities can both be minimised, with the ultimate goal being to improve the effectiveness and efficiency of the hatchery phase.

4.2 Individual hatchery performance by run by year

In 2008 and 2009 the Port Augusta hatchery completed 2 YTK larval runs, whereas the Arno Bay hatchery completed 4 runs in each year. As mentioned in section 3 there were different numbers and sizes of larval rearing tanks available at the two hatcheries and, in some cases, between different hatchery runs at the same hatchery due to the experimental design. The factors that were changed between trials at the CST hatcheries included:

- Tank colour
- The pattern and causal mechanism of water circulation in the tanks
- Whether the light was natural or artifical
- If the eggs were exposed to ozonated water prior to incubation
- Whether probiotics or formalin were added to the water to reduce harmful bacterial load in the live feed systems and/or larval tanks
- If the ‘green water’ was due to live algae or commercial algal paste
- Whether the live feeds were reared using a batch or semi-continuous approach
- When supplemental oxygen was first added to the air supply
- The stocking density of the larvae into the tanks
- The management strategy for adding live feeds to the culture tanks
- Whether the hatchery was dried out prior to each production season

A summary of the factors that were varied between the hatcheries and in which years is shown in Table 1.

Table 1: The major differences in hatchery operating protocols trialed over the past few seasons. Note the historical (2006 & 2007) information, and the *provisional changes to the 2010 protocol are also included. Note that some operational procedures happened in one hatchery and not the other (AB = Arno Bay; PA = Port Augusta).

	2006	**2007**	**2008**		**2009**		**2010***	
Hatchery			**AB**	**PA**	**AB**	**PA**	**AB**	**PA**
Tank sides	White/Blue	Colours	Marble		Green	Marble	Green	Marble
Formalin 3, 6, 9 DPH	No	No	No		Yes		No	
Upwelling	Air	Air	Air		Water+Air	Air	No	
Light	Natural	Natural	Natural		Artificial	Natural	Artificial	Natural
Rotifer system	Batch	Recirc+Batch	Recirc		Recirc	Batch	Recirc	
Rotifer feeding strategy	Feed concept	Feed concept	Density		Density		Density+Meal concept	
O_2 start day	13	13	13		0	13	13	
Probiotics in algal culture	No	Yes	Yes		No	Yes	Yes	
Probiotics in rotifer culture	-	Yes	Yes		Yes		Yes	
Green water	Live algae	Paste+Live algae	Paste	Live algae	Paste		Paste	
Larval stocking density	80-120	80-120	80-120		40	80-120	80-120	
O_3 eggs	No	No	No		Yes		No	
Misting fans	No	No	No		Yes	No	Yes	
Shade cloth	No	No	No		Yes	No	Yes	
Hatchery dry out	No	No	No		No		Yes	

In order to provide an overview of the various hatchery related performance indicators, the results for each hatchery are presented and discussed as follows:

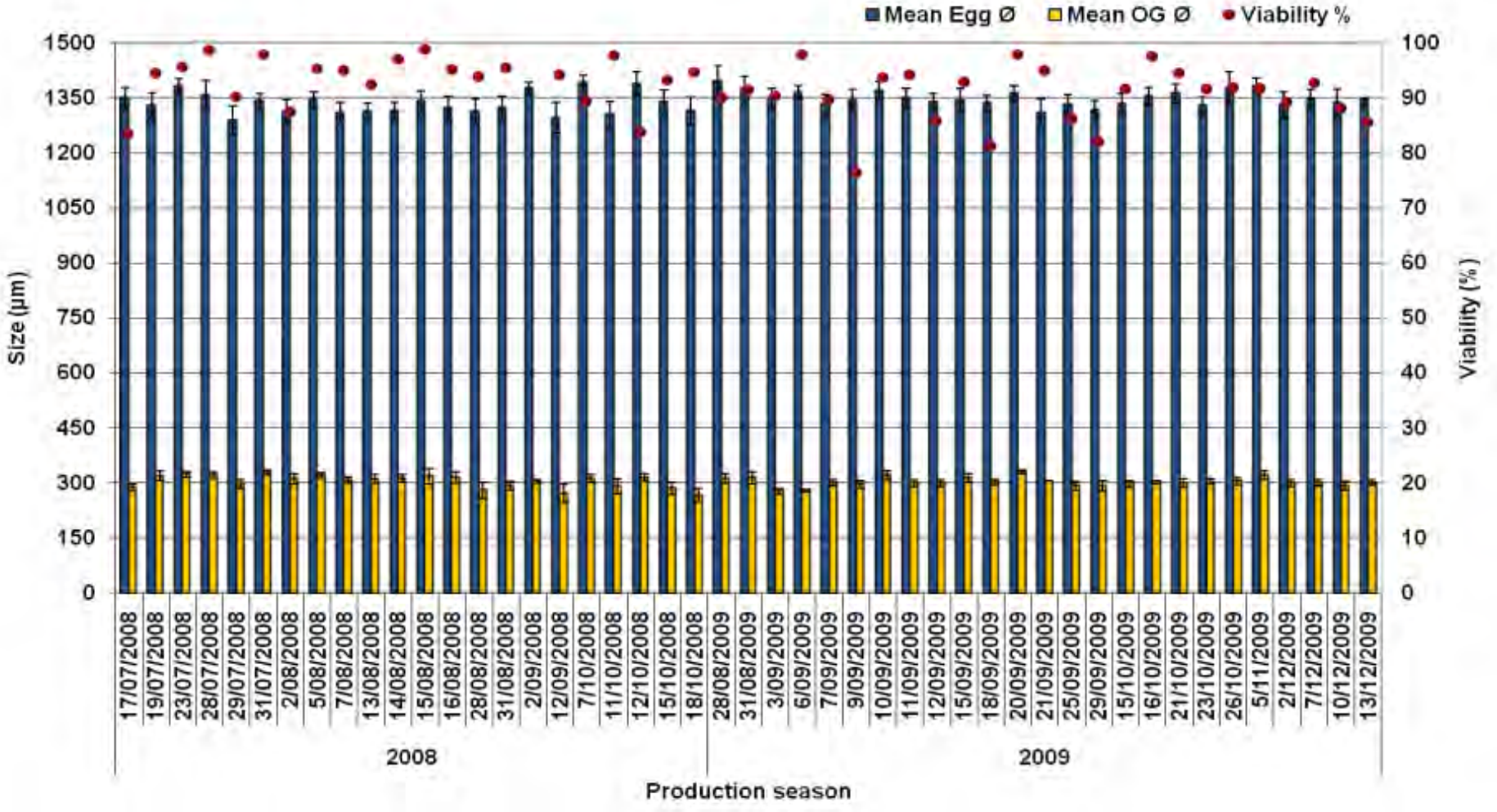
- At the year level. Data from the individual runs provide the replication. Data on egg production, egg quality parameters and incubation are shown at this level only.
- At hatchery run level. Information from individual tanks provides the replication. At this level it is possible to assess the effects of factors such as: tank setup, abiotic parameters, different live feed regimes.

Note: Larval survival was calculated by 'working backwards' from the the number of fingerlings that were transferred to sea-cages, to which was added the total number of mortalities and culls for deformities throughout the larval rearing and nursery stages.

4.3 Year level

4.3.1 Egg production and egg quality

As a result of the perceived poor hatchery results over the last few production seasons there has been increasing speculation that the quality of eggs produced by the YTK broodstock might be decreasing, which, in turn, will affect hatchery performance. However, the data show there was actually little variation in all the egg quality parameters measured over the two year project period both between years and between runs. Some of these data (from AB YTK broodstock group 2; the source of the majority of the eggs used for commercial production runs) are shown in Figure 1.


Mean Egg Ø
Mean OG Ø
Viability %
Size (µm)
Viability (%)
1500
1350
1200
1050
900
750
600
450
300
150
0
100
90
80
70
60
50
40
30
20
10
0
17/07/2008
19/07/2008
23/07/2008
28/07/2008
29/07/2008
31/07/2008
2/08/2008
5/08/2008
7/08/2008
13/08/2008
14/08/2008
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16/08/2008
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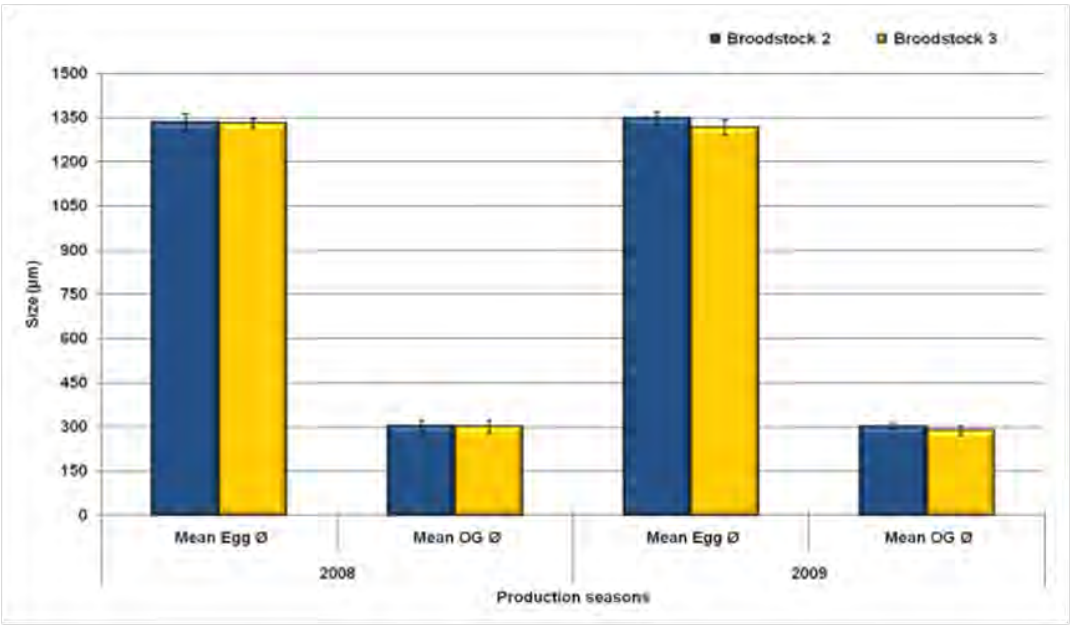
15/09/2009
18/09/2009
20/09/2009
21/09/2009
25/09/2009
29/09/2009
15/10/2009
16/10/2009
21/10/2009
23/10/2009
26/10/2009
5/11/2009
2/12/2009
7/12/2009
10/12/2009
13/12/2009
2008
2009
Production season

Figure 1: Arno Bay broodstock group 2 egg quality parameters measured during different production runs in consecutive years during the project. Egg and oil globule diameters (µm) for each batch are shown by the histograms and include standard deviation. Viability (% successful larval development) for each batch of eggs is shown by the dots.

This small variability is again observed when the same egg quality parameters are compared between two different groups of AB broodstock over both years of the project (Figures 2a & b; broodstock group and year both P>0.05, not significant).

A



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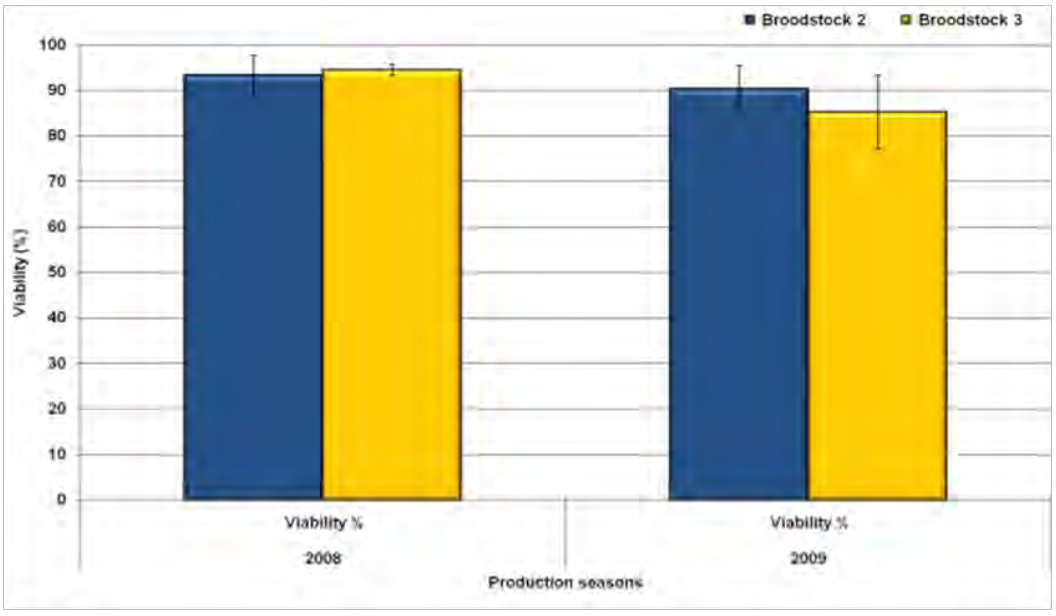


Figure 2. (a) Average egg diameter and oil droplet diameter (±SD), and (b) percentage egg viability (±SD), from Arno Bay broodstock groups 2 and 3 in 2008 and 2009.

Table 2: Comparison of AB broodstock group 2 egg quality means. t-Test: Paired Two Sample for Means . P=0.01.

AB broodstock group 2	Difference in means	t Statistic	t Critical two-tail
Egg diameter	not significant	-2.52	2.08
Oil droplet diameter	not significant	0.25	2.83
Viability of eggs	not significant	1.79	2.85

These findings suggest there is no reason to believe that the variability in larval performance observed in the hatchery is due to broodstock factors affecting egg quality. Continued monitoring of these parameters from all hatchery runs will aid in identifying good quality eggs for production.

Another useful measure of egg quality is the biochemical composition of the eggs; however, expense and long delays before all the results are known are issues. It is suggested that several random batches of eggs be obtained from all broodstock groups across a production season for analysis, to see if there any long term changes in biochemical composition. The biochemistry data from the YTK eggs sampled as part of this project are reported in TAFI/IMAS document.

Broodstock staff are responsible for the egg collection, ozonation and incubation processes in the hatchery. Ozonation of eggs was trialed in 2009 in order to reduce or eliminate the risk that potential pathogens might be transferred from the broodstock into the hatchery via the surface of eggs. Counts of colonies grown on TCBS agar plates showed that this was indeed the case, and ozonation did reduce the bacterial load associated with eggs prior to incubator stocking. The data are presented in section 4.6.2.

As noted in section 3.2, this project provided evidence that the methodology initially used to estimate egg numbers was inaccurate. Hatching success (% of stocked eggs that produce a live free swinning larvae) was oftentimes calculated to be in excess of 100% (Figure 3). With advice from the hatchery R&D staff and the TAFI and SARDI collaborators, the egg enumeration method has been replaced with a more robust and accurate procedure.

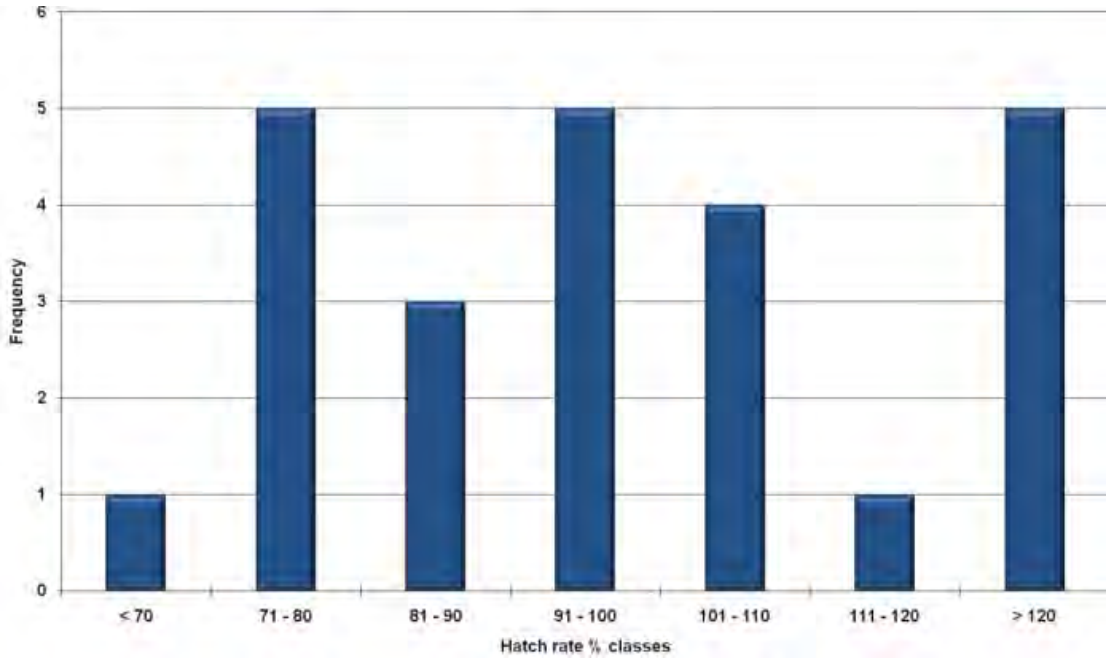


Figure 3: Frequency histogram of apparent hatching rates (%) of different batches of eggs stocked into incubators at Arno Bay in 2009. Note the high number of apparent hatch rates over 100%.

Due to the demostrated inaccuracy of measuring hatch rate in this project it is not possible to assess the subtle effects that certain treatments (eg ozonation, formalin) might have on hatching success; however, less subtle (ie gross) effects would still be apparent. Hatching rates (when estimated correctly), could correlate to larval viability rate, and, if so, could be used as an indication of the hatchery performance that could be expected for a particular batch of larvae prior to hatching.

4.3.2 Tank setup

Our collaborators at TAFI/IMAS have reported that tank setup conditions can influence the survival and deformity rates of marine finfish larvae such as Striped Trumpter (Cobcroft and Battaglene, 2009). The colour of the sides of the tanks was suggested to influence the extent to which larvae exhibited 'walling' behaviour and this could result in high levels of jaw deformity. Despite using several colours on the sides of the larval rearing tanks in these R&D trials, no particular effect (either improvement or further deterioration) in either walling behaviour or rates of jaw deformity was recorded. The interaction between tank colour and lighting is an area that could be researched further with YTK larvae, but until then there is little justification to change tank colour in the production section of the hatchery.

As with tank colour, there was no consistent beneficial effect of using artificial light instead of natural light on larval performance measures determined in this project.

However, again there was some suggestion that artificial lighting should be further researched as there were many indirect benefits noted during the trials. These included more conducive staff working practices perhaps reducing fatigue and the risk of errors.

The only tank related factor trialed in this project which provided some indication that it was beneficial to larval performance outcomes was the hydrodynamic setup. The detailed results of the 2008 trial will be show in section 4.6.1; but briefly, larval survival rates were found to be significantly higher in tanks where water flow, rather than airstones, was used to create the upwelling movement. For this reason CST utilised a water upwelling approach in two successive R&D hatchery runs at AB in 2009; however, these trials did not produce the same results, suggesting further research is needed in this area.

4.3.3 Abiotic parameters

The environmental parameters such as water temperature, pH, dissolved oxygen (DO) and exchange rates are monitored and recorded daily by the hatchery staff. Target maximum and minimum levels of these variables are established by hatchery management prior to a production run. For much of the work described in this report those targets were: water temperature at 2DPH was 24.5˚C, pH was 8.0 and DO was a minimum of 90% saturation. The amount of water exchange was increased as the larvae age. Although target values are set at a particular level, it is expected to see variation in the actual values obtained, with that variability sometimes challenging the acceptable range (Table 3).

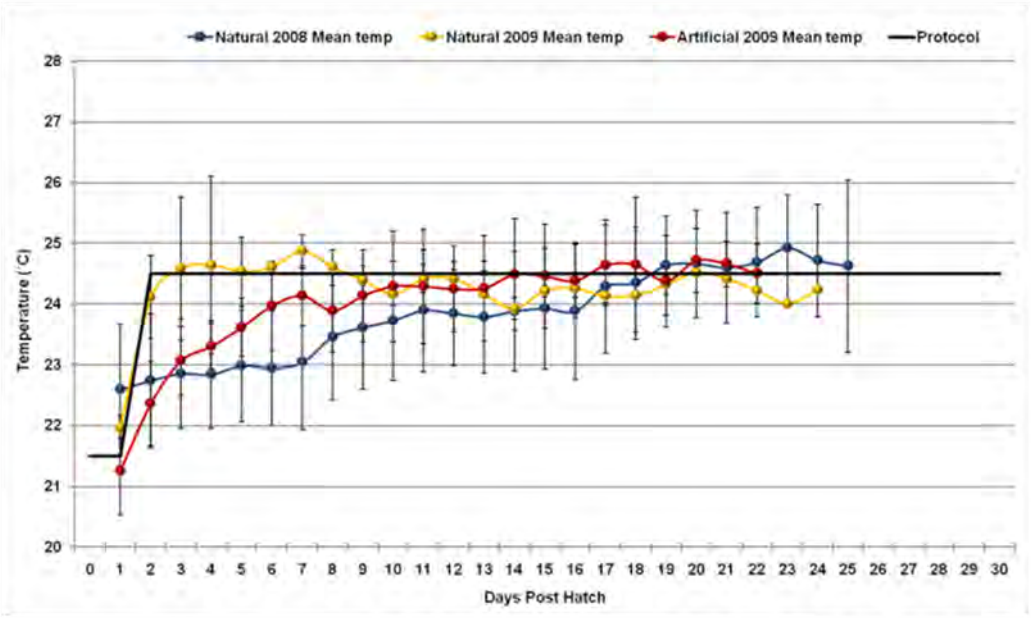
Table 3: An overview of environmental parameters showing the maximum and minimum values of: temperature (˚C), dissolved oxygen (mg/l) and pH for the 2008 and 2009 production runs at both hatcheries.

Hatchery	**AB**		**PA**	
Year	**2008**	**2009**	**2008**	**2009**
$Temp_{max}$	26.4	25.6	25.2	27.6
$Temp_{min}$	21.1	21.0	19.8	20.8
DO_{max}	9.1	8.6	8.6	10.1
DO_{min}	5.5	5.9	5.1	5.5
pH_{max}	8.3	7.8	8.3	7.8
pH_{min}	7.5	6.2	7.2	6.9

These annual maxima and minima data are useful but do not reflect the substantial variability in readings that exist between runs, within runs and even on a daily basis. Most water quality parameters (temperature, pH, salinity and DO) were measured manually three times a day and if found to be outside the range of acceptable limits were adjusted as much as possible to get back towards the recommended operating level. This sampling protocol, as well as the constant data logging records of the Oxyguard® system, together comprise a huge database that needs to be processed before it can be displayed and interpreted effectively.

For all intents and purposes, temperature would seem to be the most critical of these parameters. It may have the most significant effect on larval performance as seen in trials conducted by SARDI (see SARDI component in Appendix C), but at the time of writing CST continues to operate at 24.5°C because in our experience it remains the best practice.

Figure 4 shows the average daily water temperatures measured in the larval rearing tanks across all hatchery runs at both hatcheries in 2008 and 2009 relative to DPH. Temperature is considered to be quite critical to larvae development but, can be difficult to maintain and does fluctuate quite regularly; for example, between day and night, and during heatwaves. It is evident that there are mean differences of up to 2^{o}C between hatcheries and between years in how closely they can match the target temperature profile.



A

B

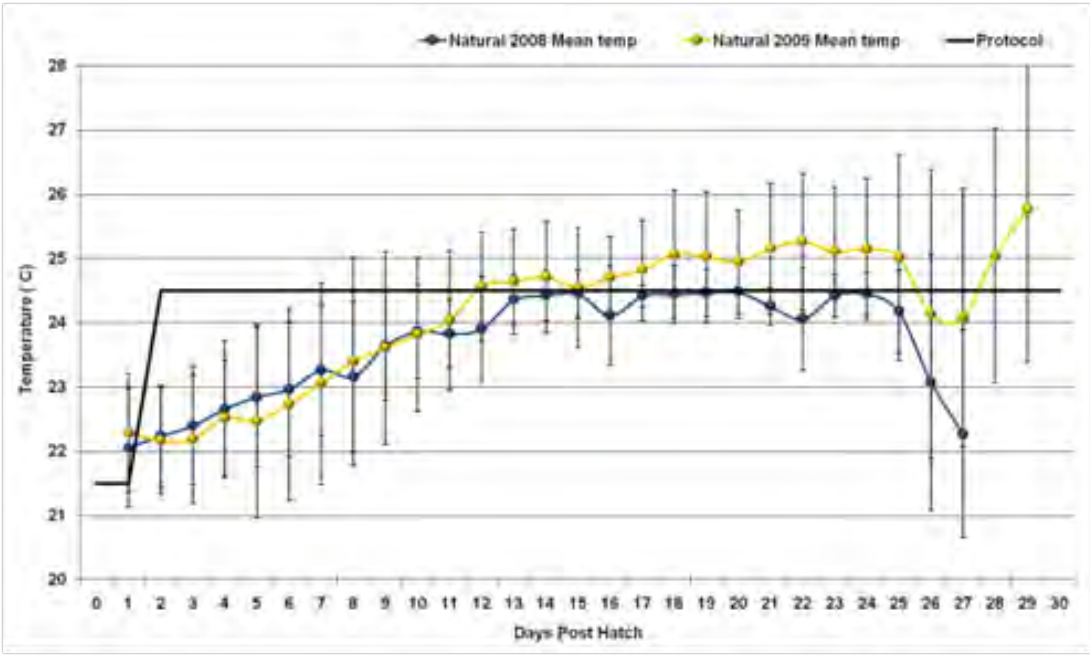


Figure 4: Mean (± SD) daily water temperatures in larval rearing tanks at (a) AB and (b) PA hatcheries with respect to days post hatch. In 2009 one run at AB was carried out under artificial light, and this is shown separately. The target temperature protocol is shown by the black line.

4.3.4 Live feed

The effort required to meet the live feed demands of the R&D and production larval rearing sections is substantial and requires a significant number of capable people to meet the demands. In 2009, CST implemented recommendations from collaborators and past R&D trials, and made several changes to feeding strategy that led to even higher demands on the live feeds section. It should be noted that additional research

effort within this CRC project was occuring in parallel at SARDI. Their work focused on optimising feeding rates, co-feeding and weaning strategies of YTK larvae (see SARDI section).

4.3.4.1 Rotifers

As mentioned above, the numbers of rotifers the live feeds section needed to produce for each production run was substantial. In 2008, each hatchery run required 25-30 billion rotifers to be grown and fed out; in 2009, the average number decreased to 14-22 billion per run (Figure 5). The differences between years and between hatcheries were due to internal (eg feed management strategy) and external (eg low water temperature) factors, both of which will be discussed later.

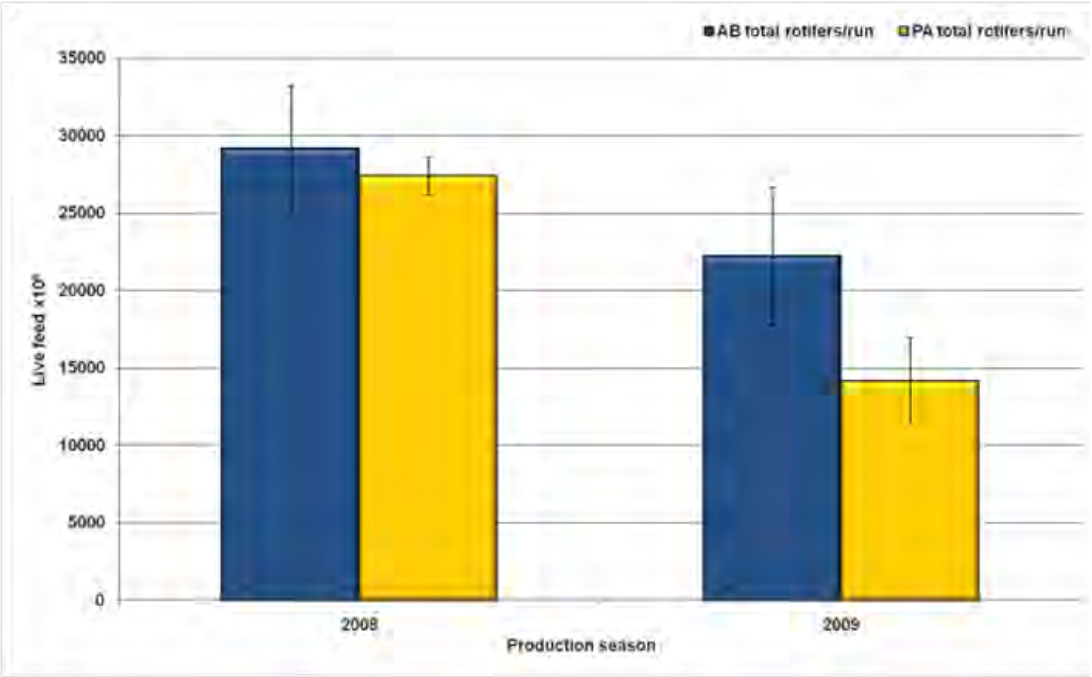


Figure 5: Mean (± SD) number of rotifers used per production run at the Arno Bay and Port Augusta hatcheries in 2008 and 2009.

4.3.4.2 *Artemia*

The cost of producing and enriching *Artemia* nauplii is a significant contribution to the budget line in the hatchery. The mean number of *Artemia* used per hatchery run is shown in Figure 6. Again, internal and external factors affected the numbers of Artemia required for individual runs, but the mean values were between 9.5 and 16.5 billion per run.

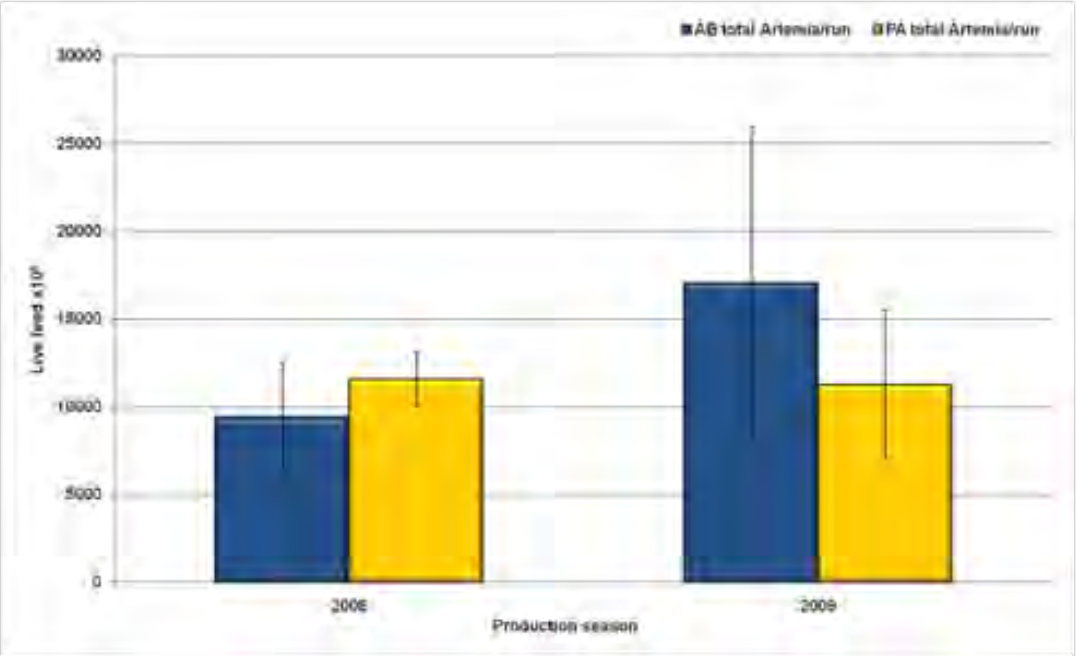


Figure 6: Mean (± SD) number of *Artemia* used per production run at the Arno Bay and Port Augusta hatcheries in 2008 and 2009.

4.3.5 Larval rearing

At the AB hatchery there was a little change in mean overall larval survival from 7.5% in 2008 to 8.6% in 2009. There was no significant difference in the survival rates between the different hatchery runs at AB within each year, but the variation in survival between runs in 2009 was larger than previously observed (Figure 7). At PA the mean larval survival rate across all hatchery runs in 2008 was 12.5% but this decreased significantly to 6.2% in 2009 (Figure 7; t-test, $P<0.05$). The variability in the survival rate of larvae at PA between years, and between hatchery runs within a year, was less than that observed at AB.

As mentioned in the Methods section, towards the end of the larval rearing phase a random sample of 100 larvae were taken, preserved in buffered formalin and sent to TAFI for deformity assessment. The mean rates of commercially significant deformities increased at AB from 27% in 2008 to 32% in 2009. In PA the deformity rate in 2008 was 31%, and in 2009 it was 32% (Figure 8).

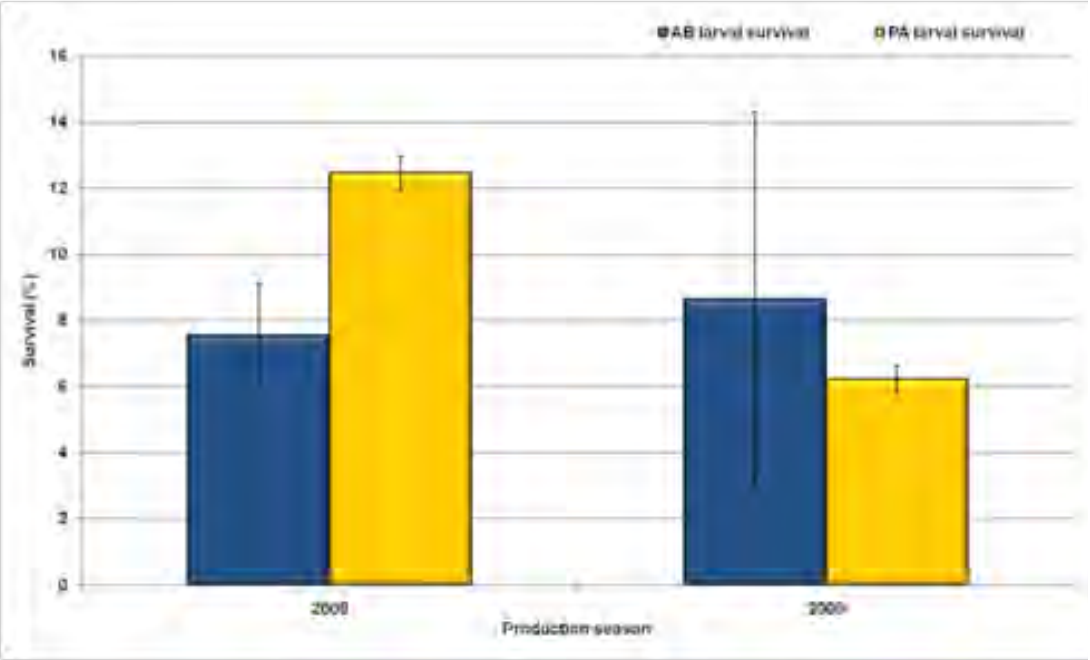


Figure 7: Mean (± SD) YTK larval survival rates at the Arno Bay and Port Augusta hatcheries in 2008 and 2009.

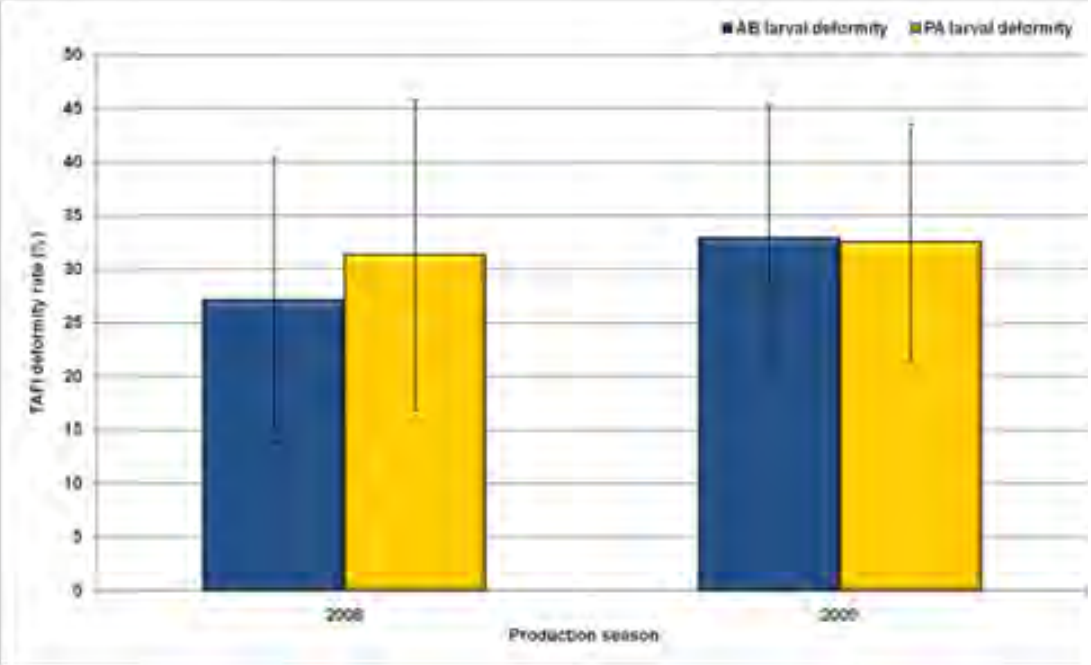


Figure 8: Mean (± SD) YTK larval deformity rates at the Arno Bay and Port Augusta hatcheries in 2008 and 2009.

Table 4: Comparison of the between year survival rates of larvae from the Port Augusta and Arno Bay hatcheries at the rearing and nursery stages, and at transfer to sea-cages. t-Test: Paired Two Sample for Means. P=0.05.

	Difference in means	**t Stat**	**t Critical two-tail**
Port Augusta			
Larval survival	Significant	68.32	12.71
Nursery survival	Not significant	-0.10	12.71
Transfer survival	Not significant	3.27	12.71
Deformity culls	Not significant	-1.49	12.71
Arno Bay			
Larval survival	Not significant	-0.46	4.30
Nursery survival	Not significant	2.71	4.30
Transfer survival	Not significant	2.69	4.30
Deformity culls	Not significant	-2.27	4.30

At this stage it is evident that neither the (a) larval survival rate, nor (b) larval deformity rate, showed any significant signs of improvement over the period 2008-9, suggesting that the changes made to commercial rearing protocols have not been effective in meeting the project objectives.

4.3.6 Nursery

At the end of the nursery phase the juveniles are transferred to the sea cages and the total number of fish transferred is counted. At this point the survival rate from numbers of larvae stocked to time of transfer can be determined. The results again suggest that neither the survival rates nor deformity rates improved in the commercial production runs during the 2008-2009 project timeframe (Figures 9 & 10). At the AB hatchery the transfer survival rate was 5% in 2008 and 3% in 2009 (t test; not significantly different). Similarly, the transfer survival rate showed a not significantly different decrease at the PA hatchery from 6% to 3%.

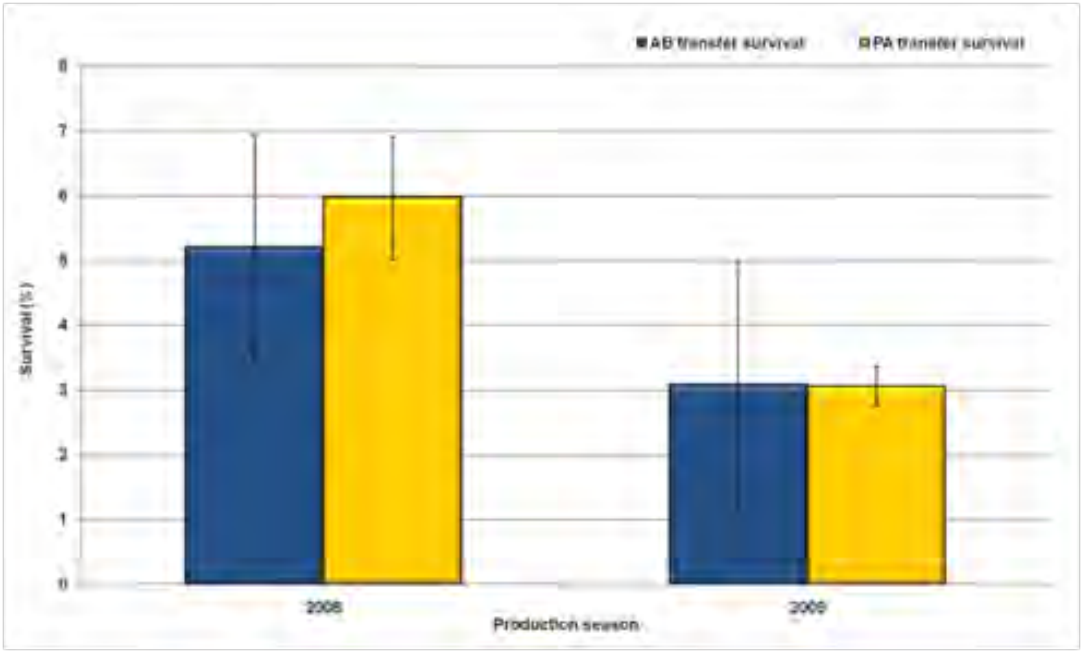


Figure 9: Mean (± SD) survival rates at transfer to sea-cages in AB and PA hatcheries in 2008 and 2009.

The deformity rate is calculated by dividing the total number of deformity culls by the initial number of juveniles that entered the nursery (ie after the hand sorting process). It is clear, once again, that deformity rates did not improve during the project period and, indeed, increased at AB from 18% in 2008 to 39% in 2009, and at PA from 24% to 30%.

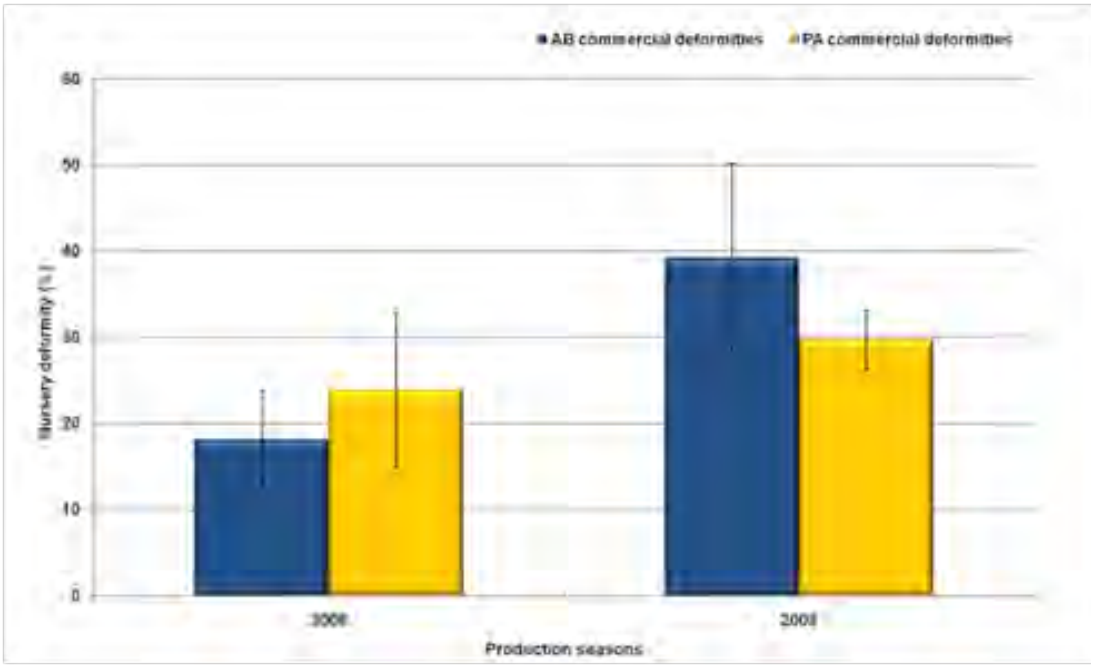


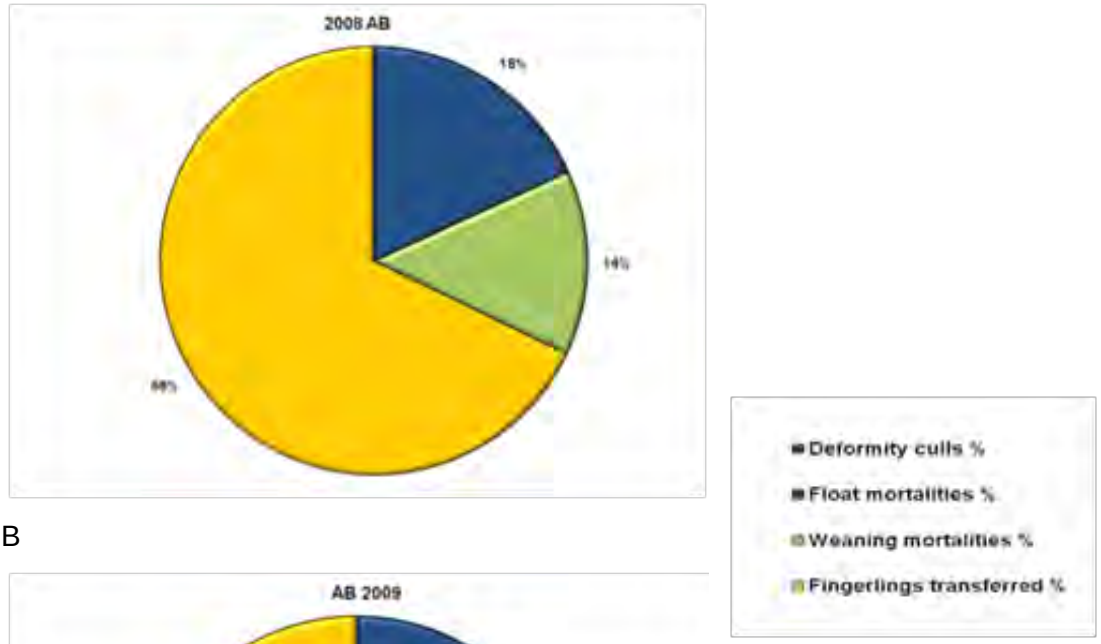
Figure 10: Mean (± SD) deformity rates at transfer to sea-cages in AB and PA hatcheries in 2008 and 2009.

A detailed breakdown of the nursery production performance of both hatcheries is shown in Figures 11 & 12. At the AB hatchery in 2008 the mean of the various production runs indicated that 68% of the weaned juveniles that entered the nursery were transferred to sea (Figure 11a). Of the remainder, 18% were culled because of deformities and 14% lost as weaning mortalities. However, in 2009 the pattern was quite different, with only 37% of the fish being stocked into the sea-cages (Figure 11b). The majority of the nursery losses (39% of fish) were the result of culling due to deformity.

In comparison, at PA the average number of fingerlings transferred to the ocean was a little below 50% in both years (Figure 12 a&b). Deformity culls increased from 24% to 30%, whereas weaning mortality decreased from 28% to 12% in 2008 and 2009, respectively.

In 2009 there was a major issue with the number of fish without swimbladders at both hatcheries, and for this reason before each tank was hand sorted the juveniles were 'floated' to separate the fish with swimbladders from the fish without (which were culled). The proportion of fish culled due to poor swimbladder inflation was 7-9% (Figures 11b & 12b). Float checks were only carried out on a few tanks in 2008 as there was not a high incidence of the condition.

A



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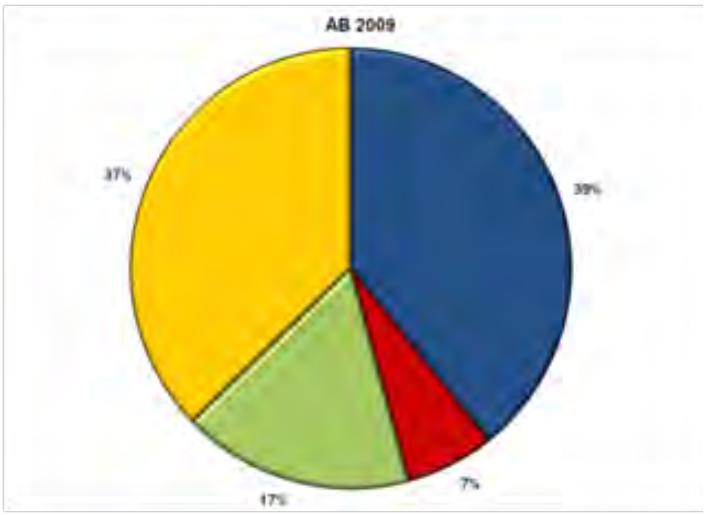
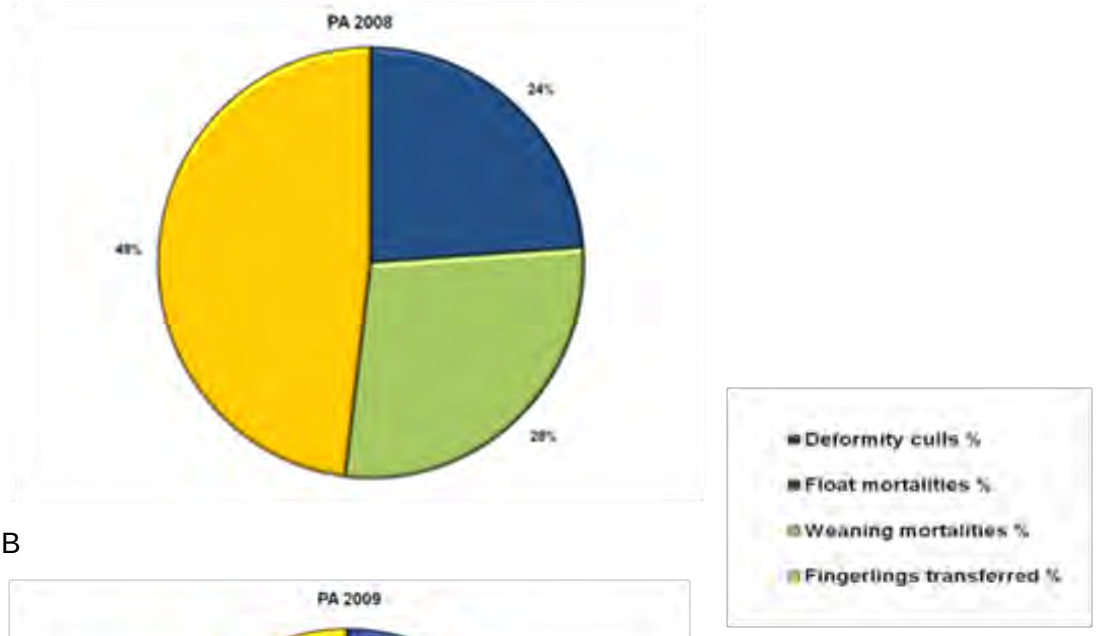


Figure 11: Pie chart representing the mean production performance figures of the newly weaned juvenile fish transferred into the nursery at the AB hatchery in (a) 2008 and (b) 2009 seasons.

A



B

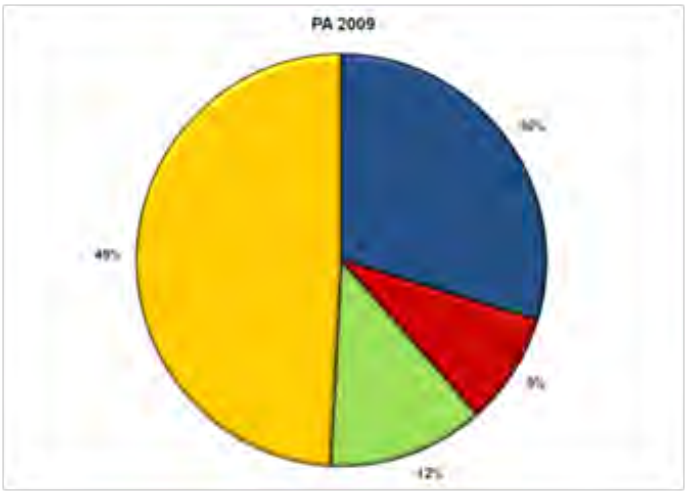


Figure 12: Pie chart representing the mean production performance figures of the newly weaned juvenile fish transferred into the nursery at the PA hatchery in (a) 2008 and (b) 2009 seasons.

The swimbladder inflation window starts at ~2DPH and continues to be measured until 10DPH after such time as the larvae become too pigmented to visualise the swimbladders. If poor swimbladder inflation rates are observed before 10DPH then a decision could be made to float every tank in that hatchery run to cull the fish without a swimbladder. Thus, by monitoring swimbladder inflation rates the hatchery staff can anticipate how the tanks are to be managed. Unfortunately, despite monitoring swimbladder inflation rates at the AB hatchery in 2009, the apparent high levels of swimbladder inflation to 10DPH (80%) were at odds with the findings at the nursery stage (only 45%)(Figure 13).

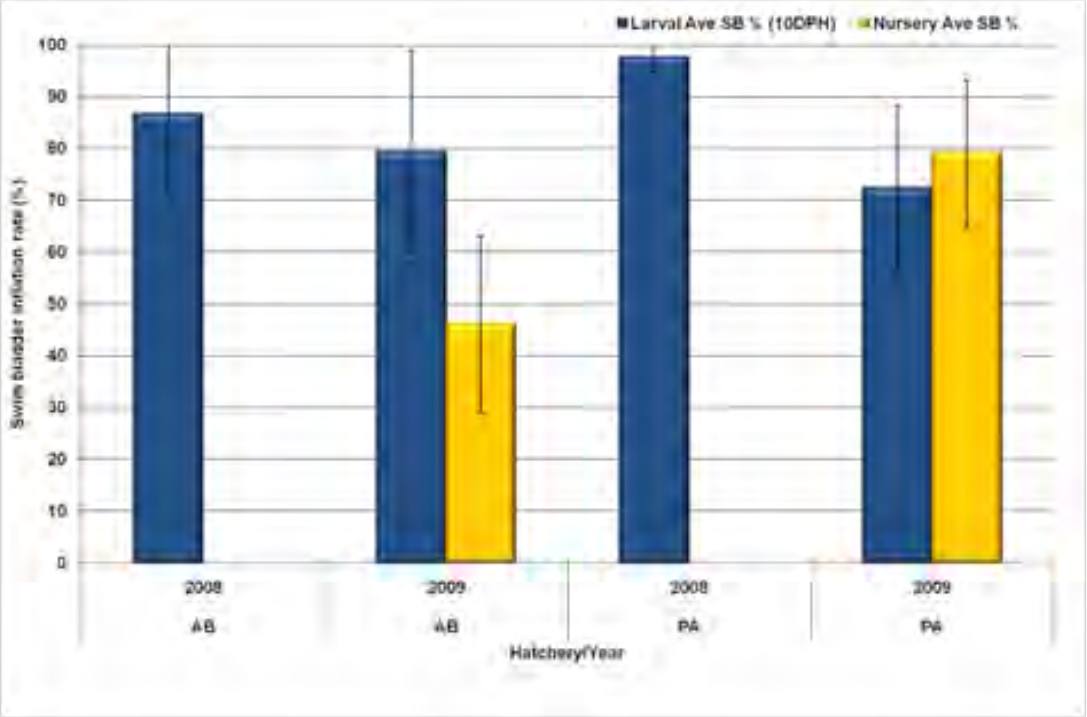


Figure 13: Mean (± SD) incidence of swimbladder inflation to 10DPH (Larval phase – blue bars) and at the float check in the nursery phase (yellow bars) for both hatcheries in 2008 and 2009.

There are several reasons why inaccurate swimbladder inflation figures could have been obtained in the larval phase. Firstly, insufficient training in identifying an inflated swimbladder could lead to under-and over-reporting. Another reason might be that the larvae were not randomly dispersed in the tanks and the sampling protocol selected more larvae that had undergone swimbladder inflation. In this instance the exact cause(s) for the discrepency cannot be identified. However, the consequences of poor swimbladder inflation rates are expensive and wasteful throughout the remainder of the production cycle.

It has been found that ‘skimming’ the water surface to remove any oils or other barrier materials is critical during the swimbladder inflation window. Although skimming is not mutually exclusive from the other subtle triggers and conditions, the relaxed practice (for example: through poor placement or maintenance of skimming devices), of this procedure could easily be the main reason for poor swim bladder inflation.

4.4 Variability in larval survival and deformity rates at the hatchery run level

Individual hatchery run results for rates of: survival, deformities, swimbladder inflation, feed and growth, will be shown. The replication within a hatchery run is at the the level of the tank.

4.4.1 Larval mortalities

As described in the Methods section, estimates of the daily mortality in each tank was recorded by siphoning the bottom of the tank. It must be acknowledged that such counts are not particularly accurate due to larval decomposition and sampling error, but they are important because they do give an indication of major mortality events as they occur (Figure 14 a,b,c,d).

With regards to the daily mortalities, each year AB generally showed that the majority of the mortalities occurred from ~2-10DPH whereas PA mortalities occurred later from ~8-18DPH. One issue here is a potential trigger that causes a spike in the number of mortalities. Temperature is possibly the most obvious as well as the presence of bacteria although no concrete reason for mass fall out events can be given, although with the abundance of samples taken for histology as well as being viewed daily suggested no obvious cause for these events either. Importantly, future research should include a certain amount of effort in determining why and when these events occur.

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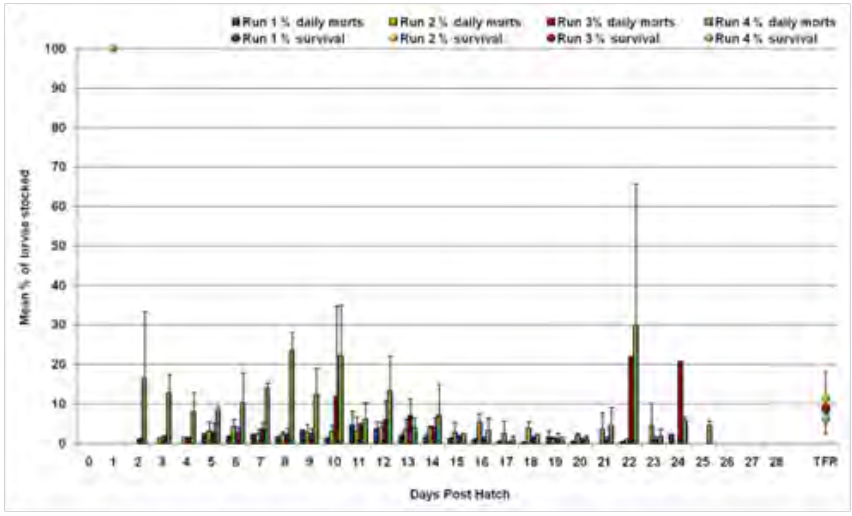
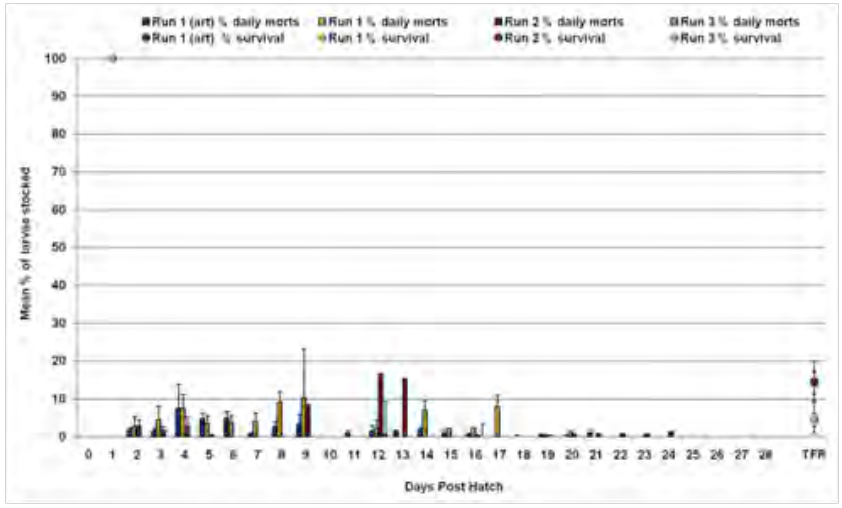
Figure 14: Mean (± SD) daily mortality rate in each hatchery run (histogram bars) as a percentage of the initial number of larvae stocked. Mean (± SD) transfer to sea survival rates for each run are shown by the dots at TFR on the x axis. (a) AB in 2008, (b) AB in 2009, (c) PA in 2008, and (d) PA in 2009.

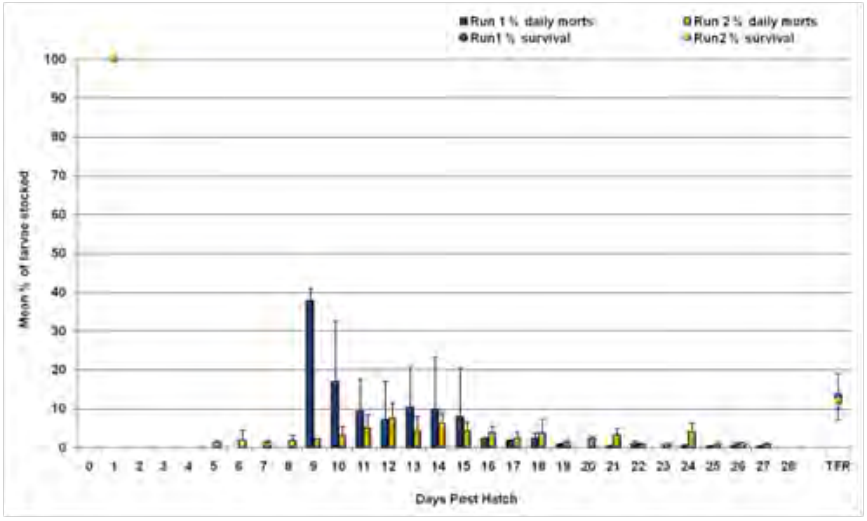
A


Run 1 % daily morts
Run 2 % daily morts
Run 3% daily morts
Run 4 % daily morts
Run 1 % survival
Run 2 % survival
Run 3 % survival
Run 4 % survival
Mean % of larvae stocked
Days Post Hatch
TFR

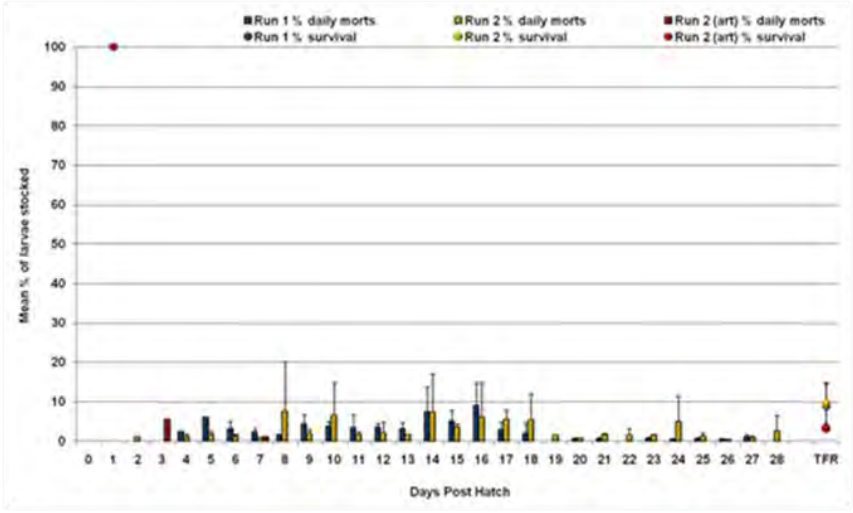
B


Run 1 (art) % daily morts
Run 1 % daily morts
Run 2 % daily morts
Run 3 % daily morts
Run 1 (art) % survival
Run 1 % survival
Run 2 % survival
Run 3 % survival
Mean % of larvae stocked
Days Post Hatch
TFR

C


Run 1 % daily morts
Run 2 % daily morts
Run1 % survival
Run2 % survival
Mean % of larvae stocked
Days Post Hatch
TFR

D


Run 1 % daily morts
Run 2 % daily morts
Run 2 (art) % daily morts
Run 1 % survival
Run 2 % survival
Run 2 (art) % survival
Mean % of larvae stocked
Days Post Hatch
TFR

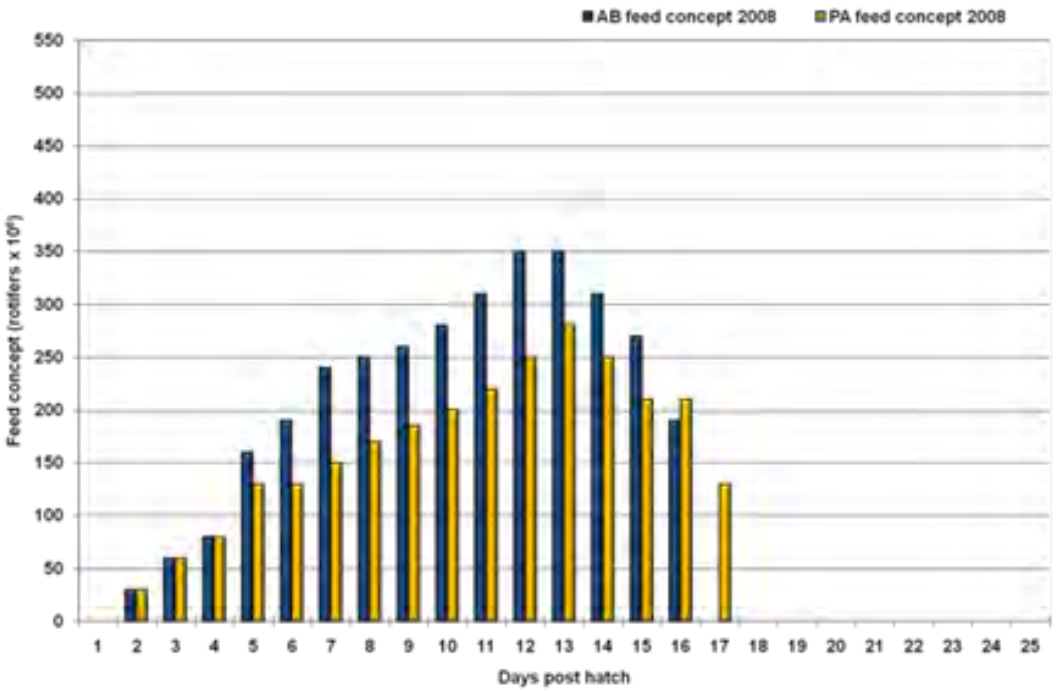
4.4.2 Live feeds

The feeding strategy and rates are set by management before the start of each production run. Thus, the amount of live feed each tank received at each feed was calculated daily according to the intended feeding protocol. The information below, however, shows a very different picture for both rotifer and *Artemia* feed rates, particularly at AB, whereas the PA hatchery more closely matched the intended protocol.

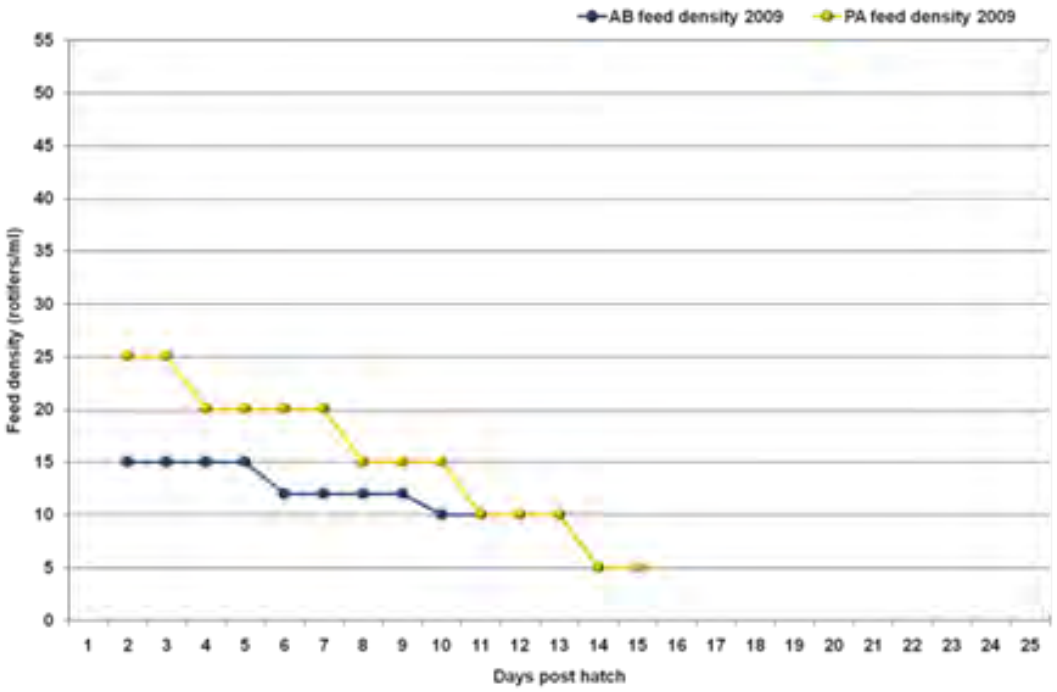
4.4.2.1 Rotifers

At CST, rotifer cultures are maintained throughout the year and are the first prey organisms that YTK larvae receive at 2DPH. Rotifer cultures are open to the atmosphere, contain high levels of dissolved organic and inorganic nutrients, and consequently will always carry a high non-rotifer microbial load (bacteria and other protists). Under good conditions, a rotifer culture can be maintained for weeks, but it can also quickly and unexpectedly 'crash' in response to identifiable (eg changes in ambient temperatures, aeration system failures, contamination and occasionally human error), and unidentifiable causes. At CST a large number of clean rotifers are produced each day through the efforts and understanding of the management and staff.

As mentioned in the Methods section, two rotifer feeding strategies were used during this project. In 2008, both hatcheries used the 'feed concept' strategy whereby the total number of rotifers each tank was allocated each day was calculated (in millions/tank), with the numbers increasing periodically during the rotifer feeding period. The lower feed levels that were allocated at the PA hatchery reflect the smaller tanks at that site relative to AB (Figure15a). In 2009, the 'feed density' strategy was used and required the number of rotifers in individual tanks to be topped up to the required density at each feed. Using this feeding strategy the data needs to be presented as rotifers/mL (Figure 15b).



A



B

Figure 15: Feeding targets as used during the project at (a) AB and PA in 2008 using the 'feed concept' strategy, and (b) AB and PA in 2009 using the 'feed density' strategy.

In the graphs below the mean tank 'feed concept' values and the 'feed density' values are both shown for each hatchery run. The 'feed concept' values have been presented as 1, 2 or 3 cumulative feeds according to the number of times the tanks were fed in the day. Due to the differences in tank size used the amount of live feed each tank received has been standardised as the mean total amount of rotifers per day/mL is also shown.

In 2008 rotifers were fed for a lot longer period than planned in the protocol due to a managerial decision to continue feeding rotifers at a decreasing rate beyond what was planned. This decision meant that the larvae were fed rotifers from 2DPH to the day of transfer (24DPH) instead of 14DPH, leading to the larger total number of rotifers used for each run in 2008 than in 2009.

What can be seen from the graphs below (Figure 16a-d) is that at the AB hatchery it was common for more rotifers to fed to each tank than what was stipulated in the protocol. Thus, the actual feed rates at AB in 2008 and 2009 do not closely match the intended feeding targets, and they also vary between hatchery runs. Given this, it will be difficult to separate any effects of the variation in feeding regime from the effect of any other factor(s) when assessing the impact on survival, deformities and growth rates.

Feeding practices at PA appeared to be much closer to the intended target regime, except when, in 2009, rotifer production was constrained (Figure 17). Note also the greater level of consistency between hatchery runs within each year.

The effect that live feed rates and regimes have had on the survival rates and deformity levels have not been assessed in depth at CST. However, work on the timing and amount live feeds have on larval survival and deformity has been conducted by SARDI and is addressed as such in Appendix C.

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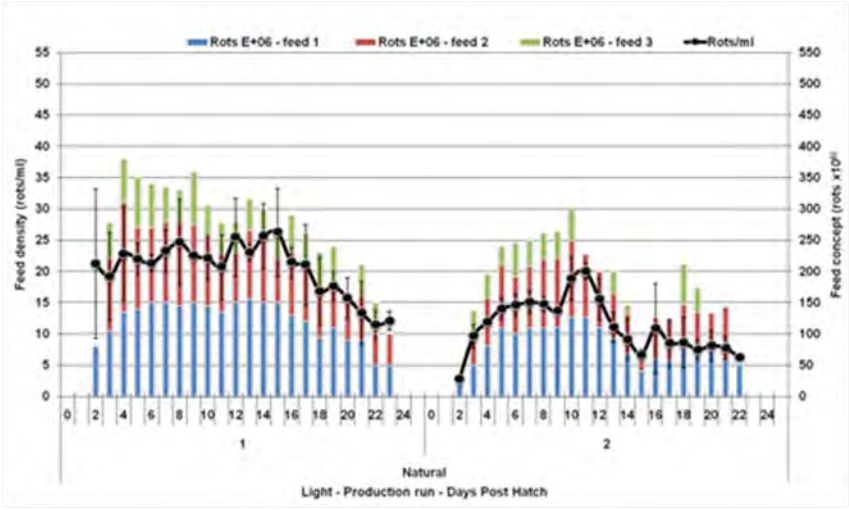
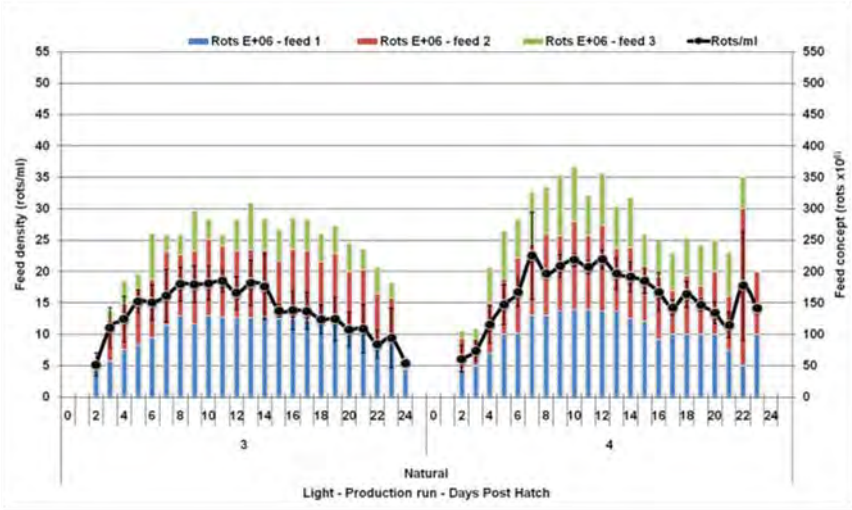
Figure 16: Rotifer feed regimes at AB shown as mean (± SD) rotifer density (rotifers/mL; black line) and numbers of rotifers added per feed (x10^6 rotifers/tank; over up to 3 feeds - blue, red and green histogram bars) per day during larval rearing. (A) and (B) 2008 hatchery runs 1-4, and (C) 2009 illumination trial, and (D) hatchery runs 2 and 3.

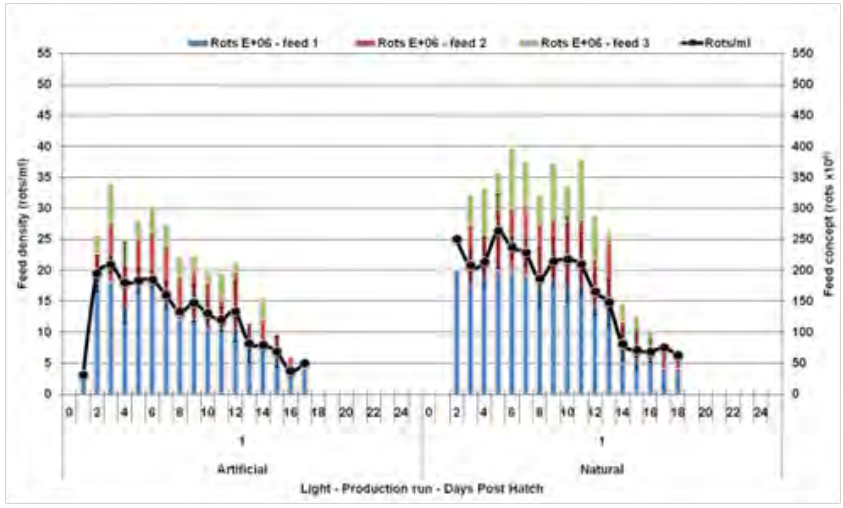
A


Rots E+06 - feed 1
Rots E+06 - feed 2
Rots E+06 - feed 3
Rots/ml
Feed density (rots/ml)
Feed concept (rots x10⁶/l)
1
2
Natural
Light - Production run - Days Post Hatch

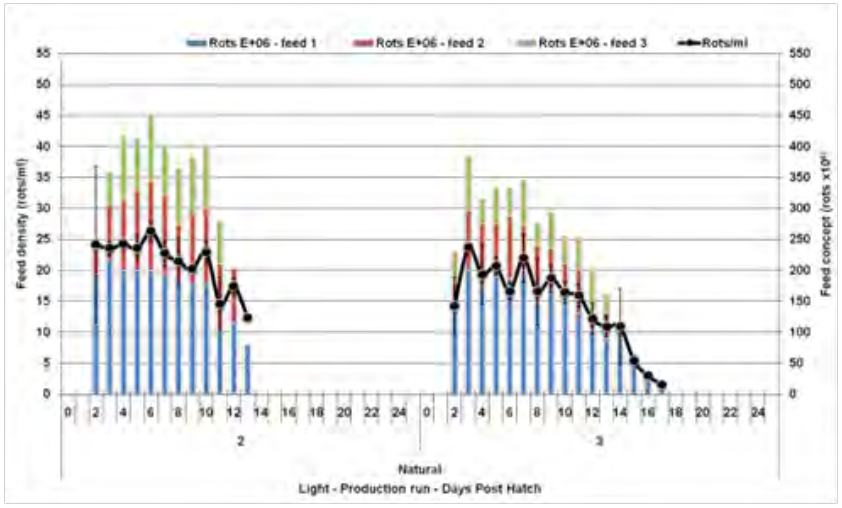
B


Rots E+06 - feed 1
Rots E+06 - feed 2
Rots E+06 - feed 3
Rots/ml
Feed density (rots/ml)
Feed concept (rots x10⁶/l)
3
4
Natural
Light - Production run - Days Post Hatch

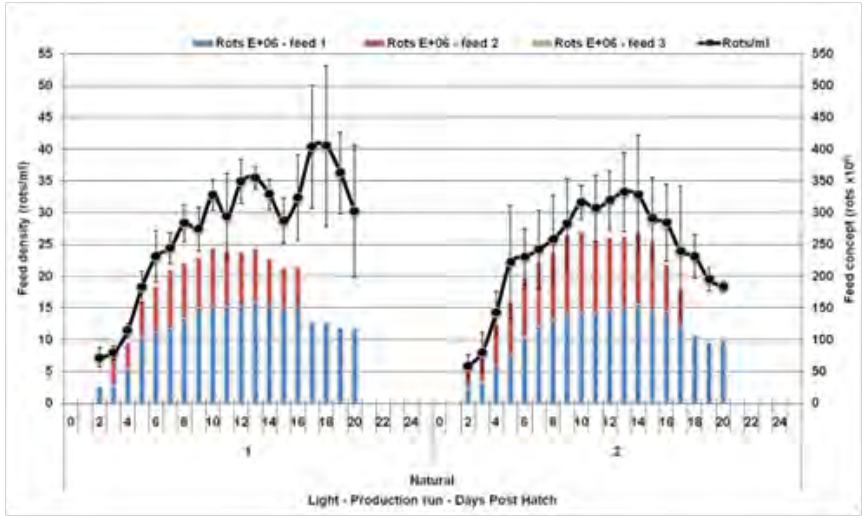
C


Rots E+06 - feed 1
Rots E+06 - feed 2
Rots E+06 - feed 3
Rots/ml
Feed density (rots/ml)
Feed concept (rots x10⁶/l)
1
1
Artificial
Natural
Light - Production run - Days Post Hatch

D


Rots E+06 - feed 1
Rots E+06 - feed 2
Rots E+06 - feed 3
Rots/ml
Feed density (rots/ml)
Feed concept (rots x10⁶/l)
2
3
Natural
Light - Production run - Days Post Hatch

A



B

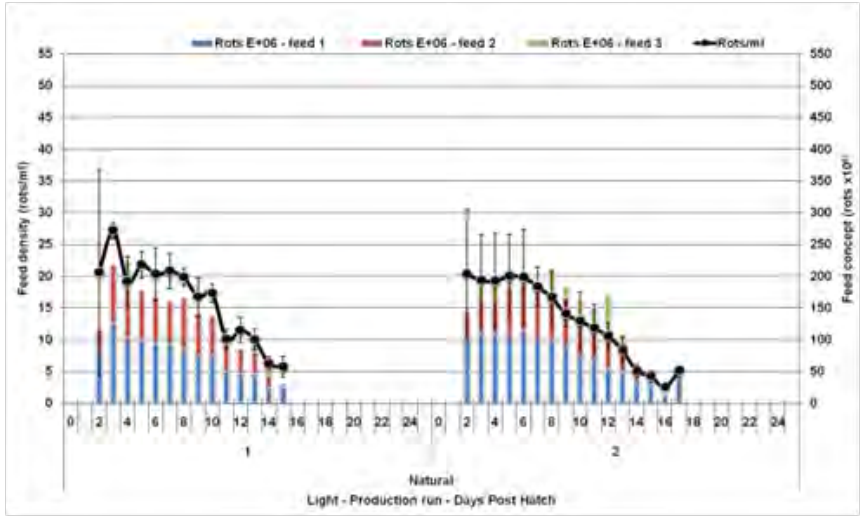
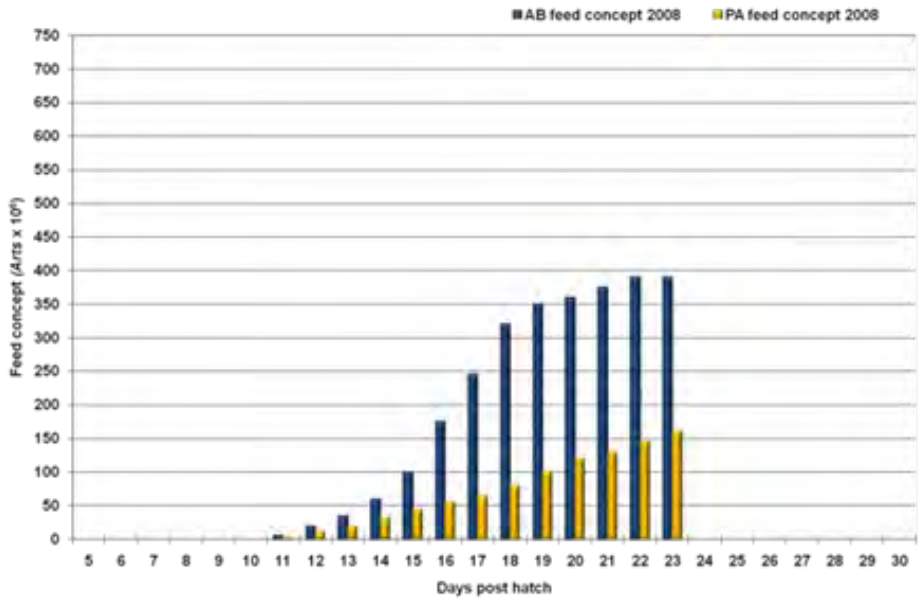


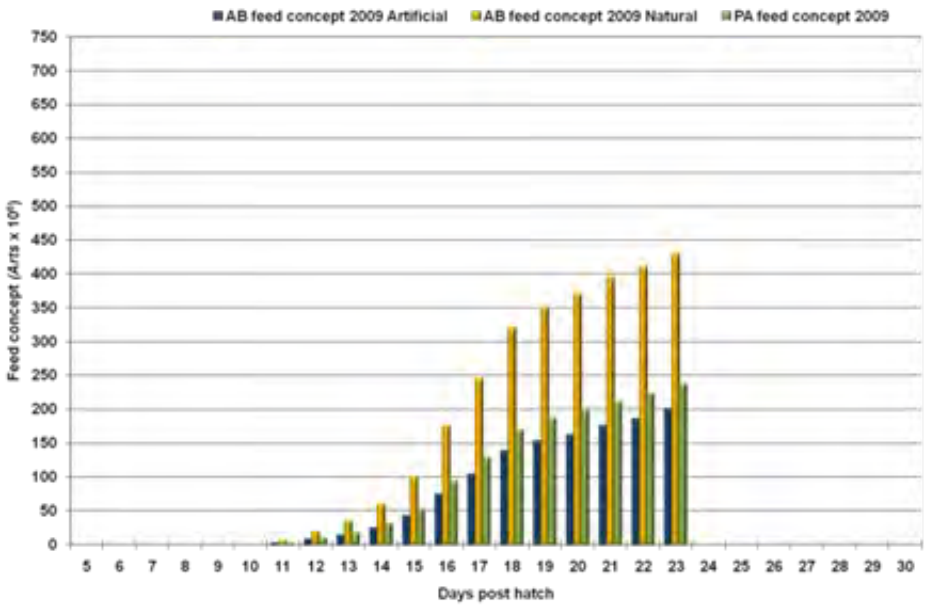
Figure 17: Rotifer feed regimes at PA shown as mean (± SD) rotifer density (rotifers/mL; black line) and numbers of rotifers added per feed (x10^6 rotifers/tank; over 1 or 2 feeds – blue and red histogram bars) per day during larval rearing. (A) 2008 hatchery runs 1 and 2, and (B) 2009 hatchery runs 1 and 2.

4.4.2.2. *Artemia*

The *Artemia* feeding regime follows the 'feed concept' approach but differs from the rotifer regime in that there can be more feeding events in a day. The *Artemia* feed strategy is outlined by management prior to the production runs (Figure 18). The reason PA larvae appear to receive fewer *Artemia* than at AB is because of the smaller tanks at that site. In the case of the AB run 1- artificial light in 2009, the experimental tanks were stocked with larvae at approximately half the density of the tanks under natural light, therefore they were allocated less food.



A



B

Figure 18: *Artemia* feed regimes showing total number of *Artemia* added per day (x10^6 *Artemia*/tank) during larval rearing. (A) 2008 and (B) 2009 hatchery runs and illumination trial, at AB and PA hatcheries.

In order to standardise the total amount of feed each tank received in a day the total amount of feed received (*Artemia* x 10^6) was divided by the volume of the tank and converted to *Artemia*/mL. Importantly, it must be understood that this value reflects the total amount of feed received and is not the same as the target density feed because the calculation does not include residual counts. As was observed with the rotifer feeding, the actual *Artemia* feeding regimes at AB did not closely match the intended target protocol, and there were some large variations in the feed rates between runs in a particular year (Figure 19).

Also reflecting the observation made with roftifer feeding, the PA hatchery much more closely followed the planned feed rates, and showed a high level of consistency between runs and years (Figure 20).

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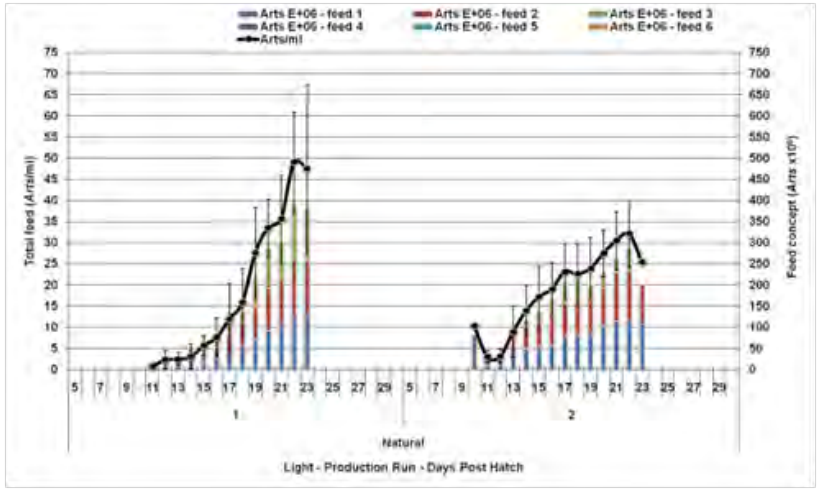
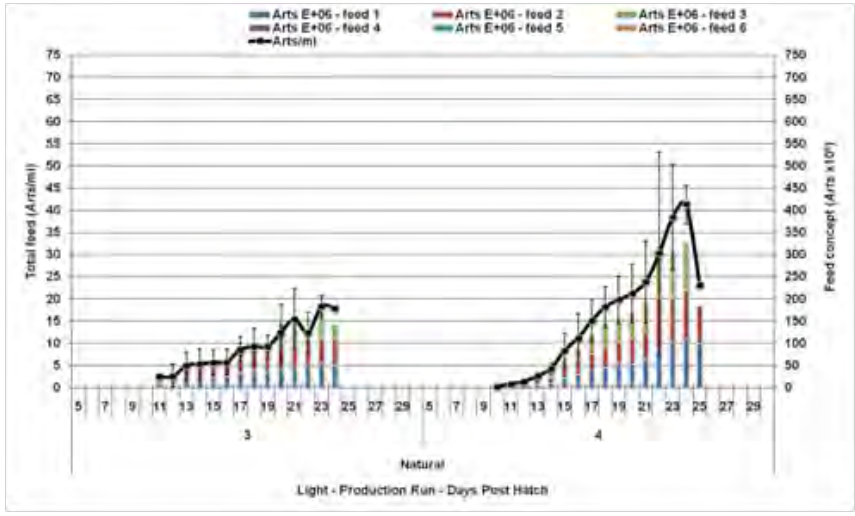
Figure 19: *Artemia* feed regimes at AB showing total number of *Artemia* added per day (x10^6 *Artemia*/tank) and mean (± SD) total *Artemia* density (*Artemia*/mL; black line) during larval rearing. (A) and (B) 2008 hatchery runs 1-4, and (C) 2009 illumination trial, and (D) hatchery runs 2 and 3.

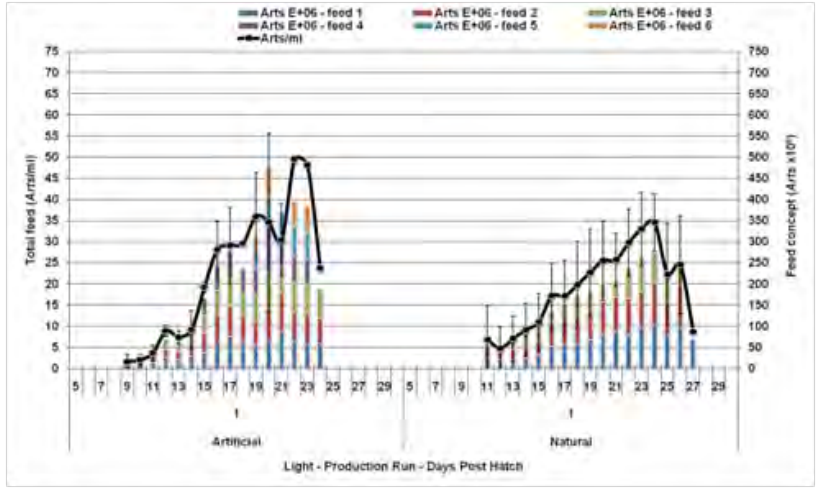
A


Arts E+06 - feed 1
Arts E+06 - feed 2
Arts E+06 - feed 3
Arts E+06 - feed 4
Arts E+06 - feed 5
Arts E+06 - feed 6
Arts/ml
Total feed (Arts/ml)
Feed concept (Arts x10⁶)
1
2
Natural
Light - Production Run - Days Post Hatch

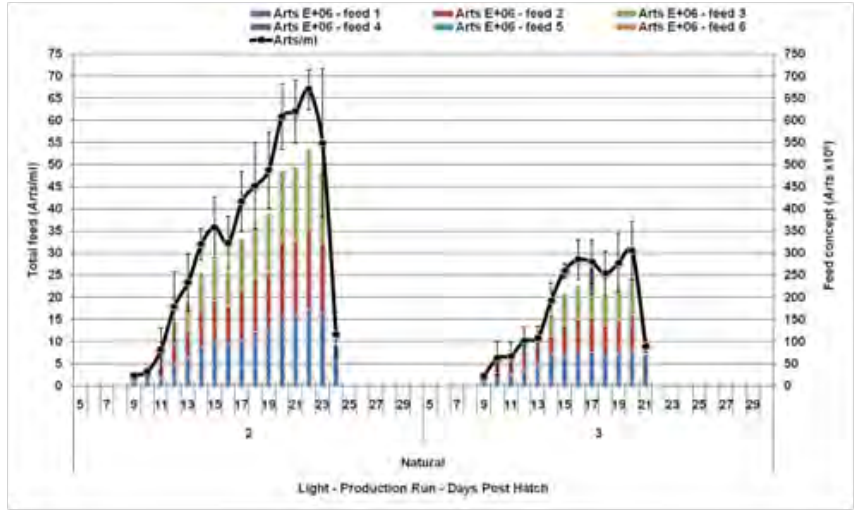
B


Arts E+06 - feed 1
Arts E+06 - feed 2
Arts E+06 - feed 3
Arts E+06 - feed 4
Arts E+06 - feed 5
Arts E+06 - feed 6
Arts/ml
Total feed (Arts/ml)
Feed concept (Arts x10⁶)
3
4
Natural
Light - Production Run - Days Post Hatch

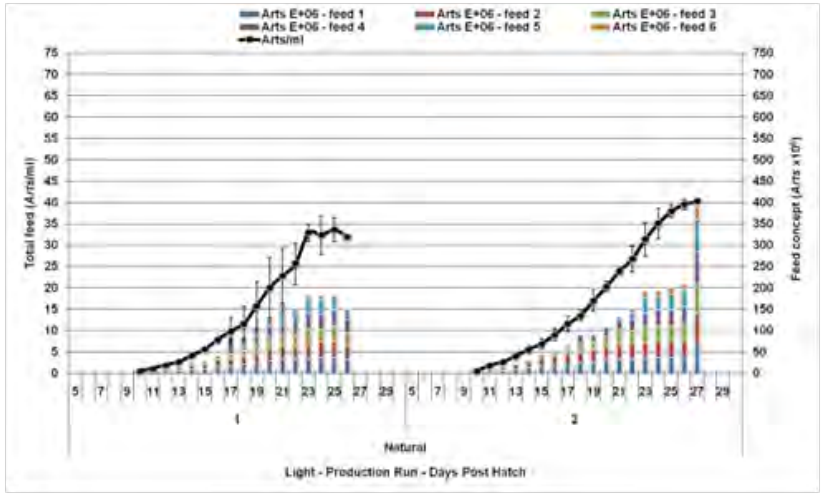
C


Arts E+06 - feed 1
Arts E+06 - feed 2
Arts E+06 - feed 3
Arts E+06 - feed 4
Arts E+06 - feed 5
Arts E+06 - feed 6
Arts/ml
Total feed (Arts/ml)
Feed concept (Arts x10⁶)
1
1
Artificial
Natural
Light - Production Run - Days Post Hatch

D


Arts E+06 - feed 1
Arts E+06 - feed 2
Arts E+06 - feed 3
Arts E+06 - feed 4
Arts E+06 - feed 5
Arts E+06 - feed 6
Arts/ml
Total feed (Arts/ml)
Feed concept (Arts x10⁶)
2
3
Natural
Light - Production Run - Days Post Hatch

A



B

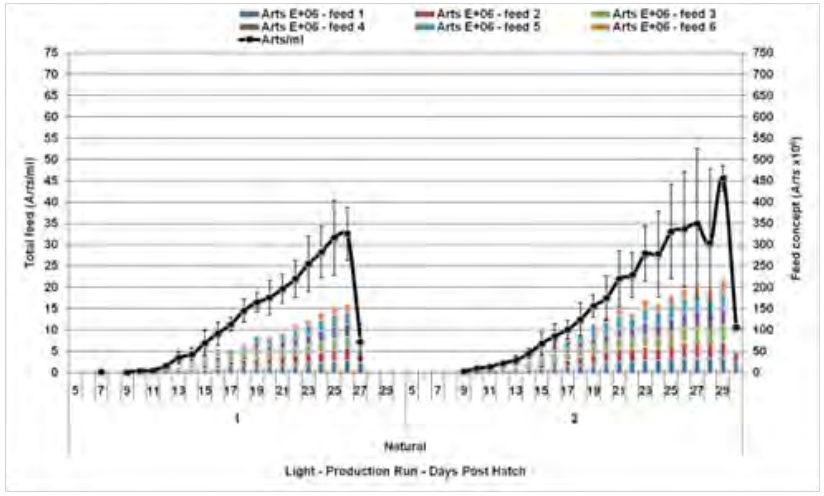


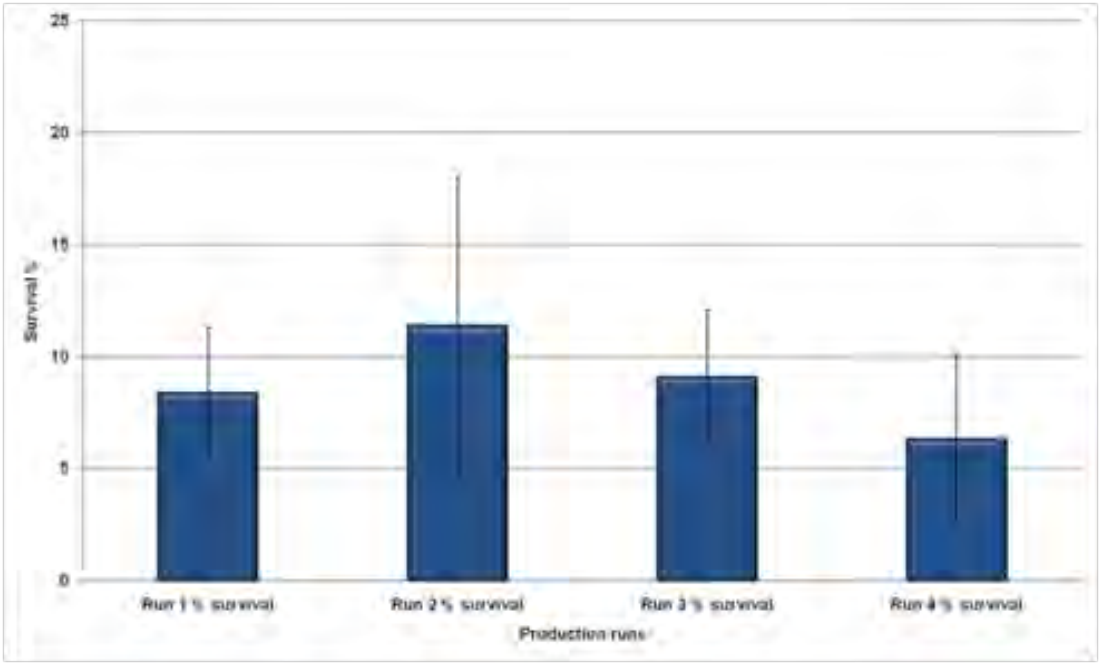
Figure 20: *Artemia* feed regimes at PA shown numbers of *Artemia* added per feed (x10^6 /tank) per day and mean (± SD) total *Artemia* density (*Artemia*/mL; black line) during larval rearing. (A) 2008 hatchery runs 1 and 2, and (B) 2009 hatchery runs 1 and 2.

4.4.3 Larval rearing

This findings from this phase of the project will be presented and evaluated with a greater level of detail than was justified for other phases. Survival and deformity rates at the end of larval rearing are key indicators of production performance. As was shown in section 4.3.6, when the annualised production performance data in 2008 and 2009 were calculated (Figures 9 & 10), there was no suggestion of any improvement at either hatchery regardless of what conditions were changed.

In the AB hatchery survival and deformity rates at the run level were remarkably different in successive years. In 2008, the mean survival rates per run were relatively stable being always above 5% but never exceeding 15% (Figure 21a). Similarly, the mean deformity rates in 2008 were consistent and were always 25-30% (Figure 21b). These findings probably reflect that in 2008 some small changes were made to the production protocols before the season started, but nothing new or different was trialled during the production runs.

A



B

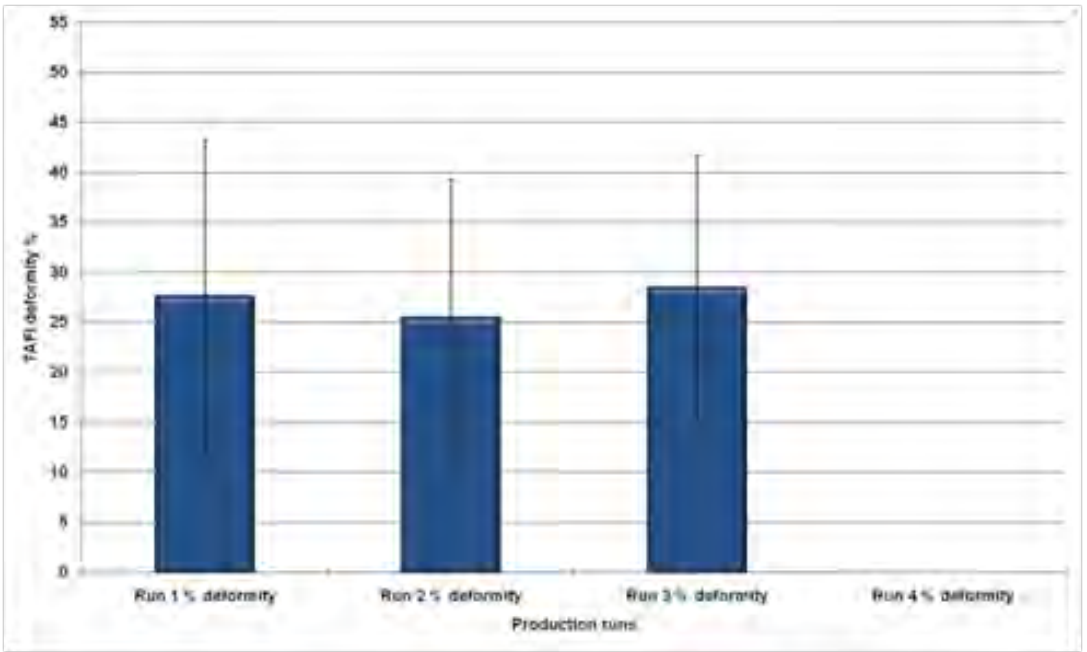
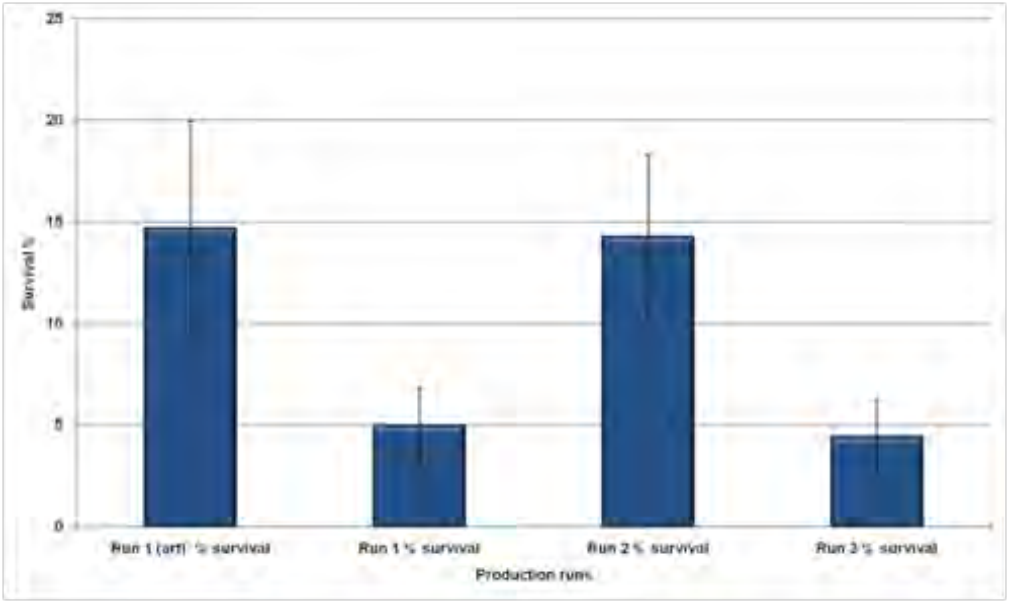


Figure 21: Mean (± SD) (a) survival rates, and (b) deformity rates based on TAFI classification, over each of the four hatchery runs carried out at the AB hatchery in 2008.

In contrast, the results obtained in 2009 were more variable with survival rates from less than 5% to 15% in successive runs (Figure 22a), and even more issues with deformity rates ranging from 22 to 45% (Figure 22b). The reason(s) for these differences is not known but it seems likely that the variable (ie non-complient) feeding rates and the somewhat *ad hoc* formalin treatments, were symptomatic of a general lack of adherence

to established practices and processes across the board.

A



B

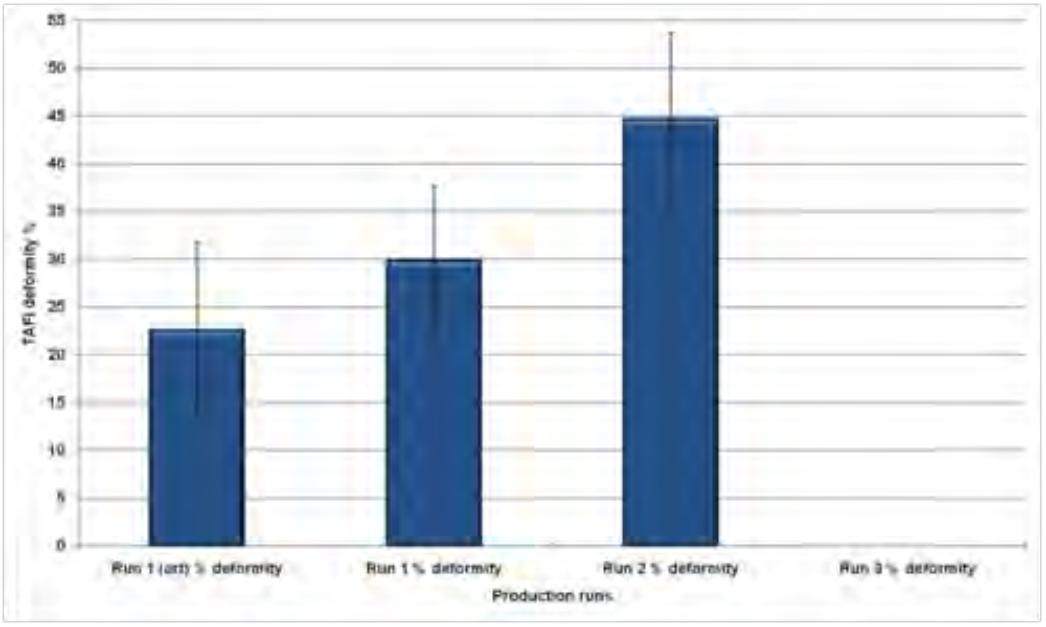


Figure 22: Mean (± SD) (a) survival rates, and (b) deformity rates based on TAFI classification, over each of the three hatchery runs carried out at the AB hatchery in 2009. Note: in run 1 some tanks were reared under artificial light (art) and the remainder under natural light conditions.

Despite the shortcomings of the 2009 season at AB, there were some useful observations that could help in the future. For example, the most successful run that had a mean survival rate per tank of 15%, and a lower deformity rate of 22%, was the first run conducted under artificial lights. Apart from the source of tank illumination, however, there were several other factors that may have contributed to this success (eg the stable indoor environment, the lower stocking density, and a water upwelling system trialled in three of the tanks; see case study 1).

In contrast, at AB the 2009 Run 1 'natural light', that was carried out at a similar time to the artificial light run, was very poor with a survival rate of just 5% and a deformity rate of 30% (Figure 22a&b). Other differences, however, included that this run used various types of air upwelling patterns in the tanks. It may also have been that the temperature variation in the tanks under natural light was more pronounced than when artificial lights were used (Figure 23).

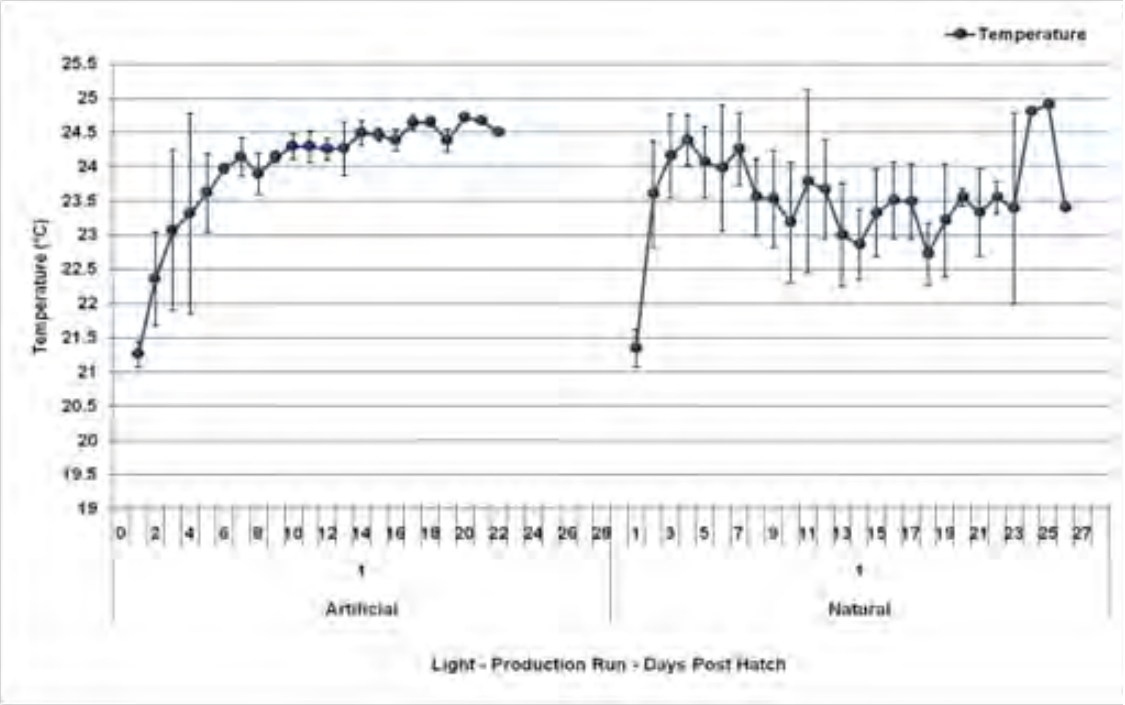
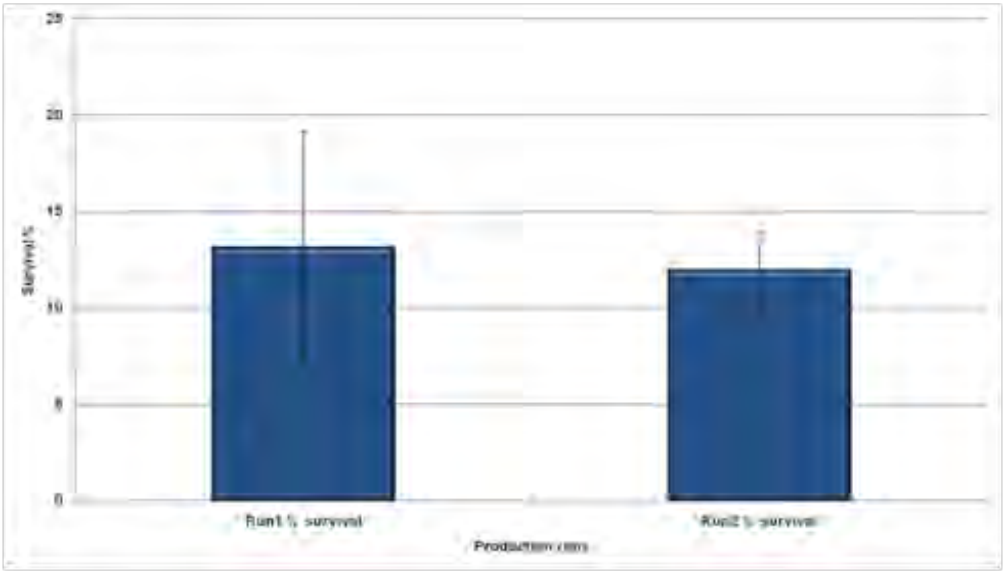


Figure 23: Mean (± SD) daily water temperature in the larval rearing tanks illuminated by (left) artificial, and (right) natural light, during Run 1 at the AB hatchery in 2009.

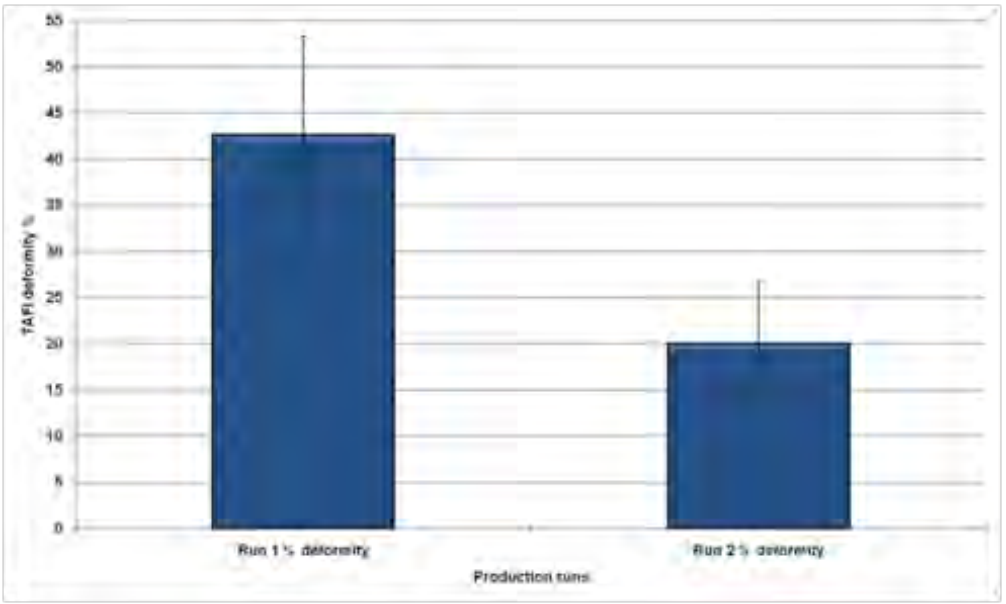
Contradicting this, however, were the results of 2009 Run2 'natural light' with a survival rate around 15% (Figure 21a). Interestingly, for this run all the tanks were fitted with water-driven upwelling systems in place of air, perhaps suggesting this was the main factor affecting larval survival. Alternatively, extremely high *Artemia* feeding rates were used in this run compared to other trials, so maybe it's more important to increase feed rates. Unfortunately, however, the high survival rate was essentially nullified by this run having the highest mean deformity rate of near 45% (Figure 21b). So, the lessons learned from this trial included: only changing one parameter at a time, having appropriate control treatments/groups, and strictly following the assigned experimental protocol. Whilst these criteria and behaviours might be assumed in specialist R&D only facilities, they may conflict with the beliefs and intentions of some of the staff in a more commercial facility, and a greater level of two-way communication and supervision will be required.

The reliability of the PA hatchery is indicated by the consistency in the mean survival and deformity rates from run to run, and from year to year (Figure 24). This is despite PA struggling with some of the biotic and abiotic conditions associated with its location at the top of Spencer Gulf and being immediately next to a power station, that result in higher water temperatures and higher salinities than at AB, and which can, at times, change unpredictably.

Upon evaluating the performance indicators from the 2 production runs carried out in 2008 it can be seen that mean survival rates for both were 12-13% (Figure 24a). The mean deformity rate in the first run was high (42%) and in the second (20%), a significant improvement (Figure 24b).



A



B

Figure 24: Mean (± SD) (a) survival rates, and (b) deformity rates based on TAFI classification, over each of the two hatchery runs carried out at the PA

hatchery in 2008.

The 2009 season also showed the same level of consistency but there was no significant improvement in the mean survival rates or deformity rates. Mean survival rates decreased in this year to 8-9% for both runs and deformity rates remained relatively high, at 31-34% (Figure 25a&b).

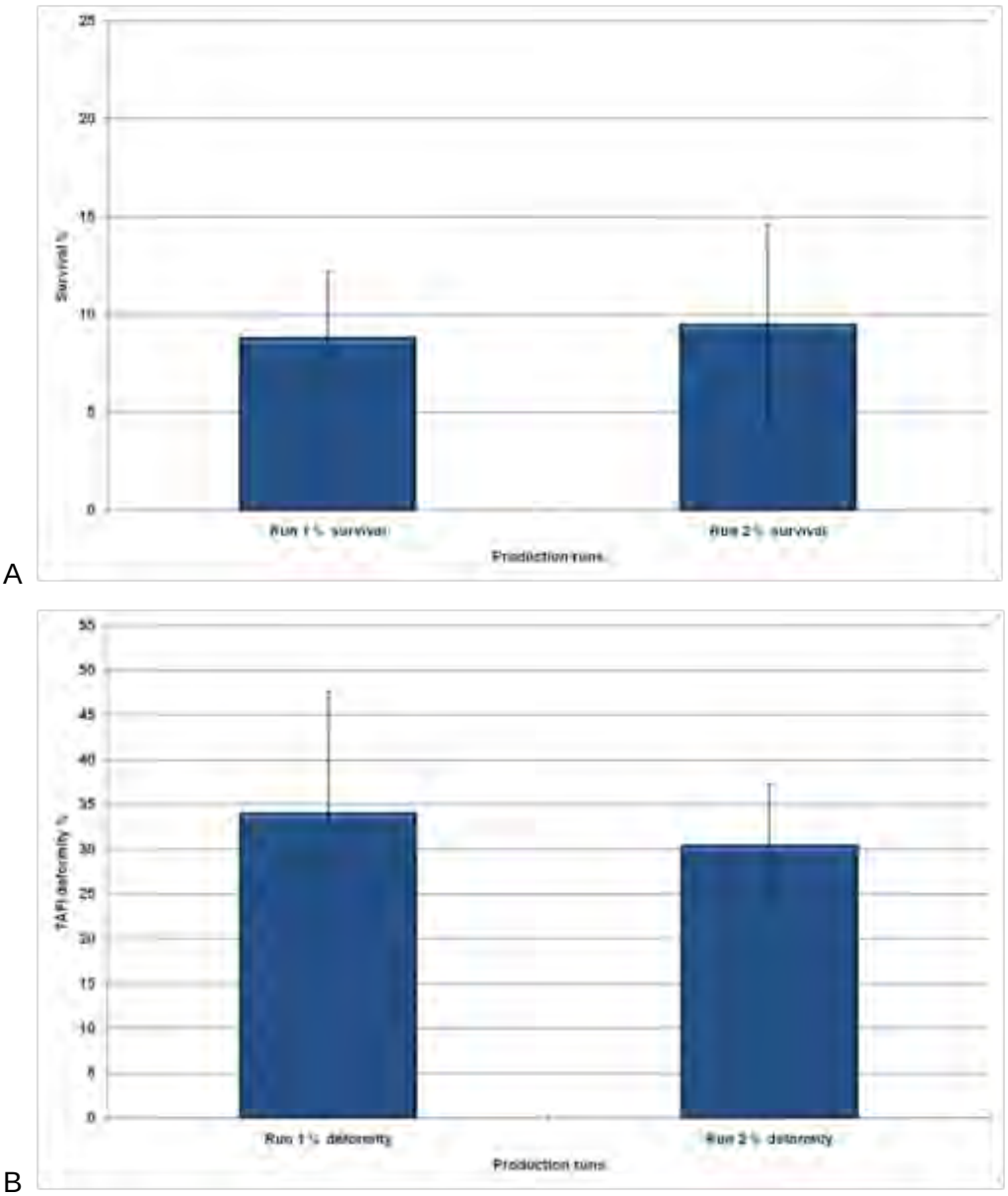


Figure 25: Mean (± SD) (a) survival rates, and (b) deformity rates based on TAFI classification, over each of the two hatchery runs carried out at the PA hatchery in 2009.

In summary, it appears that the PA hatchery is more likely to consistantly deliver modest outcomes in terms of survival (~10%) and deformity rates (~30%) that can be factored into production plans to meet target numbers of YTK juveniles. However, there was nothing in any of the trials that suggested improvements in either survival or deformity rates could be anticipated in the near future.

4.4.4 Growth

Growth is obviously an important measure of fish performance. It probably goes without saying that if the larval and juvenile fish grow faster then they can be moved out of the hatchery sooner. A variety of growth measurements were taken from every hatchery run during both production season in this project. The first parameters, yolk sac and oil globule diameters, were measured daily for the first three days to give some indication of nutrient reserve absorption rates. It is not yet clear how these data should be interpreted as it can be argued that rapidly absorbing nutrient reserves might indicate either fast growing and developing larvae (a good outcome), or poor quality nutrient reserves (a bad outcome). For this reason these results are not presented in this report. However, it is appropriate to mention that the persistence of the oil globule at 4-5DPH (after the fish have been offered rotifers for a couple of days) has some production significance, and it indicates that the larvae are off to a bad start for some reason, and that further difficulties can be anticipated.

Although several growth measurement was taken, standard length was used as the primary measurement of growth (Figure 26). These measurements suggest that, regardless of the production run or future survival and/or deformity rates of the batch, there is very little variation in standard length of larvae until about 10DPH, when the *Artemia* feeding phase begins. These observations raise several questions including: why should growth rate during rotifer feeding be so consistant when the numbers and nutritional quality of the rotifers can vary so much between runs and between tanks? And, why does this relationship breakdown when the feed changes to *Artemia*?

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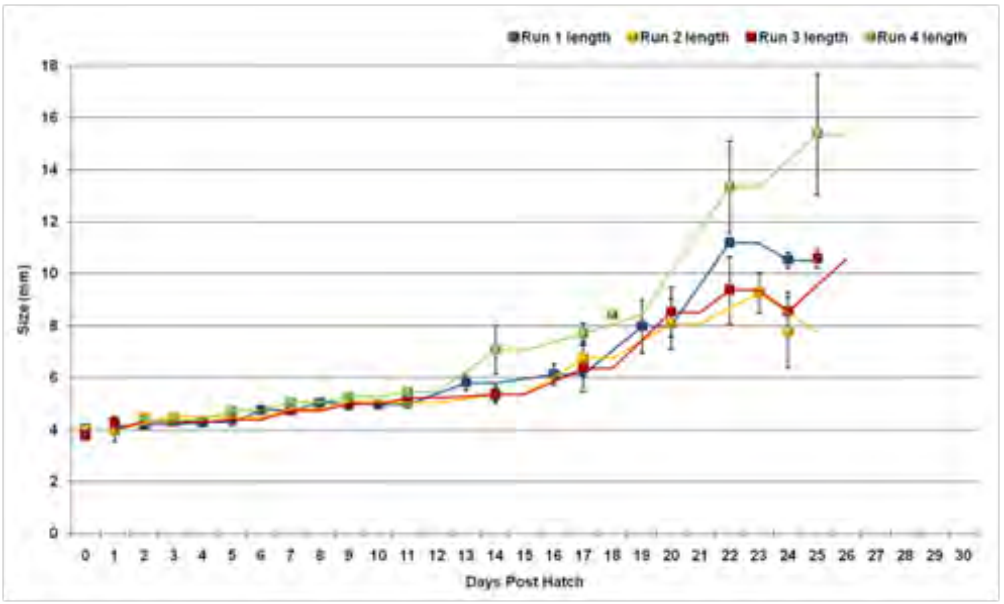
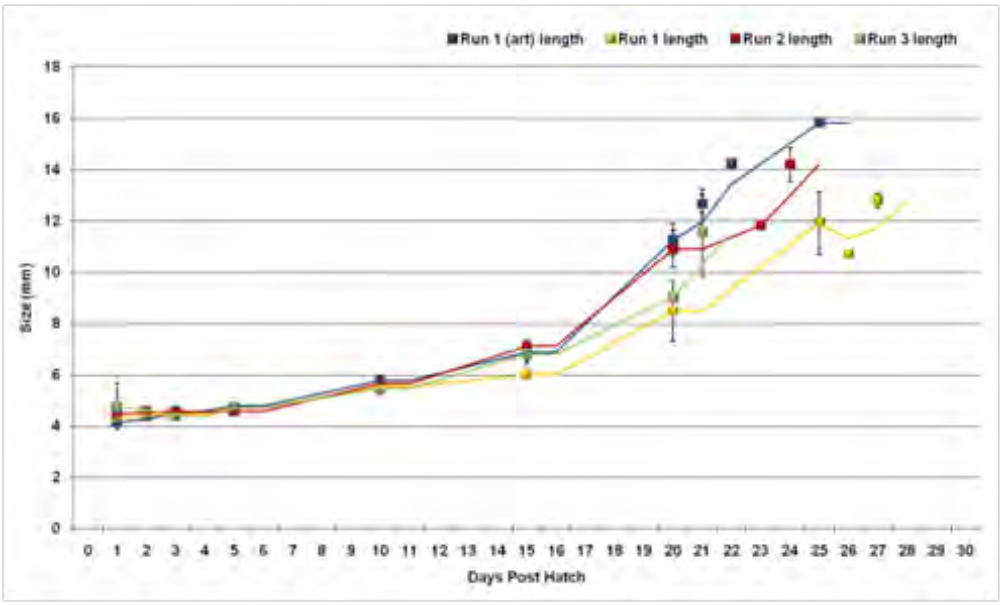
Following page

Figure 26: Mean (± SD) standard length of YTK larvae during the hatchery phase (0-28DPH) in each hatchery run at AB during (a) 2008 and (b) 2009, and at PA in (c) 2008. Note: there were only 3 days sampled in Run 2 at PA in 2008, so the blue line is not an accurate representation of the actual growth curve.

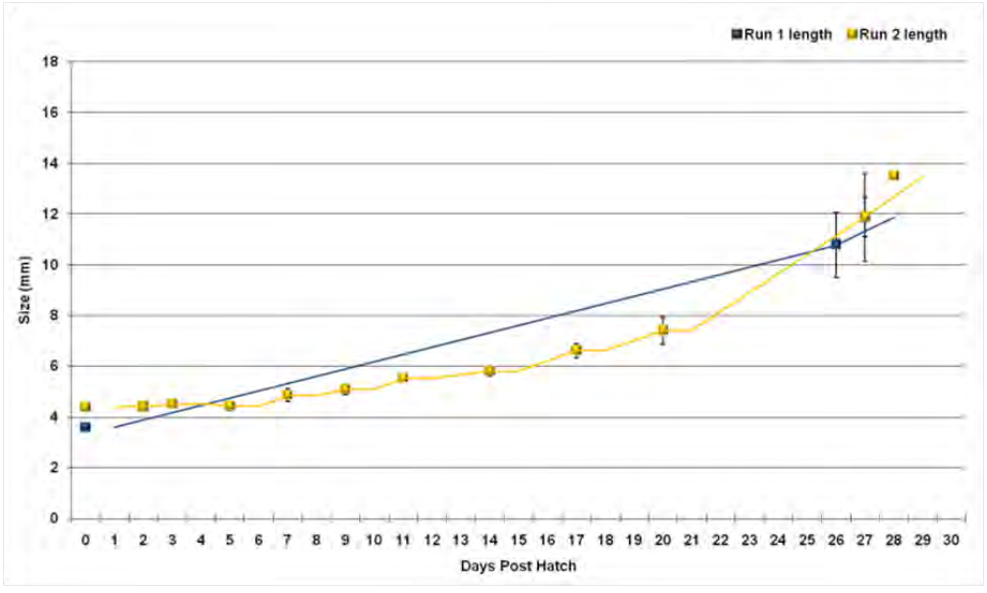
A


Run 1 length
Run 2 length
Run 3 length
Run 4 length
Size (mm)
Days Post Hatch

B


Run 1 (art) length
Run 1 length
Run 2 length
Run 3 length
Size (mm)
Days Post Hatch

C


Run 1 length
Run 2 length
Size (mm)
Days Post Hatch

4.4.5 Swimbladder inflation

As mentioned previously, in 2009 the mean swimbladder inflation rates measured in the first 10DPH at both hatcheries did not correlate to what was found later in the nursery phase. The run by run swimbladder inflation results for 2008 and 2009 at AB and PA hatcheries are shown in Figure 27. No, or only partial, swimbladder inflation has significant consequences during growout as the fish have difficulty with buoyancy control and can expend energy in swimming to maintain their position in the watercolumn.

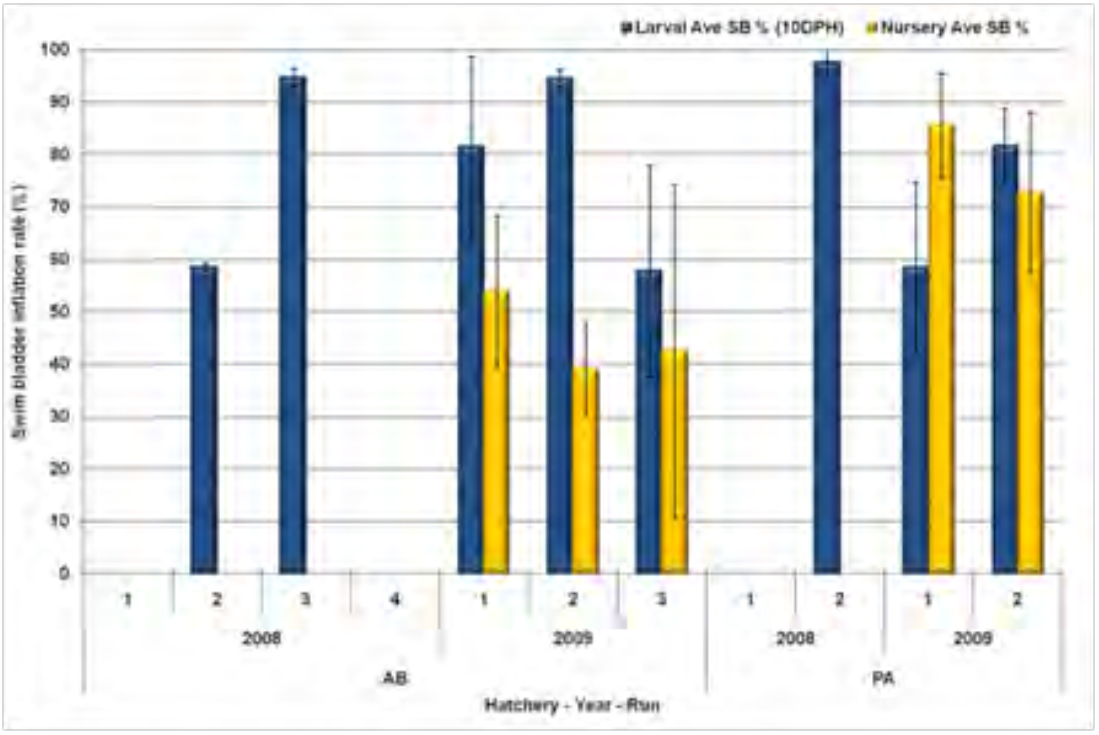


Figure 27: Mean (± SD) swimbladder inflation percentage as assessed at 10DPH (blue bars) and after transfer to the nursery tanks (yellow bars – 2009 only), over several hatchery runs at both AB and PA sites in 2008 and 2009.

The daily swimbladder inflation data to 10DPH for each hatchery run at both hatcheries in both years is shown in Figure 28. As indicated before, although there was tank to tank variation in the observed frequency of swimbladder inflation (shown by the height of the SD lines), it is not known whether this was due to actual differences between replicate tanks, misidentification of correct swimbladder inflation by one (or more) observers, or non-random distribution of larvae with and without swimbladders.

The main operational factors affecting swimbladder inflation rates are:

- Aeration/water upwelling must not be too vigorous
- Air driven surface skimmers need to be working effectively

- Additional skimming of the water surface during daylight hours using paper towels at the rate of every 30-45 min from 2-7DPH then every 1-2 hours 8-10DPH
- The effectiveness of the skimming practices must be monitored to ensure any surface film is removed (a good guide is whether centrally rising air bubbles will drift to the side of the tank)
- The nature of tank illumination (light intensity and setup; but further research is required for YTK larvae).

Other factors (unproven) may include: the bacterial load in the tank water, and the quality of the larvae.

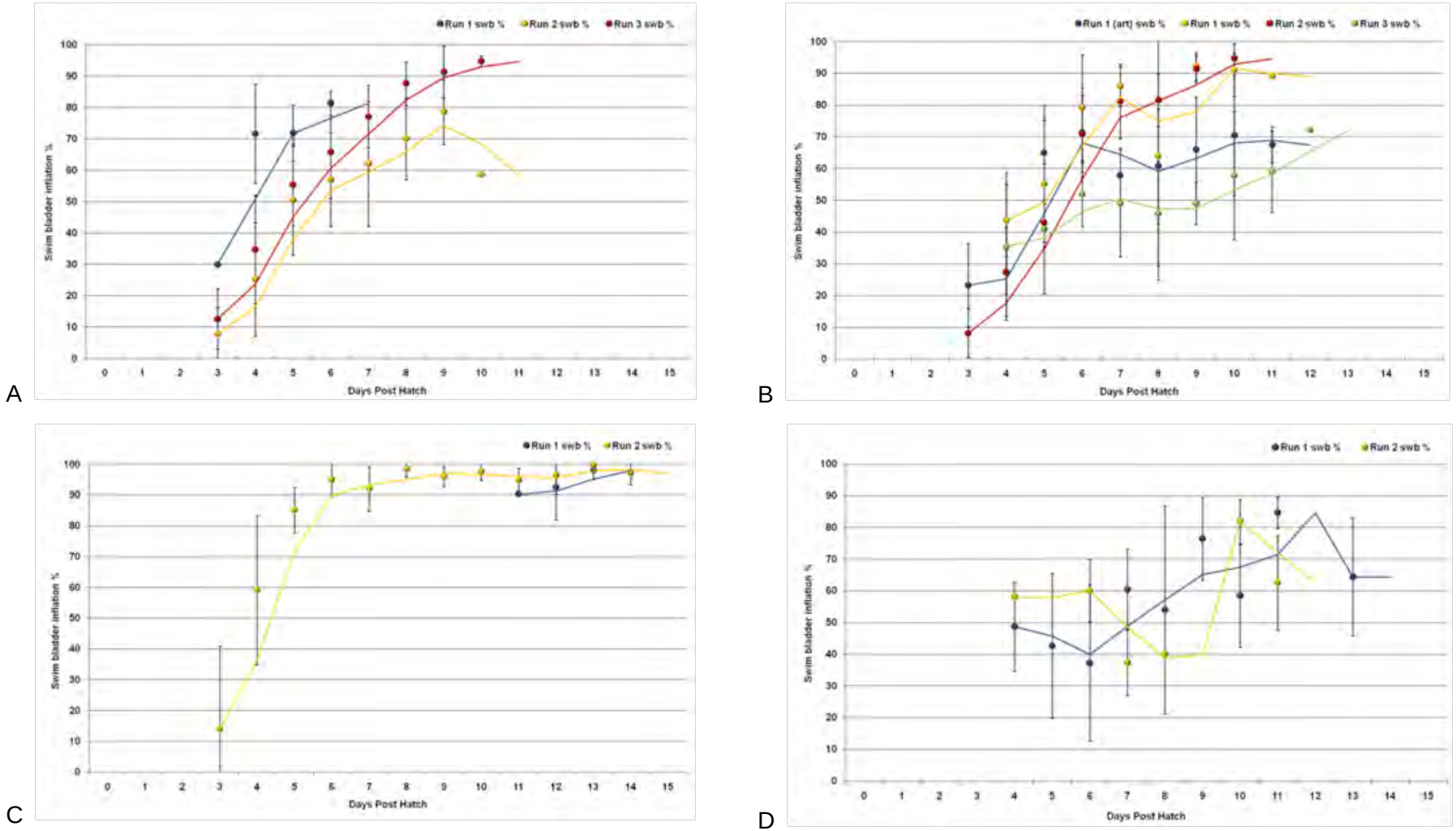


Figure 28: Mean (± SD) daily swimbladder inflation percentage as assessed during the larval rearing phase (to 10-14DPH) during separate hatchery runs at AB in (A) 2008 and (B) 2009, and at PA in (C) 2008 and (D) 2009.

4.5 Bacteriology

Bacterial monitoring has always been in place at both hatcheries and serves to monitor the bacterial load in each of the different sections at different stages during the production run. However, even if abundant populations of harmful bacteria are discovered in some locations by this surveillance approach, there are no quick fixes that won't also be harmful to larval survival. For this reason the hatchery managers and staff take preventative measures, including: hatchery dry out periods between production seasons, pigging the all hatchery water intake lines before each run, ensuring the hatchery water is sand filtered, sock filtered to 1µm and then UV sterilised, and finally, probiotic formulations are added to the live feeds as a means to control harmful bacteria in those cultures. Nonetheless, despite all these processes it is inevitable that bacteria colonise the various open systems, however it is hoped that the bacteria that do grow are benign and outcompete or suppress the growth of harmful species.

Table 5 shows some results from a bacterial surveillence survey from a hatchery. The number of colony forming units growing on the TCBS media from water samples gathered from across the hatchery are differentiated by colour and counted. During the course of this project some of the colonies grown from water samples, surface swabs, live food cultures and larvae were further isolated and and sent to Gribbles Pathology for formal identification (Table 6).

Table 5: Results from a bacterial surveillence survey from a CST hatchery. The colony forming units growing on the TCBS media from water samples were differentiated by colour and counted.

SAMPLE	Yellow	Green	Black	White
Bio 1 –c	286	11	4	0
Bio 2 –c	300	22	3	5
Bio 3 –c	113	53	2	7
Bio 4 –c	43	11	2	5
Sump –c	250	17	4	12
Inlet –c	0	0	0	0
Bio 1 –p	330	3	2	12
Bio 2 –p	400	0	0	40
Bio 3 –p	100	5	0	6
Bio 4 –p	37	7	0	40
Sump –p	18	5	0	0
Inlet –p	0	0	0	0
L rots culture water	2000	0	8	30
L rots 100X dilution	2000	0	5	0
S rots culture water	2000	1500	6	18
S rots 0 dilution	200	1000	50	5
Algae feed	0	0	0	0
CS feed	3	1	0	0
Control	0	0	0	0
Sterilised water	0	0	0	0

Table 6: A list of bacteria genera or species isolated from plates inoculated with water samples (section) and larval surfaces (larva) taken from the Port Augusta and Arno Bay hatcheries in 2008 that were subsequently identified by Gribbles Pathology.

	Section					
Species	**Broodstock**	**Algae**	**Rotifer**	**Artemia**	**Larvae**	**Larva**
Shewanella putrefaciens			✓		✓	✓
Photobacterium sp.	✓					
Aeromonas sp.		✓	✓			
Aeromonas hydrophila			✓			
Morganella morganii			✓			✓
Lactococcus lactis			✓			
Microbacterium sp.						✓
Flavobacterium sp.						✓
Pseudomonas sp.						✓
Vibrio sp.					✓	✓
Staphylococci sp.						✓

4.6 Case studies and trials

There is large amount of work that R&D personnel do behind the scenes, either on request from management or for better understanding to help justify changes in the protocols. In 2009, significant headway was made in setting up an R&D situation at AB under artificial lights. This is described below in a case whereby the survival and deformity rates for individual tanks are shown and discussed in detail. Before this happened R&D personnel conducted a variety of small trials during the production seasons. Two of these trials are shown below.

4.6.1 Case Study 1. Artificial light: Water vs. Air hydrodynamic setup

The setup in of the tanks for 2009 AB run1-artificial differed from the normal setup in several ways. Firstly, and most obviously, the six tanks were set up under artificial lights. Secondly, the larval stocking densities were approximately half of the normal stocking density (~40 larvae/L). And thirdly, three tanks were setup with air stones around the central standpipe, while the remaining three tanks were setup with a circular water inflow system which propelled a current of water across the floor of the tank thereby creating the hydrodynamic circulation in these tanks. The idea was to try to prevent the so called 'weaker' larvae from settling on the bottom by forcing them off the floor with the water current created using this setup. The details and design of this system were still being developed and further research would help fine tune the design and effectiveness.

The survival and deformity rates observed in these tanks are shown in Figure 29. Survival rates between the 3 replicate tanks for each treatment were reasonably consistent. The mean survival rate in the tanks with air-driven circulation was around 10%, whereas for the tanks setup with water-driven circulation it was 19%. For deformity rates, the air upwelling tanks were quite variable and ranged between 12 and 38% (mean 24%); in contrast, water upwelling tanks were more similar (18 to 24%), with a mean of 21%. So, the three tentative conclusions that were drawn for this trial were that: (1) water-driven circulation systems seemed to reduce larval mortality, (2) water-driven circulation systems gave more consistent results, and (2) that abiotic culture conditions could clearly affect larval survival rates.

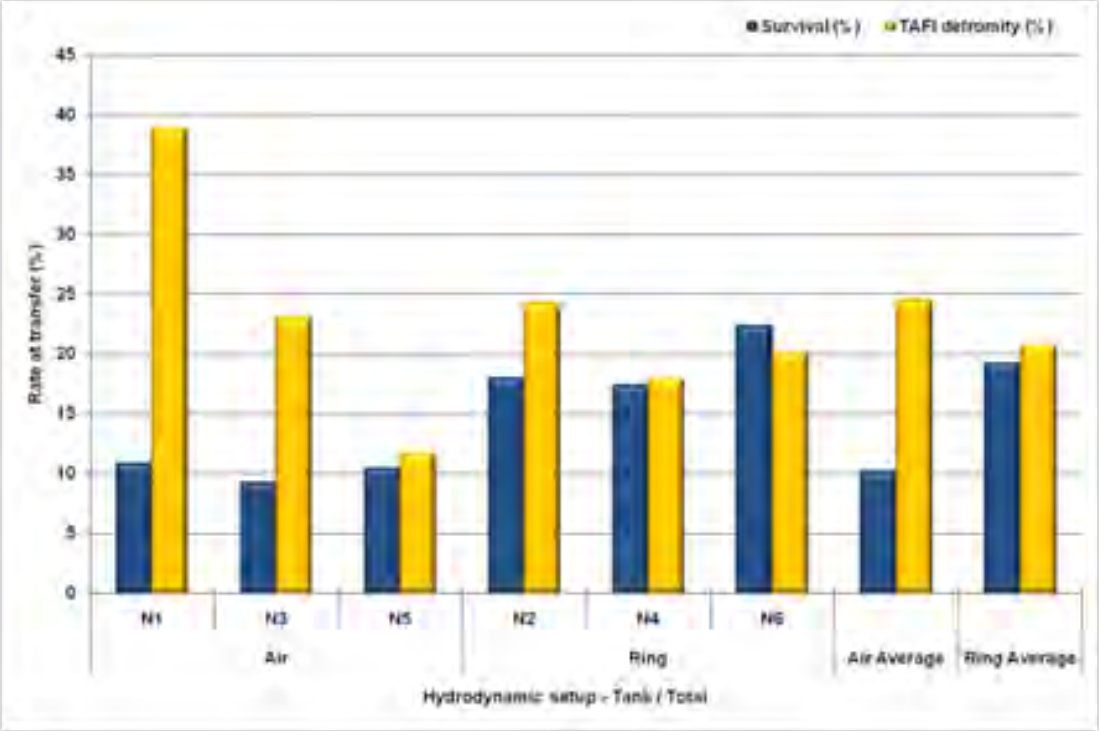


Figure 29: Larval survival (blue bars) and deformity rate (yellow bars) for individual tanks set up with Air- and Water- (Ring) driven hydrodynamic circulation patterns (n=3 tanks each), and the average of survival and deformity rate for each treatment (right hand side). All 6 tanks were under artificial light.

These data suggested that there could be merit in using a water upwelling system to improve survival rates during larval rearing, but further scrutiny of the data suggested that it comes at a price. The swimbladder inflation data shows that water-driven upwelling system had a negative impact on the larvae's ability to inflate their swimbladders (Figure 30). Clearly, the tanks setup with the ring system had lower swimbladder inflation percentages at 10DPH (mean 53%), compared to a mean of 83% in the air-driven system tanks. It could have been the case that the force that the water was being pumped around the tanks, was too strong to allow the larvae to inflate their swimbladders. Despite this, there was enough data to suggest that additional research and tweaking of the system to be less violent or forceful may result in the larvae being able to inflate at the acceptable rates as well as improve survival rates.

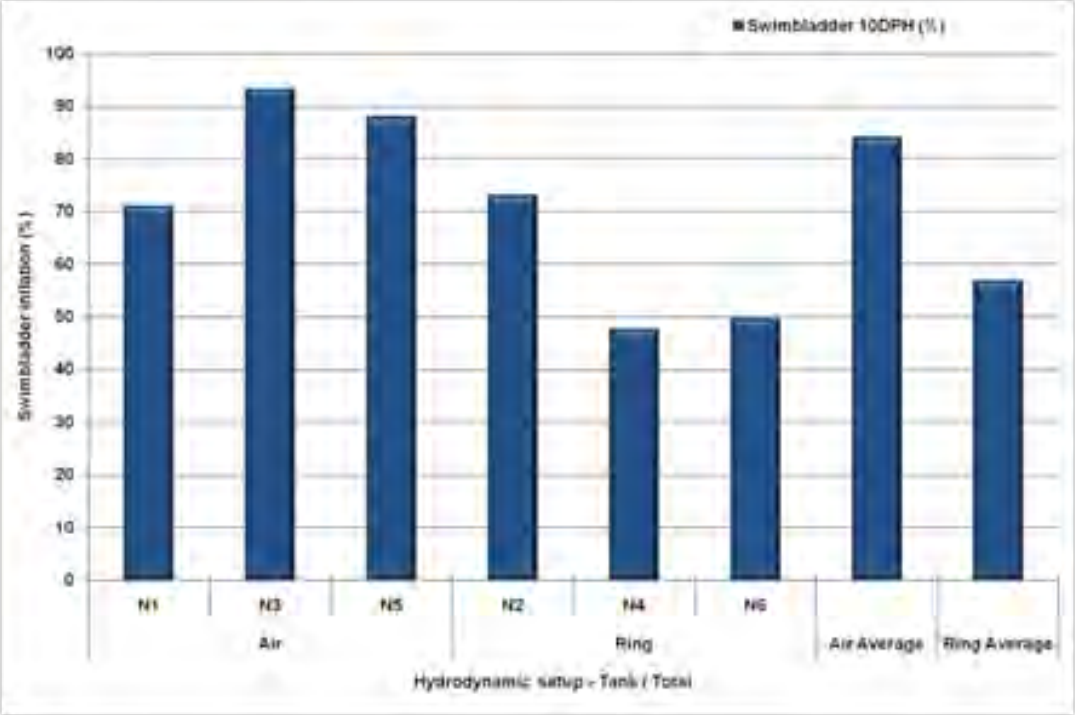


Figure 30: Larval swimbladder inflation rates (%) to 10DPH for individual tanks set up with Air- and Water- (Ring) driven hydrodynamic circulation patterns (n=3 tanks each), and the average of rate for each treatment (right hand side). All 6 tanks were under artificial light.

4.6.2 Case Study 2: Egg ozonation trial

Until the time when these trials were carried out YTK eggs used for commercial larval rearing were traditionally collected from the egg traps beside the broodstock tank and then rinsed with clean seawater to remove organic waste material. The eggs were rinsed for 5 to 10 minutes until a visual assessment suggested they were 'clean'. Obviously, this procedure does not have any specific anti-microbial effect. The aim of this trial was to improve egg quality by use of ozonated water, an egg disinfecting technique, before incubation.

Two studies were conducted using the same approach. Eggs were rinsed in clean seawater to reduce organic contamination, before they were collected in a net that was then immersed in seawater that had been ozonated at a prescribed dose (ppm; measured using the colorimetric palin test) and duration. Ozonation was converted to contact time (CT) that was calculated by:

Exposure dose (ppm) x Exposure time (mins) = CT

After ozonation the eggs were washed in 1µm filtered UV sterilised seawater and put into incubator tanks.

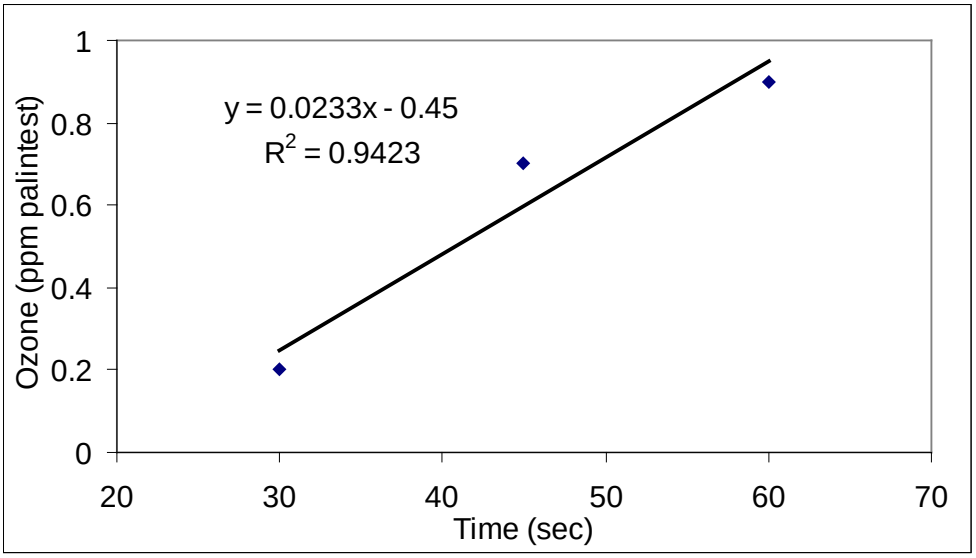


Figure 31: A dose-response curve of the ozone concentration achieved with different ozone generation times in a 20 L bucket of seawater. This relationship was used to calculate the time required to generate a specific ozone dose for treatments.

Test 1 7/8/2008

Broodstock group 2 spawned at 0530 h. Eggs remained in the egg collector at a water temperature of 21.8˚C until the time of trial. Research technicians determined a fertilisation rate of 95% from a small sample of eggs collected at 0800 h. A total of 700,000 eggs, assessed to be in mid-stage blastula, were removed from the egg collector at 0930 h (4.0 HPF), rinsed in clean seawater and then exposed to an ozone concentration of 0.65ppm for 70 sec providing a CT of 0.76. After this treatment eggs were rinsed in 1µm filtered UV sterilised seawater and incubated in a 1,400 L tank with a water temperature of 21.3˚C and flow rate of 27 L/min. This trial had no control treatment.

Test 2 22/9/2008

Broodstock group 2 spawned at 1230 h. Eggs remained in the egg collector with a water temperature of 21˚C until time of trial. No fertilisation rate was recorded. A total of 700,000 eggs, assessed to be at the 32-cell stage were removed from the egg collector at 1500 h (2.5 HPF), rinsed, then exposed to an ozone concentration of 0.65ppm for 70sec providing a CT of 0.76. After ozone treatment the eggs were rinsed in 1µm filtered UV sterilised seawater and incubated in a 1,400 L tank with a water temperature of 21.3˚C and flow rate of 27L/min. In this trial a control group of 700,000 eggs was included in the design.

In both trials the bacterial load (TCBS media) on the eggs was measured on day 0, 1 and 2. All eggs in both trials seemed to develop and hatch well irrespective of the ozonation treatment and the bacterial counts. Ozonation was effective at eliminating bacteria from eggs on day 0

(Table 7). However, it appeared that bacteria re-colonised the incubating eggs as soon as 1 and 2 days after treatment. These bacteria formed yellow-green colonies. In trial 1 the day 2 counts were 1,000 cfu, which was higher than in trial 2. In trial 2, the counts on ozonated and control eggs were very similar, and in the same range as the average microbial load measured in commercial production runs using the standard egg cleaning protocol.

Table 7: Microbial load (colony forming units) on YTK eggs to determine the effect of ozonation with a contact time (CT) of 0.76 (Trials 1 and 2), mean (± SD) results from several production runs (no ozone) are also shown for comparison. Bacteria cultured on TCBS at 30°C for 24 h.

	CFU					
	Trial 1		**Trial 2**		**Production Run**	
Day	**Ozonated**	**Control**	**Ozonated**	**Control**	**AVG.**	**S.D.**
0	0	-	0	2	5	2
1	2	-	0	0	10	8
2	1000	-	52	53	11	15

These limited trials do not suggest any substantial benefit of ozonation on reducing microbial load on YTK eggs, however it must be noted that only one CT condition was trialled, and one of the trials did not have an appropriate control treatment group for comparison.

4.6.3 Case Study 3: Swimbladder and buoyancy trial

The aim of this investigation was to characterise the distribution of larvae in larval rearing tanks according to swimbladder inflation and buoyancy during the critical mortality drop-out window from 8 to 15 DPH.

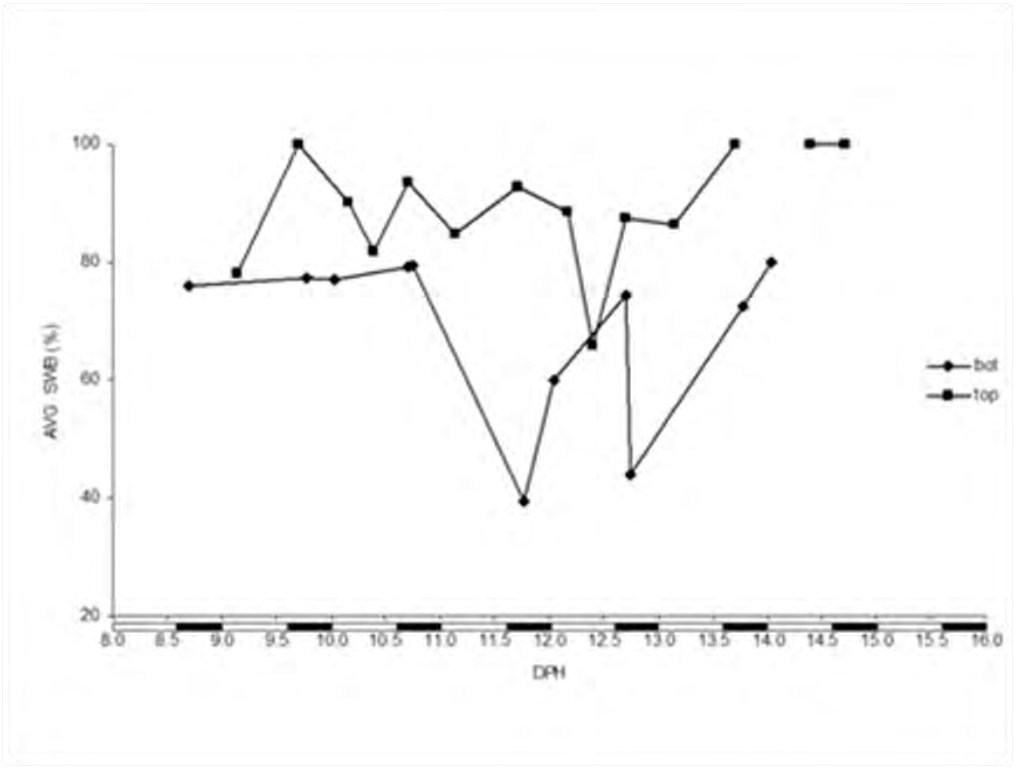
Materials and Methods

Larvae were sampled from six larval rearing tanks from the surface layer or bottom layer of the water column randomly throughout each 24 h period during this 8-15DPH window. The larvae were collected from the bottom layer by siphoning into a 20 L bucket that was fitted with filter mesh on the side wall; and from the surface layer simply by dipping with a 2 L sample jug. The larvae from both samples were in turn poured through a small sieve and back-flushed into a Petri dish with working AQUI-S solution. Once anesthetised, the larvae were sorted into 2 Petri dishes containing seawater (38 ppt salinity) based on the presence and absence of a swim bladder as determined under a dissecting microscope. The larvae from each Petri dish were then poured into a 70 mL sample jar containing seawater and the number of larvae floating, suspended in mid-water and at the bottom of the container were counted.

Results

The proportion of larvae with an inflated swimbladder and that floated at the top and bottom of the tanks across the sampling period are shown in Figure 31. More fish with inflated swimbladders were found at the top of the tank than at the bottom, but in most instances even 60-70% of the fish that were are the bottom of the tank had inflated swimbladders (Figure 31a). The majority of larvae (70-90%) sampled in this study did not float (Figure 31b); the larvae sampled in the surface layer of the tank had a higher proportion of floaters than those found at the bottom of the tank. Clearly, the presence of a swimbladder and the propensity of a larva to float are not closely related.

A



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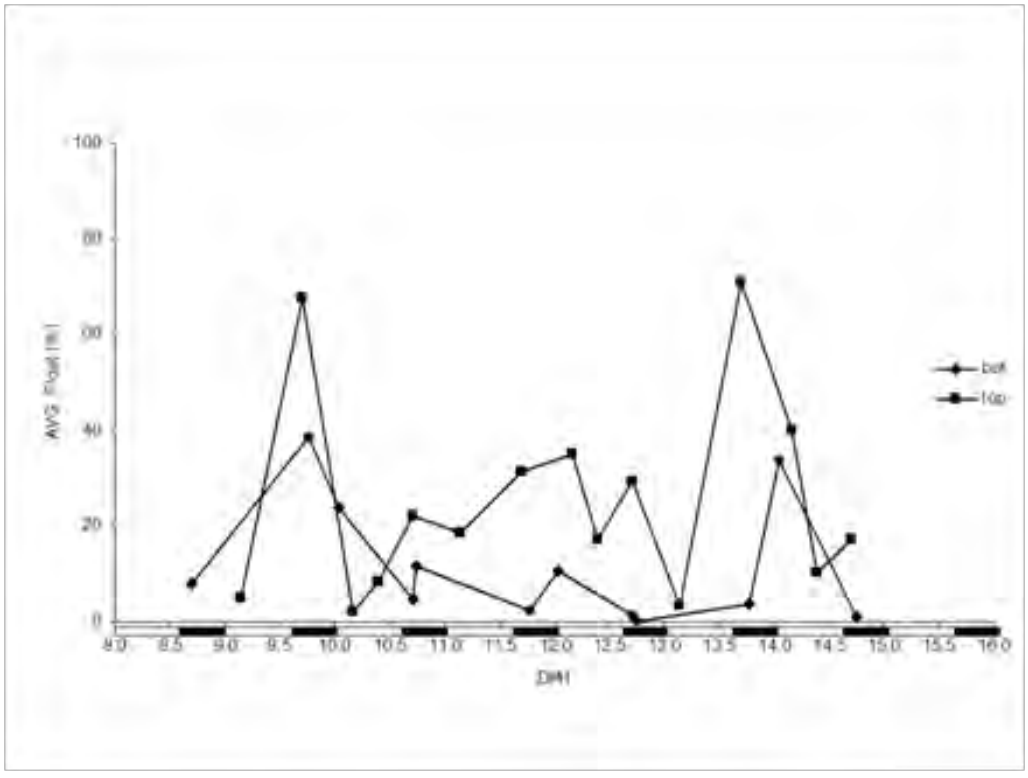

Figure 31: Random sampling from the surface and bottom layers of larval rearing tanks showing (a) the mean swimbladder inflation (%SWB) rate, and (b) the mean percentage of larvae that floated in 38 ppt salinity seawater. The *x* axis shows days post hatch (DPH), and the light and dark phases of each day.

Discussion

Larval distribution within the water column is known to vary during a normal 24 h period; during periods of darkness the larvae tend to be reasonably even distributed through the water column, and during periods of illumination they tend to aggregate in the top third of the water column. Larvae are believed to be phototactic, becoming more active during periods of illumination and swimming upwards to the water surface. This can occur under both natural and artificial light regimes.

All larvae without a swim bladder sank irrespective of whether sampled from the surface or from the bottom (data not shown). The results of this trial seem to suggest that swimbladder inflation does not dictate where larvae will be found within a tank, either during the light or dark parts of the photoperiod. Similarly, larvae that float can be found both at the surface and at the bottom of the tanks, suggesting that their buoyancy does not override their 3 dimensional movement. Together, these results indicate that the 8-15DPH larvae either do not show a strong propensity to stratify within a larval rearing tank according to either physiological condition or outside lighting cues, or that they are not able to exhibit a preference to stratify because of the physical constraints within the system. It is probably appropriate to re-investigate this study using a more gentle or static hydrodynamic system.

5. Further Development

This 2008-2009 project clearly demonstrates that, at that time, it was difficult to setup and run controlled replicated R&D trials at the CST hatchery sites at Arno Bay and Port Augusta. As a result of this project, and other 2007-2009 trials, CST acknowledged the shortcomings in the capacity of the existing facilities, the capability and expected workload of the staff, and it has since made a significant investment to address these areas. A new R&D hatchery building and tank system was constructed at Arno Bay, and new specialist staff have been recruited to improve the practices and processes in line with what is expected of a R&D hatchery facility. Thus, the shortcomings identified in this report reflect the circumstances as they were at the time, and do not reflect the current (2011) and future situation.

Whilst most the trials reported in this project were confounded by changes in more than one variable, and/or had no appropriate control(s), the data did throw light on several critical areas of hatchery practice. These were:

- The use, training in and quality control checks of accurate sampling methods for estimating mortality and deformity rates – the key performance criteria for the hatchery. Not only should external collaborators be used to benchmark existing practices and procedures, but internally there should be a level of cross-checking of data, especially in areas where staff turnover is high.
- Adherence to the planned feeding strategy. It was clear, again perhaps more so at AB, that staff did not always stick to the protocols. It can be difficult for some commercially-focussed technicians to follow a R&D experimental strategy and throw excess live feeds away; but it is better to do this than add it to a rearing tank and confound the interpretation of a post-hoc analysis of the success (or otherwise) of a particular run. On the other hand, reducing the demands on the live feed section will reduce the chance that the staff in that section will be ‘forced’ into using marginal quality feed when supplies are limited.
- Swimbladder inflation is obviously an area that needs additional work to improve the understanding of when and how this occurs. This will allow hatchery staff to better anticipate the needs of the larvae at this time and to provide the optimal conditions that allow it to happen successfully.
- Abiotic conditions do appear to affect larval survival and deformity rates. Of the factors that were varied in this project the two that appear to be most interesting are tank hydrodynamics (method and purpose), and illumination. Tank wall colour did not seem to be as important, although it is possible that it could become important if, and when, the hydrodynamics and illumination conditions are better resolved.

6. Conclusion

This component of the overall project has shown that:

- The data collected by the hatchery staff on each tank, on each day, of each hatchery run carried out each year, can be a valuable R&D resource. However, it is necessary to remind all staff that the planned/target protocols and strategies (even if they conflict with past prectices) must be adhered to if those data are to have their greatest value.
- To be used effectively, however, that database must be interrogated and evaluated by someone who is not involved in the day-to-day care of the larvae (to avoid the possibility that familiarity with the data collection practices will lead to incorrect assumptions), and that person must be aware of the intended or planned rearing protocol(s) so that any deviations can be rectified and/or reported as soon as possible.
- Proper training of staff in all sampling and assessment practices and processes is critical to the quality of the data. An ongoing program of cross-checking or quality control of sampling and assessment practices would be beneficial to detect and address any issues at the time.
- Ongoing interactions with collaborators who only do R&D in specialist hatchery facilities (eg DAC, TAFI/IMAS and SARDI) will help to further improve on site R&D practices and promote an openness to considering ‘better ways’ to do things as technologies improve.
- Biotic and abiotic conditions do affect larval survival and deformity rates both between hatchery runs, and between replicate tanks within a single hatchery run, in the commercial YTK hatchery. At the moment there appears to be a trade-off between high larval survival and high juvenile deformity rates (which ultimately will be culled out), and low larval survival and low juvenile deformity rates. Neither outcome is satisfactory, and R&D must continue both on-farm and in specialist facilities to resolve the current situation.

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Appendix 1 – Intellectual Property

There was some new intellectual property arising from this project. This has been incorporated into new and/or revised SOPs that are utilized in both CST hatcheries.

Appendix 2 – Staff

Clean Seas Tuna

Alex Czypionka

Morten Deichmann

Mike Thomson

Konrad Czypionka

Allan Mooney

Arno Bay Hatchery team

Port Augusta hatchery team

Collaborators

TAFI/IMAS - Prof Stephen Battaglene, Dr Jenny Cobcroft

SARDI – Wayne Hutchinson, Dr Bennan Chen, Steven Clarke

Flinders University – Assoc Prof Jian Qin

Darwin Aquaculture Centre – Jerome Bosmans and Glenn Schipp

Appendix 3a. Hatchery R&D Sampling Schedule

	R__ / B__ / BS__	R__ / B__ / BS__	R__ / B__ / BS__	R__ / B__ / BS__
	Tank #:	Tank #:	Tank #:	Tank #:
	Date And Check	Date And Check	Date And Check	Date And Check
Day -3: Day of Spawn				
> Fertilisation Rate				
Day -2: Eggs stocked in incubator				
> Viability Rate				
> Egg Data Measurements - 30 Eggs				
> Egg Biochemistry (5mL, 3 reps)				
> Bacteria (Incubator)				
Day -1: Eggs in incubator				
> Bacteria (Incubator)				
Day 0: Eggs Hatching				
> Hatch Rate				
> Bacteria (Incubator)				
Day 1: Larvae moved to larval tanks				
> Stocking Density				
> 20 T L, S L, YS L, YS H, OG Ø				
> 20 Histopathology (Includes 10 for TAFI)				
> 20 Malformation				
> Larval DNA (3ml)				
Day 2:				
> 20 T L, S L, YS L, YS H, OG Ø				
> 20 Histopathology				
Day 3:				
> 20 T L, S L, YS L, YS H, OG Ø				
> 20 Histopathology				
> Swimbladder Assessment				
> Rotifer Biochemistry (5mL, 3 reps)				
Day 4:				
> 20 Histopathology				
> Swimbladder Assessment				
Day 5:				
> 20 Histopathology (Includes 10 for TAFI)				
> 20 Malformation				
> 20 T L, S L				
> Swimbladder Assessment				
Day 6:				
> 20 Histopathology				
> Swimbladder Assessment				
Day 7:				
> 20 Histopathology				
> Swimbladder Assessment				

Day 8:				
> 20 Histopathology				
> Swimbladder Assessment				
Day 9:				
> 20 Histopathology				
> Swimbladder Assessment				
> Larvae Biochemistry (20 larvae, 2 reps)				
Day 10:				
> 20 Histopathology (Includes 10 for TAFI)				
> 20 Malformation				
> 20 T L, S L				
> Artemia Biochemistry (5mL, 3 reps)				
> Swimbladder Assessment				
Day 15:				
> 20 Histopathology (Includes 10 for TAFI)				
> 20 Malformation				
> 20 T L, F L				
Day 20:				
> 20 Histopathology (Includes 10 for TAFI)				
> 20 Malformation				
> 20 T L, F L				
Day of Larvae Transfer to Nursery				
> 20 Histopathology (Includes 10 for TAFI)				
> 100 Malformation				
> 20 T L, F L				
> Wet Weight check				
Day of Hand sort (Per Nursery Tank)				
> 50 Malformation				
Day of Count/Transfer to raceway (Per Nursery Tank)				
> 50 Standard Lengths				
> 50 Wet Weights (1 rep)				

Appendix 3b. R&D Hatchery Sampling Protocols

General Notes

- The R&D sampling is outside of what the production workers do, these samples need to be taken in a professional manner and in line with production standard procedures. To do this we need to communicate with the hatchery staff constantly.
- Hatchery staff must be fully aware of all of our sampling activities.
- No fish sampled from the egg incubators, larval rearing tanks or nursery tanks are to be returned to these tanks as this would pose a biosecurity risk.
- When taking samples remember that these fish are production fish and mortalities from our samples must be minimised.
- To know when to take the appropriate samples please refer to the `Hatchery R&D sampling schedule'.

Egg Fertilization and Standard Lengths

- Communicate with the hatchery staff to find out when they think the fish will spawn.
- Samples should be taken 3 hours after spawning on day -2, this will be at night so talk to the broodstock manager and he will inform you on how to take the samples so the fish are not disturbed.
- Take a sample of eggs out of the egg collectors in the broodstock room using a sterile beaker.
- Using a pipette remove some of the eggs from the beaker and place onto a Sedgwick -Rafter counting cell, and use the pipette to remove the water. Repeat this step until the counting cell is full of eggs.
- Using the compound microscope (record the specific microscope and the magnification you use to ensure accurate conversion factors are applied), measure the diameter of 30 random eggs and also the diameter of their oil globule as seen in Figure 1.1 below.
- The diameters should be recorded onto the `CST_YTK_Egg and Oil globule diameter measurement' sheet.
- Still using the same eggs, count all of the eggs across the width of the slide using a hand counter and at the same time count all the unfertilized eggs.
- This data is to be recorded on the `CST_YTK_Fertilization Rate Datasheet'.
- This process is to be replicated three times at different areas of the slide, counting no less than 60 eggs per replicate.

Normal Blastomere Rate

- No less than 12 hours after the fish have spawned the normal blastomere rate must be recorded (normally done at 8am).
- A sample of eggs is to be taken from the egg collectors with a sterile beaker.
- Using a pipette remove some of the eggs from the beaker and place onto a Sedgwick-Rafter counting cell, and use a pipette to remove the water. Repeat this step until the counting cell is full of eggs.
- Under the compound microscope (record the specific microscope and the magnification you use to ensure accurate conversion factors are applied), count all the eggs across the width of the slide using a hand counter and count any eggs with poorly formed blastomeres (see Appendix 3c below).
- These data to recorded on the `CST_YTK_Normal blastomeres Rate Datasheet'.
- This process is to be replicated three times at different areas of the slide, counting no less than 60 eggs per replicate.

Egg Dry Weight

- Using eggs from the blastomere sample, rinse with ammonium formate and then separate exactly 20 eggs and place them onto 47mm filter paper.
- Fold the filter paper so that the eggs remain in the filter paper and wrap with alfoil.
- Place the alfoil into a 10mL or 5mL vial and place in liquid nitrogen or -80 freezer, or can be placed into a normal -18 freezer for a short period of time until the sample can be taken to the -80 freezer in Pt Lincoln.

Larval Standard Length During Hatching

- A sample of larvae is to be taken out of the incubator tank with a sterile beaker at 6am.
- Filter the sample using a 250µm sieve.
- Rinse sieve into a Petri dish using AQUI-S working solution (see below for instructions on how to make up AQUI-S working solution).
- Once all the larvae have been transferred to the Petri dish allow anaesthetic to work for 1 minute.
- Transfer 20 larvae to the Sedgwick-Rafter counting cell and measure the total length of the larvae as well as the length of the yolk sack and oil globule under the compound microscope (record the specific microscope and the magnification you use to ensure accurate conversion factors are applied).
- This process is to be done three times, at 6am, 9am and again at 12pm

Larvae Dry Weights

- Using 10 larvae that have been anaesthetised in AQUI-S working solution, rinse with ammonium formate, and then place them onto 47mm filter paper.
- Fold the filter paper so that the larvae remain in the filter paper and wrap with alfoil.
- Place the alfoil into a 10mL or 5mL vial and place in liquid nitrogen or -80 freezer.
- Do not place samples in normal -18 freezer as this destroys the sample.

Histopathology

- Collect a random sample of live larvae form the tank using a siphon (20 larvae/tank/day).
- It is important to ensure that the sampled larvae are not bent or damaged in any way, if the larvae are too large for the fine tip of the pipette then cut it off to make it wider.
- Use 1L jug to sample larvae from deep in the tank.
- Anaesthetise the sampled fish using AQUI-S working solution.
- When fish are anaesthetised, use the pipette to transfer larvae to a 70mL container of 10% formalin.
- Label jar with a sticky label.
- Every 5th day an extra 10 larvae per tank should be fixed in 10% neutral buffered formalin (NBF). After 24h in fixative, transfer these extra 10 fish per tank to histology cassettes - between 3 and 5 larvae to each cassette depending on fish size.
- Write sample ID on cassette in pencil.
- Place cassette in a container with excess 10% NBF.
- Complete data sheet `CST_Histopathology_sample_datasheet'

Malformation Sampling

- Collect a random sample of larvae or juveniles from the tank:
    - use a 1L jug to scoop from the surface for larvae <25 days old. Carefully pour onto a screen (about 250µm mesh) then transfer to AQUI-S working solution.
    - For nursery juveniles, use a dip net, and anaesthetise the sampled fish in AQUI-S working solution. This is a critical to prevent the fish from flaring their operculum in the fixative.
- When fish have slowed down, transfer larvae by pipette to a jar with 10% NBF. Use forceps for larger fish, trying to move fish by the tail to avoid damaging head or spine. Try not to add too much salt water to the 10% NBF jar.
- Put a paper label inside jar – sample details written in pencil.
- Complete data sheet `CST_YTK_Malformation_sample_Datasheet'.

Larval Standard Lengths

- Using the leftover fish from the larval density sample, place 20 fish onto the Sedgwick-Rafter counting cell.
- Using the compound microscope when the larvae are small and the dissecting microscope when the larvae are larger, measure the length of the fish from head to tail (record the specific microscope and the magnification you use to ensure accurate conversion factors are applied).
- This data is to be recorded onto the `CST_YTK_Growth, gut content and feeding behaviour' data sheet.

Swim bladder formation

- Days 3-10

- 60 fish
- Morning and afternoon

Biochemistry

Eggs and live feeds

- Collect live feed or egg sample onto a screen (provided by production staff).
- Rinse with 0.5M ammonium formate (instructions below).
- Transfer rinsed and drained sample to a 5 mL vial.
- Replicate three times.
- Write sample details on vial in marker pen and cover with clear sticky tape

Larvae samples

- Larvae must be collected before feeding (in the dark, early morning) to ensure the guts are empty.
- Collect a random sample of larvae (20 larvae/tank) using a sample jar to scoop larvae from the tank.
- Anaesthetise the sampled fish in AQUI-S working solution, then rinse with ammonium formate.
- Transfer 20 larvae (very important that the number is correct) to a 45 mm diameter filter paper, fold over the paper to keep fish inside, and wrap in a small square of Aluminium foil.
- Write sample details on foil in marker pen
- Replicate twice.
- Quickly place samples in liquid nitrogen, or on dry ice.
- Complete data sheet.
- Hold samples in liquid nitrogen, or transfer to a -80°C freezer (or to -30°C freezer in Port Lincoln Office).

Working solutions

AQUI-S

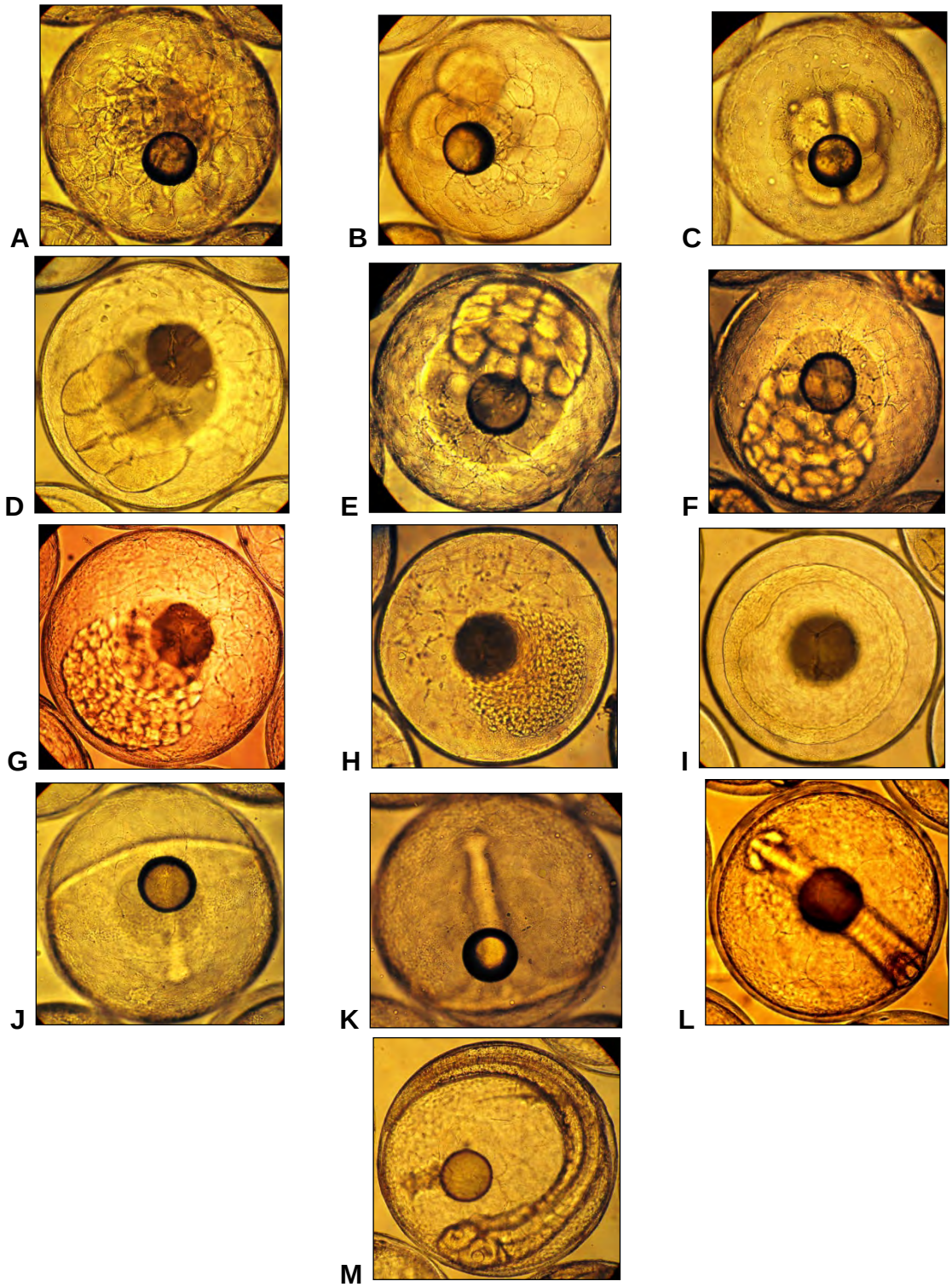
- Directions to make AQUI-S working solution (0.2mL per 7L):
- Using a 4L container with lid, fill with 3.5 litres of clean sea water
- Using a 1ml syringe transfer 0.1ml of AQUI-S to the 3.5L of seawater and shake well

AMMONIUM FORMATE

- Place 32g of ammonium formate into a 1L Schott Duran bottle
- Add 1L for filtered fresh water or demineralised water to bottle
- Shake well till dissolved

Appendix 3c. YTK Egg Development Reference Sheet

YELLOWTAIL KINGFISH EGG DEVELOPMENT



Early developmental stages of Yellowtail Kingfish embryos at 21˚C and 37ppt salinity.
(A) pre-cleavage 0.3 HPF; **(B)** 2-cell stage 0.8 HPF; **(C)** 4-cell stage 1.2 HPF;
(D) 8-cell stage 1.5 HPF; **(E)** 16-cell stage 2.1 HPF; **(F)** 32-cell stage 2.5 HPF;
(G) early-stage blastula 3.5 HPF; **(H)** mid-stage blastula 4.5 HPF; **(I)** gastrula 10.5 HPF;
(J) late-gastrula 15.5 HPF; **(K)** appearance of embryo 17.5 HPF; **(L)** 20 myomere embryo 24.5 HPF;
(M) advanced embryo 42.5 HPF.

Improvements in Yellowtail Kingfish Larval Survival and Juvenile Quality

Appendix C – SARDI and Flinders University Component

Wayne Hutchinson, Ben Nan Chen, Jian Qin and Steven Clarke

Project No. 2009/749.10

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

This project was conducted by SARDI and Flinders University

The Australian Seafood CRC is established and supported under the Australian Government's Cooperative Research Centres Program. Other investors in the CRC are the Fisheries Research and Development Corporation, Seafood CRC company members, and supporting participants.

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Non-Technical Summary

PROJECT 2009/749.10 Improvements in Yellowtail Kingfish larval survival and juvenile quality (SARDI and Flinders University component)

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Non Technical Summary

Yellowtail Kingfish (YTK) eggs were provided by the Clean Seas Tuna Ltd hatchery at Arno Bay (AB) during 2008 and 2009 and the larvae were hatched and reared at the SARDI facility at West Beach, Adelaide. Three studies were carried out to determine the effects of different biotic and abiotic culture conditions on the key production performance criteria of larval survival, growth, jaw malformation and swimbladder inflation. The abiotic factor examined was water temperature during early larval development, the biotic factors were live food (rotifers and *Artemia* naupii) densities, and the optimal time post-hatch to begin weaning the larvae from *Artemia* onto microparticulate diets.

The project showed that:

- To reduce mortality rates and the incidence of jaw malformation water temperatures need to be less than 23^{o}C for several days post-hatch, but after 18 DPH the temperature can be increased to 25^{o}C. The larvae appear to develop a greater tolerance to the higher water temperatures over time, and the warmer temperatures seem to improve larval growth.
- Rotifer densities of 20-25 mL^{-1} from 2 DPH were best for larval survival (up to 28%) when larvae were stocked at 60 L^{-1} (compared to the commercial stocking rate of ~100 L^{-1}). A regime whereby rotifer density was increased from 15 mL^{-1} at 2 DPH, to 40 mL^{-1} at 10 DPH, also resulted in a high survival rate (29%) of YTK larvae. The incidence and extent of jaw malformations was high (50-65%) and not significantly affected by the different rotifer density treatments. A high constant rotifer density of 40 mL^{-1} proved to be deleterious to larval survival.
- Constant *Artemia* densities between 15 and 35 mL^{-1} from 12 DPH resulted in high survival rates (up to 56%) but with no differences between the densities tested. As was found with rotifers, a high *Artemia* density (45 mL^{-1}) was deleterious to larval survival. There was no benefit of persisting with a low density (5 mL^{-1}) of rotifers out to 20 DPH. No *Artemia*

density treatment evaluated in this project made a substantial change to the overall high incidence of jaw malformation (77-95%).

- The best weaning outcomes (3.3% survival) were observed when microparticulate diet was introduced after 22 DPH. This was several days after the larvae were observed ingesting the diet, and structural and functional developments of the gut have occurred. Later weaning times also tended to lower the incidence of jaw malformations of commercial significance (to as low as 10%). Attempts to undertake early weaning of YTK larvae in this system are likely to have deleterious consequences for production performance.
- R&D must continue, both on-farm and in specialist facilities, to resolve the current situation whereby YTK larval survival and juvenile quality are too low.
- Information from this project on the optimal water temperature, live feed densities and feeding durations, and weaning strategy has been incorporated into the Standard Operating Procedures (SOPs) used by CST YTK hatcheries.

Acknowledgements

The authors wish to thank:

SARDI hatchery technician Paul Skordas for his assistance in carrying out these larval fish rearing experiments.

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All the folks from Clean Seas Tuna, especially Mike Thomson, Morten Deichmann and Alex Czypionka.

Our collaborators at TAFI/IMAS, Stephen Battaglene and Jenny Cobcroft.

John Carragher (Logifish Consulting) for reviewing and finalising the draft report.

1. Background

This section of the final report describes the work done by SARDI and Flinders University researchers in the SARDI hatchery at West Beach in Adelaide. The work described was carried out in the 2008 and 2009 YTK spawning seasons, therefore the procedures described herein reflect the YTK larval rearing practices at that time. As a result of this project, and the others carried out by our CRC collaborators, many of these practices and procedures will since have been modified as necessary. In this way, the hatchery manuals and SOPs developed by CST are very much 'living documents'.

As mentioned earlier in the report, the survival rate of YTK from hatched larvae to weaned juveniles is very low (~5-10%), with mortality mostly due to (a) deaths in the tank, and (b) culls due to deformities of the spine, jaws and opercula (Cobcroft *et al*., 2004). A number of biotic and abiotic factors have been shown to be associated with higher levels of mortalities and deformities at the hatchery stage in other species, and it seems likely that some of these factors are also likely to be important for YTK larval rearing success. The aim of the current project was to determine the effects of some of the most influential factors affecting larval survival, growth rate and incidence of jaw malformation in these other species, on YTK larval survival and quality. Thus, the effects of:

- water temperature in the larval rearing tanks,
- live food (rotifers and *Artemia*) density and feeding durations, and
- the timing of co-feeding and weaning onto a microparticulate diet,

were assessed via replicated tank experiments, in which the appropriate factor was manipulated whilst all other conditions were constant.

2. Need

The costs of producing juvenile YTK which are fit (ie having no or low level of deformity) to be stocked at sea for growout need to be reduced. Currently, the low survival rates and high deformity rates of YTK larvae and juveniles is constraining the growth and profitability of this industry in price-sensitive markets. This project aimed to determine the optimal levels of some key biotic and abiotic factors that might contribute to the low survival and high deformity rates, and sought industry-relevant ways in which to address them.

Notes on the design and structure of this component of the report

The researchers have reported their activities in the format of 3 manuscripts that will be submitted to international peer-reviewed journals for publication. These manuscripts have been copied into this final report largely 'as-is' with the exception of some formatting changes to suit the remainder of this document. The text of the manuscripts has been left in Times New Roman font in order to differentiate them from the remainder of the report.

At the time that this report was finalised (May 2012) the publication status of the 3 manuscripts was:

- Chapter 3: Effect of water temperature regimes on survival, growth and the incidence of jaw deformity during Yellowtail Kingfish, *Seriola lalandi*, larval rearing. Is being combined with some of Zhenhua Ma's PhD data into a more comprehensive paper.
- Chapter 4: Effect of live food feeding protocols on Yellowtail Kingfish *Seriola lalandi* larval performance: survival, growth and jaw malformation. Hass been submitted to an international aquaculture journal and is being revised prior to acceptance.
- Chapter 5: Effect of age of Yellowtail Kingfish (*Seriola lalandi*) larvae on success of weaning from live food to a formulated diet. Manuscript in preparation.

3. Effect of water temperature regimes on survival, growth and the incidence of jaw deformity during Yellowtail Kingfish, *Seriola lalandi*, larval rearing.

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3.1 Abstract

A larval rearing trial was conducted to investigate the effect of water temperature regimes on the survival and quality of Yellowtail Kingfish, *Seriola lalandi*, fingerlings. Seven different water temperature regimes were investigated for culture of larvae until 28 days post hatching (DPH). Three constant water temperature regimes (21°C, 23°C and 25°C) and four regimes in which water temperature was progressively increased from 21°C, were compared. The four increasing water temperature regimes were 1) 21°C until 8 DPH then 23°C until 28 DPH; 2) 21°C until 8 DPH, 23°C until 13 DPH then 25°C until 28 DPH; 3) 21°C until 13 DPH then 23°C until 28 DPH; and 4) 21°C until 13 DPH, 23°C until 18 DPH then 25°C until 28 DPH. Significantly higher survival of larvae was achieved at constant water temperatures of 21°C (12.76 ± 5.38%) and 23°C (8.82 ± 2.70%) compared to a constant water temperature of 25°C (0.36 ± 0.19%). There was no significant difference in survival among regimes that progressively increased water temperature during larval rearing. For regimes that included 25°C, improved survival was observed for both regimes that increased temperature to 25°C at 18 DPH. Growth of larvae was similar in all temperature regimes until 16 DPH. From 19DPH growth of larvae was significantly greater at 25°C while growth in all other treatments remained similar until the end of the trial at 28 DPH. The highest incidence of jaw deformity was found in larvae cultured at a constant water temperature of 25°C. Progressively increasing temperature treatments from 21°C to 23°C or 25°C produced juveniles with a lower incidence of jaw deformity than those cultured at a constant temperature of 25°C. Results obtained for Yellowtail Kingfish support information from other species that elevated water temperature during the early stages of ontogeny can reduce survival and increase the level of jaw malformation of larvae and ultimately the quality of juveniles produced in hatcheries. It is suggested that improved survival and lower incidence of jaw deformity of Yellowtail Kingfish larvae may be achieved by delaying elevation of water temperature until the onset of metamorphosis. After metamorphosis it is suggested that larvae may exhibit greater thermal tolerance allowing for increased growth by temperatures closer to 25^{o}C.

Keywords: Yellowtail Kingfish; *Seriola lalandi*; Growth; Larval rearing; Survival; Water temperature; Jaw malformation.

3.2 Introduction

Yellowtail Kingfish, *Seriola lalandi*, are distributed in warm temperate and sub-tropical coastal and oceanic waters of the southern hemisphere and northern Pacific Ocean (Benetti et al., 2005; Gomon et al., 2008). In Australia, aquaculture of Yellowtail Kingfish has expanded since 1998 with approximately 1,700t produced during the 2007/2008 financial year (EconSearch, 2009). However, low survival and a high incidence of deformity of hatchery reared fingerlings continue to hinder the expansion of Yellowtail Kingfish production from hatchery reared fingerlings as is also the case in New Zealand and Japan (Cobcroft et al., 2004; Kolkovski and Sakakura, 2007).

Apart from a few exceptions, fish are ectotherms and their body temperature matches that of the surrounding water (Wootton, 1990; Jobling, 1997; Crockett and Londraville, 2006). Water temperature is recognised as one of the most critical environmental factors regulating fish ontogeny, feeding, growth, and survival (Pepin, 1991; Blaxter, 1992) and one of the factors that most affects the development of fish embryos and larvae (Rombough, 1997; Abdel et al., 2004). Fish species have a temperature range that is optimal for growth and survival (Fry, 1991; Blaxter, 1992; Rombough, 1997). Exposure of fish to temperatures outside of this range represent stressful conditions primarily due to an inability of osmoregulatory mechanisms to cope with the increased water flux needed to provide the higher oxygen demands of an increased metabolic rate at elevated temperatures (Ferguson, 1988). Increasing water temperature eventually changes the tertiary structure of enzymes resulting in reduced protein function and ultimately causing denaturing and loss of enzyme activity and is responsible for setting the upper thermal limit (Somero et al., 1996; Jordaan and Kling, 2003). The consequences of adverse environmental conditions are considered to extend beyond the period of exposure of embryos or larvae due to induced deformities and effects of reduced feeding and growth (Brown and Núñez, 1998; Martell et al., 2005). Water temperature can also affect features such as morphological plasticity, locomotor activity, metabolic rate, gut evacuation rate, food conversion efficiency, feed consumption, whole-body composition and initial swim bladder inflation of marine fish larvae (Blaxter, 1992; Letcher and Bengtson, 1993; Koumoundouros et al., 2001; Trotter et al., 2003; Person-Le Ruyet et al., 2006). Consequently, identifying the preferred range of water temperature for eggs and larvae of species of interest to aquaculture will allow optimal growth and survival to be achieved within the managed artificial environment imposed by intensive fish hatcheries.

The optimal water temperature range for Yellowtail Kingfish larval rearing has not been defined. Larvae of yellowtail, *Seriola quinqueradiata*, have been reared at 22^{o}C (Sakakura and Tsukamoto,

1997) and between 19.6 °C and 22.1°C (Fukuhara et al., 1986), and *S. lalandi* between 19°C and 20°C (Cobcroft et al., 2004) and between 21°C and 23°C (Moran, 2007). In a commercial hatchery *S. lalandi* larvae are typically stocked into larval rearing tanks at 21.5°C and the water temperature is then increased to 25°C over the following 48 hours (M. Deichmann, pers. comm.).

Apart from growth and survival, of significant importance for Yellowtail Kingfish aquaculture is the high incidence of deformities for hatchery reared juveniles. High mortality, slow growth and severe deformity have been attributed to the use of inappropriate water temperatures during larval rearing a number of species of marine finfish larvae (Blaxter, 1992; Lein et al., 1997; Steinarsson and Björnsson, 1999; Koumoundouros et al., 2001). Deformities of juveniles impact commercial aquaculture through the reduced value of these fish at market size and the time and cost incurred for manual sorting prior to transfer to on-growing systems. Currently up to 50% of commercial hatchery reared Yellowtail Kingfish juveniles may be discarded prior to transfer to sea cages due to the incidence of deformities, the majority of which are jaw deformities (M. Deichmann pers. comm.). Skeletal deformities are recognised as a significant issue for marine finfish hatcheries. On average skeletal deformities affect 7-20% of hatchery produced sea bream, *Sparus auratus*, and this figure can on occasions be as high as 45-100% (Georgakopoulou et al., 2010). Fish cultured in managed aquaculture systems are particularly susceptible to developmental deformities due to exposure of developing fish to a range of suboptimal environmental conditions (Brown and Núñez, 1998) and consequently may develop deformities due to insufficient knowledge of optimal environmental preferences during the early stages of development (Sfakianakis et al., 2006; Geogakopoulou et al., 2010).

Different species of fish require different water temperature conditions for spawning, embryonic, larval and juvenile development (Herzig and Winkler, 1986; Howell and Baynes, 2004). Effects of temperature are most pronounced during embryonic and larval stages when growth is rapid (Blaxter, 1992; Kamler, 1992). Exposure to suboptimal environmental conditions tends to be much more profound when embryos or larvae are undergoing critical stages of differentiation and prior to skeletal ossification and if eggs or larvae are cultured at the extremes of temperature tolerance then developmental abnormalities often occur (Brown and Núñez, 1998; Howell and Baynes, 2004). Consequently it is possible that the incidence of deformities of Yellowtail Kingfish may be due to exposure to water temperatures that are outside of the desired range for the larvae of this species; and that this range may vary with the stage of larval development.

Information on the effect of water temperature on Yellowtail Kingfish larvae is limited. Knowledge of the optimal water temperature for each stage of larval development would maximise production of high quality fingerlings in the shortest time (van Maaren and Daniels, 2001) to minimise operational costs for commercial hatcheries. This study was conducted to investigate the effect of a range of water temperature regimes on larval survival and the incidence of jaw deformity in Yellowtail Kingfish larvae. Of particular interest was the effect of progressively increasing water temperature as larvae developed to reveal if there were any benefits to be gained by this approach as the larvae advanced in development progressed. This approach followed the premise that lower water temperature during the early stages of larval development may benefit survival and quality while tolerance to higher water temperature at latter stages of larval development may allow increased growth to be achieved to optimise production performance within the hatchery phase.

3.3 Material and methods

Yellowtail Kingfish eggs were spawned from captive broodstock maintained by Clean Seas Tuna Limited at Arno Bay, South Australia. Ozone disinfected fertilised eggs were transported to the South Australian Aquatic Sciences Centre, Adelaide and incubated at 21°C in 220 L conical bottom fibreglass incubators. At 2 days post hatching (DPH) the larvae were stocked into 28 conical bottom fibreglass experimental larval rearing tanks, each with a working volume of 170 L. Approximately 8,500 larvae were stocked into each tank to achieve a stocking density at 50 larvae L^{-1}. The vertical walls of each tank were coated with a 'marble' pattern adhesive film (Alkor-Venilia GMBH, Am Haag 8, Graefelfing, D-82166, Germany) to minimise 'walling' behaviour (Cobcroft and Battaglene, 2009). After 24 h acclimation (3 DPH) the water temperature was progressively changed to the desired level for each treatment. Water was maintained at the desired temperatures by a building management system controlling solenoid operated valves that mixed ambient, hot and cold seawater delivered in response to a thermostat located in the larval rearing tank. All seawater was filtered to a 5-µm filter and UV treated before delivery to the bottom of each tank to provide upwelling water movement. Water discharged from each tank through a 'banjo' screen (300 µm or 500 µm) at the side of each tank. An air stone was used in each tank for additional aeration and mixing. During the experiment, salinity was 37.5 g L^{-1} and the photoperiod was 14 h light: 10 h dark. A 50 w halogen light (520 BL-50 pond light, Nelson Industries Pty Ltd, Knoxfield, Victoria, Australia) fitted with a 35 w halogen bulb (Osram, Decostar® 51 ES) mounted at 85 cm above each tank provided light intensity of 978 ± 117 lx (mean ± SD) at the water surface.

Yellowtail Kingfish larvae were cultured according to three constant and four increasing temperature regimes (treatments) for 26 days from 3 DPH until 28DPH. Each water temperature treatment was replicated in four tanks (n = 4). Water temperature in each tank was recorded daily and the daily water temperature for tanks (n=4) for each treatment was used to determine total temperature exposure (degree days) provided by each treatment between 3 DPH and 28 DPH. The three constant temperature regimes were: 21°C (21C; 525 °C days), 23°C (23C; 563 °C days) and 25°C (25C; 616°C days). The four varying water temperature regimes were: (1) 21°C for until 8 DPH then 23°C until 28 DPH (21(8)23C; 555°C days); (2) 21°C until 8 DPH, 23ºC until 18 DPH then 25ºC until 28 DPH (21(8)23(10)25C; 569°C days); (3) 21°C until 13 DPH then 23ºC until 28 DPH (21(13)23C; 545 °C days); and (4) 21°C until 13 DPH, 23ºC until 18 DPH then 25ºC until 28 DPH (21(13)23(5)25C; 556 °C days) (Fig. 3.1, n = 4).

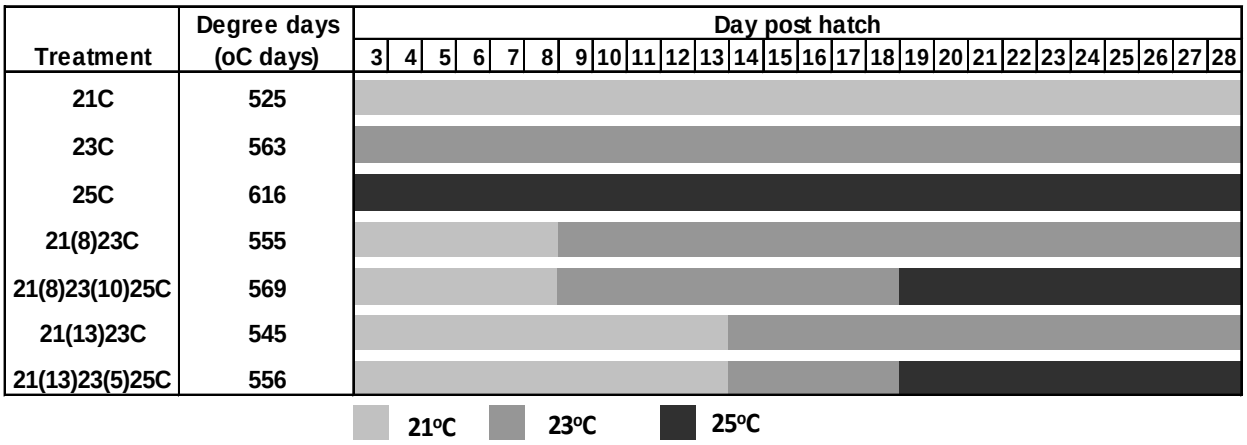

Fig. 3.1. Different water temperature regimes (treatments) investigated for Yellowtail Kingfish larval rearing and degree days (actual) between 3 DPH and 28 DPH.

Larvae were fed three times daily at 0900, 1300 and 1700 h. Rotifers were fed to fish at 20 rotifers mL^{-1} from 3 DPH until 11 DPH. Between 12 DPH and 16 DPH the amount of rotifers was progressively reduced from 10 rotifers mL^{-1} to 0 rotifers mL^{-1}. From 11 DPH *Artemia* nauplii were fed to larvae at 0.1 nauplii mL^{-1} and progressively increased to 3.7 nauplii mL^{-1} at the end of the trial. The amount of live feed added at each feeding time was calculated based on counts of residual live feed remaining in each tank when sampled approximately one hour prior to feeding. Concentrated *Nannochloropsis* sp. (Nanno 3600™, Reed Mariculture Inc., 871 East Hamilton Ave, Suite D, Campbell, CA 95008 USA) was added to each larval rearing tank six times a day at approximately 2 h intervals from 8:00h to 18:00h to maintain "greenwater" conditions. Each tank was siphoned daily into a 9 L plastic bucket to remove debris and mortalities. The number of

mortalities from each tank was estimated from the average of counts of dead larvae in three 50 mL sub-samples taken after thorough agitation of the water in each bucket.

Fish larvae were randomly sampled from each tank to estimate growth on 3, 4, 5, 6, 7, 9, 11, 13, 14, 15, 16, 19, 23 and 28 DPH. Mean standard length was calculated from measurement of 20 larvae before 19 DPH, and mean total length was measured after this time. Fish growth was determined from the absolute growth rate (AGR) as mm d^{-1} and specific growth rate (SGR) as % d^{-1} (Hopkins, 1992). AGR was calculated as: $AGR = (L_f - L_i)/\Delta t$, and SGR was determined as: $SGR = 100 \times (\text{Ln } L_f - \text{Ln } L_i)/\Delta t$, where L_f is the fish length (mm) at the end of experiment and L_i at the beginning; Δt is time interval (d). Coefficient of variation, CV (%) = 100*SD/Mean, of the final length at the end of the experiments was calculated to compare the individual size variation among treatments. On the final day of the trial Aqui-S® (Aqui-S New Zealand Ltd, Lower Hutt, New Zealand) was added to each tank at 28.6 mg l^{-1} to anaesthetise larvae before sampling and harvesting to avoid fish flaring the operculum during fixation. All remaining larvae were collected from each tank at the end the experiment and preserved in 10% neutrally buffered formalin and subsequently counted to determine final survival.

Jaw malformation was assessed under a stereo microscope (Leica, MZ6) using the criteria described by Cobcroft et al. (2004) and Cobcroft and Battaglene (2009). At the end of the experiment, 50 anaesthetised larvae were randomly collected from each tank. The appearance of the jaws of each larvae was rated on a scale of 0 to 3 according to the jaw malformation index (Cobcroft et al., 2004) modified for Yellowtail Kingfish larvae. A score of 0 indicated normal jaw formation while a score of 0.5 indicated a very minor malformation that would not be considered a malformation from a commercial perspective. Larvae were defined as malformed when the jaw score was 1 (minor), 2 or 3 (major), which would be considered a malformation of commercial significance resulting in fingerlings being culled following quality control protocols.

Statistical analyses were performed with PASW Statistics, Rel. 18.0.1. 2009, Chicago: SPSS Inc. One-way analysis of variance (ANOVA) was used to test effects of water temperature regimes on larval survival, growth and jaw malformation. Tukey HSD post hoc analysis was used to determine differences between means when significant treatment effects were detected. The level of statistical significance was set at $P < 0.05$. All values are given as mean ± SD on the mean, if not stated differently.

3.4 Results

Water temperature

The experimental system maintained water temperature near desired levels during the trial although periodic deviations occurred in some regimes. For the elevated water temperatures, the 23C treatment did not achieve the desired temperature until 11 DPH and until this time water temperature ranged between 21.0ºC and 22.5ºC. The 25C treatment did not achieve 25ºC until 6DPH but remained near this level for the remainder of the trial. Water temperatures for other treatments were controlled near to desired levels (Fig. 3.2).

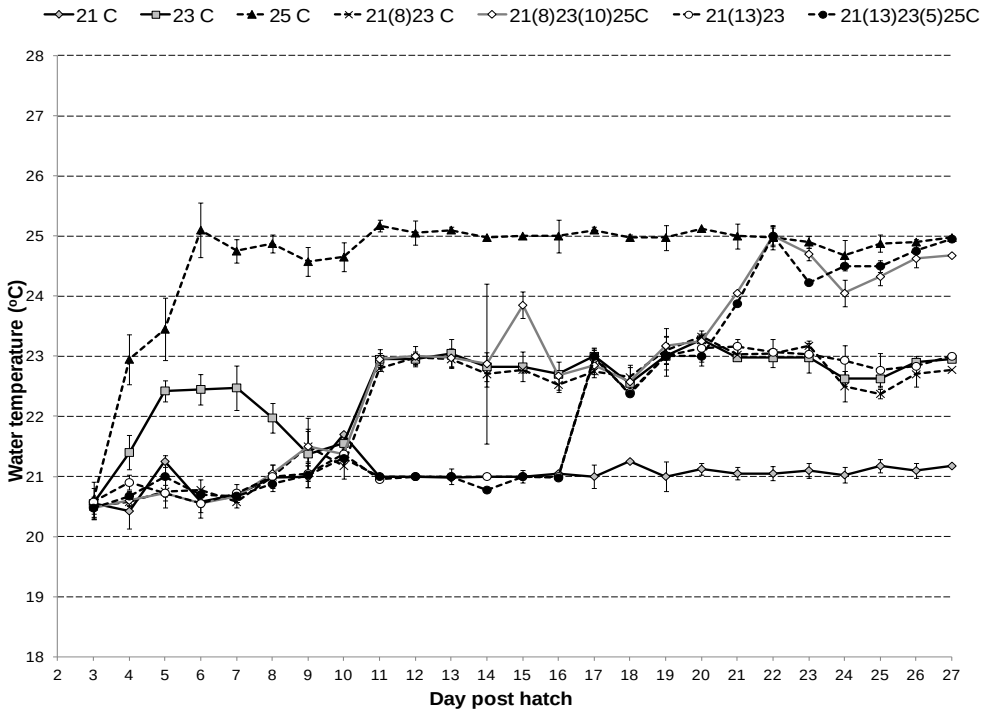

Fig. 3.2. Mean daily water temperature for each regime investigated for Yellowtail Kingfish larval rearing from 3 DPH until 28 DPH. Values are means ± SD, *n* = 4 replicates per treatment.

Survival

High mortality was observed following the onset of feeding by Yellowtail Kingfish larvae in all water temperature treatments. Apart from treatment 25C, the pattern of larval mortality was similar in all other treatments. The accumulated mortality in treatment 25C was 86.9 ± 10.3% on 9 DPH which was significantly higher ($P < 0.05$) than the mortality in all of the other temperature treatments and from this time onwards (Fig. 3.3). The highest survival of Yellowtail Kingfish larvae to 28 DPH was recorded in treatment 21C (12.76 ± 5.38%) although this was not significantly higher than the survival achieved in any of the other treatments except treatment 25C (0.36 ± 0.19%) (Fig. 3.4). Comparison of survival and the total degree days provided by each of the water temperature regimes

followed an inverse relationship that is best described by the polynomial relationship $y = -0.0046x^2 + 0.3681x - 59.85$ ($R^2 = 0.87$) (Fig. 3.5).

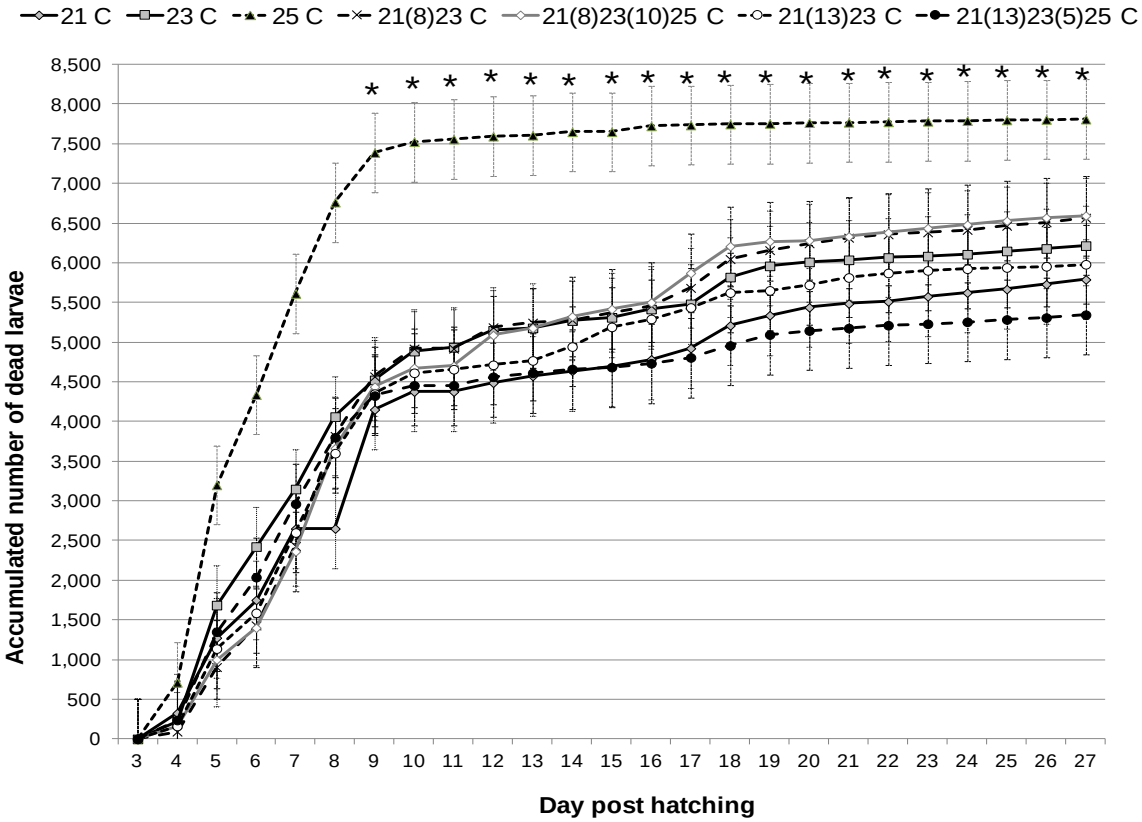

Fig. 3.3. Comparison of the accumulated mortality of Yellowtail Kingfish larvae cultured using different water temperature regimes from 3 DPH until 28 DPH. Values are means ± SD, $n = 4$ replicates per treatment. * denotes a significant difference ($P < 0.05$) from the other treatments.

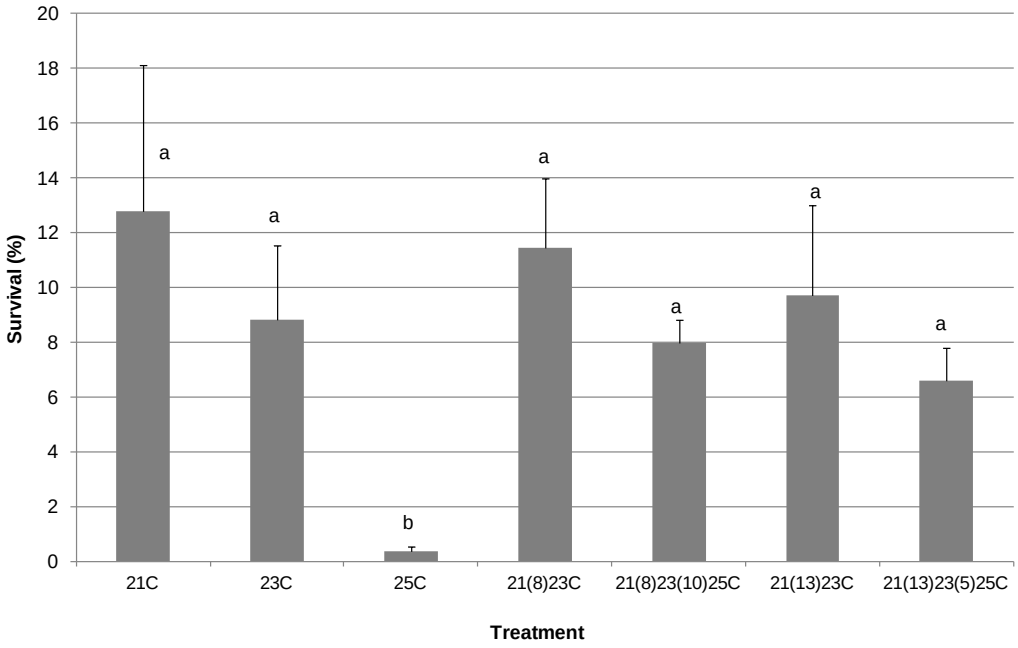

Fig. 3.4. Mean final survival (%) of Yellowtail Kingfish larvae cultured from 3 DPH until 28 DPH using different water temperature regimes. Values are means ± SD (n = 4 replicates per treatment). Treatments that share a common letter are not significantly different (P > 0.05).

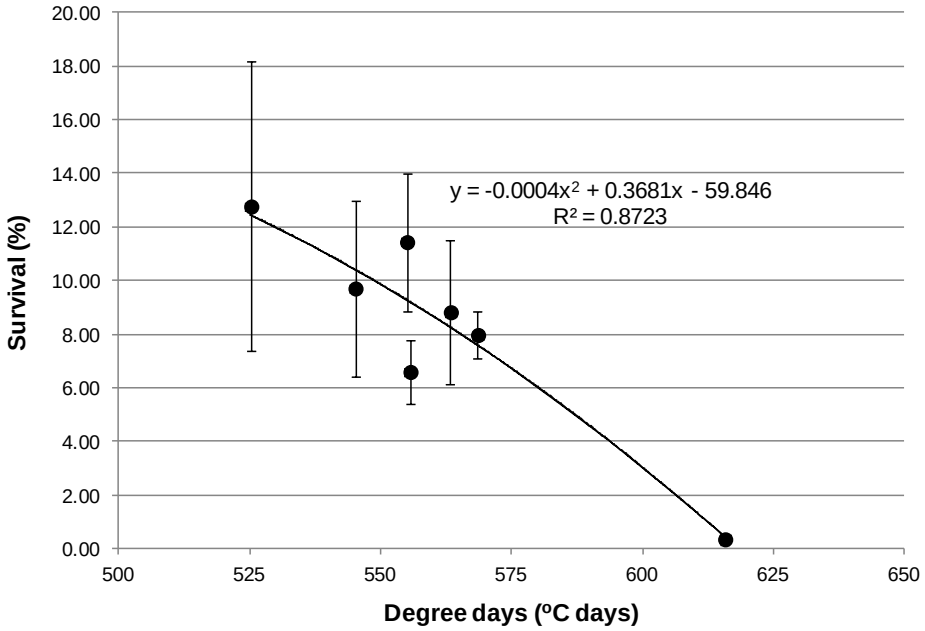

Fig. 3.5. Relationship between the mean survival (%) of Yellowtail Kingfish larvae and the mean actual degree days provided by each water temperature regime between 3 DPH and 28 DPH. Values are means ± SD (n = 4 replicates per treatment).

Growth

There was no significant difference ($P > 0.05$) in mean standard length of larvae in different temperature regimes until 19 DPH (Fig. 3.6). After 19 DPH, mean standard length of Yellowtail Kingfish larvae in treatment 25C was significantly higher ($P < 0.05$) than the mean standard length of larvae in all other temperature regimes. Similar growth was recorded for larvae cultured in all other water temperature treatments. Comparison of growth with respect to the total degree days provided by each of the water temperature regimes followed a relationship that was best described by the polynomial function $y = 0.0017x^2 - 1.8453x + 506.52$ ($R^2 = 0.98$) (Fig. 3.7).

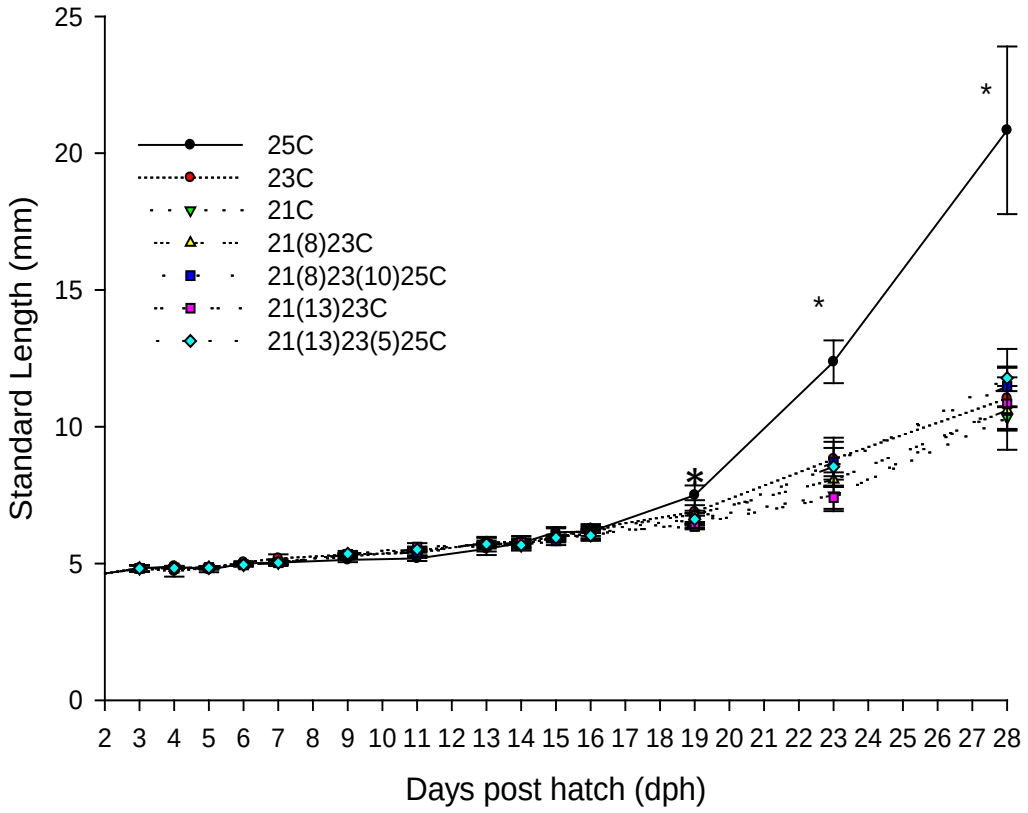

Fig. 3.6. Growth of Yellowtail Kingfish larvae cultured in different water temperature regimes from 3 DPH until 28 DPH (means ± SD, n = 4 replicates per treatment). *denotes a significant difference ($P < 0.05$) from the other treatments. Total length used to 19 DPH, standard length at 23 and 28 DPH.

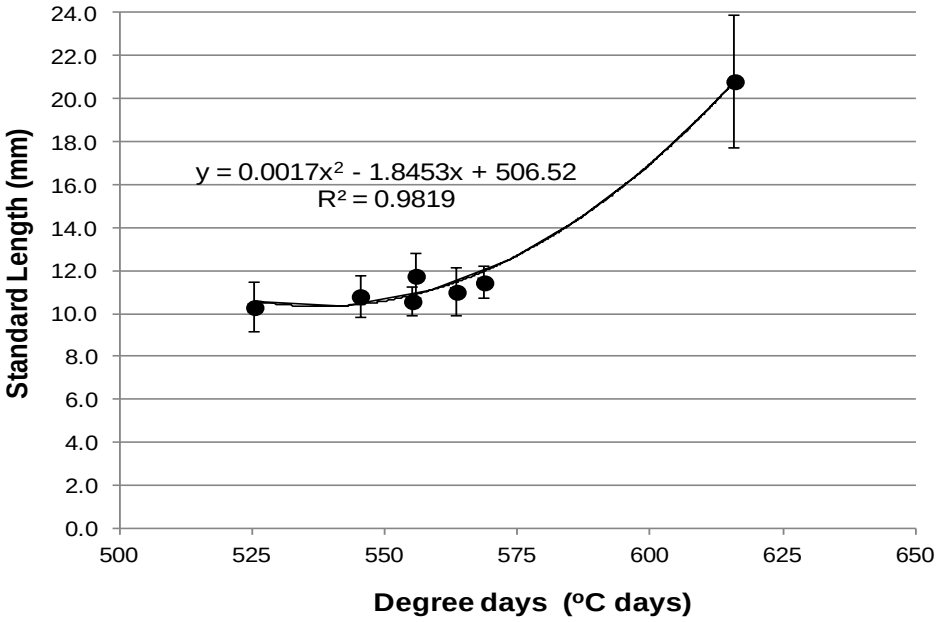

Fig. 3.7. Relationship between the mean standard length (mm) of Yellowtail Kingfish larvae at 28 DPH and the mean actual degree days (ºC days) provided by the different water temperature regimes. Values are means ± SD (*n* = 4 replicates per treatment).

Both the AGR and SGR in Treatment 25C were significantly higher than those in all other treatments ($P < 0.05$). There was no significant deference between treatments except Treatment 25C (Table 3.1). Significantly larger ($P < 0.05$) individual size variation (CV) was also recorded between larvae within Treatment 25C compared to other regimes (Table 3.1).

Table 3.1. Mean (± SD) absolute growth rate (AGR), mean specific growth rate (SGR) and mean coefficient of variation (CV) for Yellowtail Kingfish larvae cultured in different water temperature treatments from 3 DPH until 28 DPH. * denotes a significant difference ($P < 0.05$) from the other treatments ($n = 4$ replicates for each treatment).

Treatment	**AGR (mm d^{-1})**	**SGR (% d^{-1})**	**CV (%)**
21C	0.22 ± 0.04	3.10 ± 0.42	18.65±4.06
23C	0.24 ± 0.04	3.35 ± 0.39	20.77±1.80
25C	0.61 ± 0.11*	5.69 ± 0.53*	29.68±1.81*
21(8)23C	0.23 ± 0.03	3.21 ± 0.24	20.90±4.20
21(8)23(10)25C	0.26 ± 0.03	3.50 ± 0.23	16.36±0.49
21(13)23C	0.24 ± 0.04	3.29 ± 0.33	19.19±4.07
21(13)23(5)25c	0.27 ± 0.04	3.59 ± 0.34	17.11±3.76

Incidence of jaw malformation

The incidence of jaw malformation (score of 1, 2 or 3) (68.2 ± 14.3%) at 28 DPH was highest in larvae from treatment 25C (constant 25°C) but it was not significantly different ($P > 0.05$) to treatments 21C (constant 21°C) and 23C (constant 23°C). The incidence of jaw malformation from treatment 25C (constant 25°C) was significantly higher ($P < 0.05$) than the level of jaw deformity in all treatments in which water temperature was progressively increased. The lowest incidence of jaw malformation at 28 DPH was recorded for larvae from treatment 21(13)23C (22.0 ± 10.7%) which was significantly lower ($P < 0.05$) than for larvae in treatments 23C and 25C ($P < 0.05$, Fig. 3.8). Comparison of the incidence of jaw deformity with respect to total degree days provided by each of the water temperature regimes followed a relationship that was best described by the polynomial function $y = 0.009x^2 – 9.9713x + 2797.4$ ($R^2 = 0.70$) (Fig. 3.9).

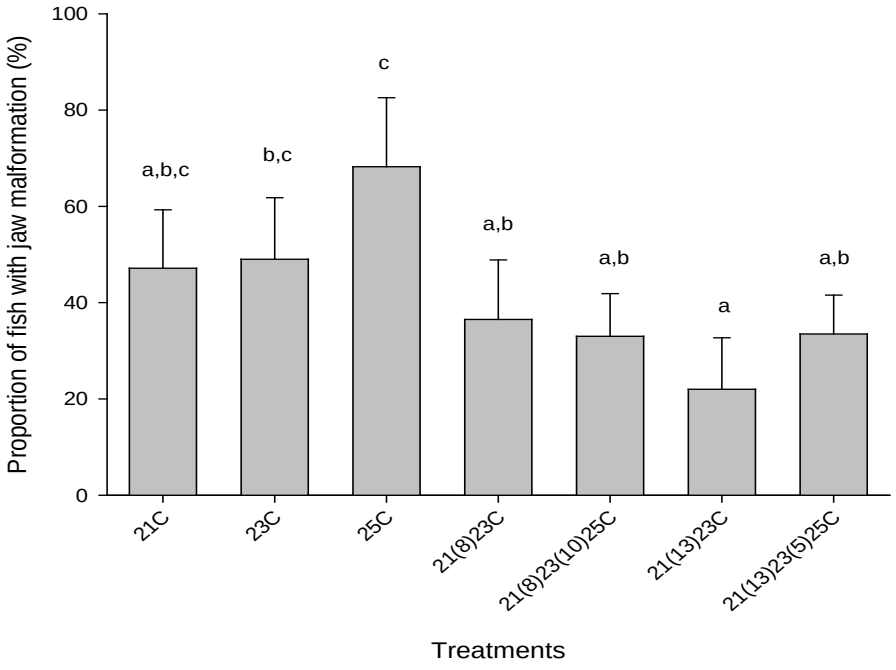

Fig. 3.8. Incidence of jaw malformation (%) for Yellowtail Kingfish larvae cultured until 28 DPH using different water temperature regimes. Values are mean ± SD, n = 4 replicates per treatment. Treatments that share a common letter are not significantly different ($P > 0.05$).

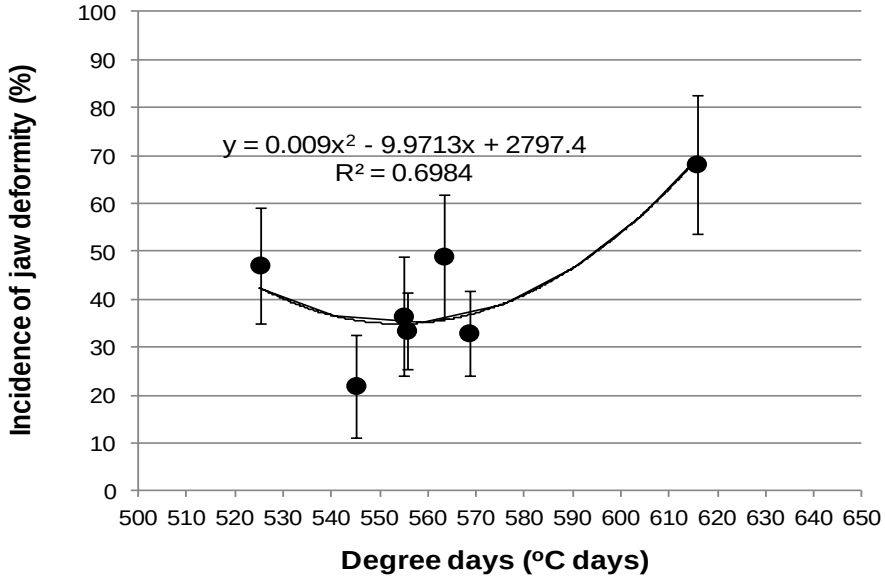

Fig. 3.9. Relationship between the mean incidence of jaw deformities (%) of Yellowtail Kingfish larvae at 28 DPH and the mean actual degree days provided by the different water temperature regimes. Values are means ± SD (n = 4 replicates per treatment).

3.5 Discussion

Survival

In this study, the lowest survival rate (0.36 ± 0.19%) occurred when Yellowtail Kingfish larvae were cultured at a constant water temperature of 25°C. For the three constant water temperature treatments the best survival was observed for larvae cultured at 21°C (12.76 ± 5.38%) and 23°C (8.82 ± 2.70%), while among the four treatments in which water temperature was increased, better survival was observed in treatments that did not exceed 23°C. When temperature regimes were expressed as total degree days there was a clear inverse relationship with survival during the culture period. These data indicate that it is likely that the optimal water temperature for early stage Yellowtail Kingfish larvae is lower than 25°C. Lower survival occurred when the optimum water temperature was exceeded as is the case with other species. For Atlantic cod, *Gadus morhua*, larvae between 73 µg and 251 µg the optimal temperature range for survival is 8.5-8.8°C (Steinardsson and Björnsson, 1999) with higher mortality occurring at lower or higher temperatures outside of this range. Highest survival to mouth opening of larval sea bream, *Sparus auratus*, occurred at water temperatures between 16°C and 22°C with increased mortality outside of this range (Polo et al., 1991). The optimal water temperature for southern flounder *Paralichthys lethostigma* is 21°C and fish survival at 25°C was significantly lower than survival at 21°C (van Maaren and Daniels, 2001). Trotter et al (2003) demonstrated higher survival when striped trumpeter, *Latris lineata*, larvae were reared at water temperatures ranging from 16°C to 19°C with low survival at 12°C and 21°C. Survival of larval spotted wolfish, *Anarchichas minor*, was negatively correlated with temperature with highest survival at 63 DPH recorded for larvae reared at 8°C. Significantly lower survival of larvae was recorded at 14°C and 16°C and similar survival was recorded for larvae cultured at 10°C and 12°C (Hansen and Falk-Petersen, 2002). Results presented for *S. lalandi* demonstrate that better survival was achieved when larvae were exposed to water temperatures of 21°C or 23°C until 18 DPH compared to 25°C. This in agreement with results presented from other species that suggest that survival is often negatively correlated with increasing water temperature during the early stages of larval development.

This trial also investigated the hypothesis that tolerance to elevated water temperature may increase at latter stages of larval development as has been concluded from other marine fish species. The high level of mortality of Yellowtail Kingfish larvae cultured at 25°C was observed early during the trial indicating that this temperature may be outside of the range that is optimal at an early stage of development. Irvin (1974) concluded that eggs of Dover sole, *Solea solea*, are stenothermal while larvae are eurythermal with temperature tolerance increasing during through development and up to

metamorphosis. Tandler et al (1989) found an inverse relationship between survival of *S. auratus* larvae and increasing duration of exposure to regimes that increased water temperature from 19.0°C to 20.5°C then 22.5°C and finally 24.5°C at different times to provide durations of exposure of 682.5, 710 or 737.5 degree days. Lein et al., 1997 concluded there is a change in temperature tolerance of Atlantic halibut larvae at 3-4 weeks after hatching suggesting that they become less sensitive to elevated water temperatures after formation of the major organs. The optimal temperature for growth of cod, *Gadus morhua*, increases from 9.7°C to 13.4°C as larvae grow from 73 µg to 251 µg indicating that growth rate and temperature optima increase with size during the larval stage (Steinarsonn and Björnsonn, 1999). Van Maaren and Daniels (2001) found that lower survival of larval southern flounder, *Paralichthys lethostigma*, occurred when water temperature was greater than 17°C until after metamorphosis. After metamorphosis, higher survival was observed at 21°C. For *S. auratus*, survival (25.0% ± 4.0%) of larvae until 17 mm mean total length (TL) was significantly higher when the initial water temperature of 16°C was increased to 19°C from 7-9 DPH (3.7 – 4.0mm TL) compared to all other treatments (i.e. constant 16°C, 19°C, 22°C, initial 22°C then 19 °C) except the treatment in which the initial water temperature was increased from 19°C to 22°C (15.0% ± 2.0%) (Georgakopoulou et al., 2010). Based upon previous experience, Tachihara et al. (1997) optimised survival of *Seriola lalandi* larvae by maintaining water temperature at 21.5°C until 10 DPH and then gradually increasing the water temperature to 24°C over 5 days. Results presented in the present study show that for treatments in which Yellowtail Kingfish larvae were exposed to 25^{o}C, higher survival was achieved in treatment 21(8)23(10)25C (7.98 ± 0.85%) and 21(13)23(5)25C (6.60 ± 1.20%) in which larvae were cultured at 25^{o}C from after 18DPH. This suggests an increase in tolerance of Yellowtail Kingfish larvae to higher water temperature may coincide with the onset of metamorphosis that has previously been observed from this time (Chen et al., 2006).

Growth

Temperature is positively correlated with food consumption and metabolic activity of fish, and consequently affects growth of larvae (Blaxter, 1992; Letcher and Bengtson, 1993). In this study, there was no significant difference in growth of Yellowtail Kingfish larvae cultured in any of the water temperature regimes until after 16 DPH (6.16 ± 0.16 mm SL). From 19 DPH the mean size of larvae in the constant 25°C regime was significantly higher than for larvae in all other treatments. From 19 DPH until 28 DPH there was no significant difference in growth of larvae cultured at the constant water temperatures of 21°C or 23°C, or at the four regimes that progressively increased water temperature as larvae developed. This suggests that there is a period of development of

Yellowtail Kingfish larvae (i.e. until 16 DPH) during which growth may be independent of water temperature as has been reported for larvae of other fish species. Klimogianni et al. (2004) found that the growth of common Pandora, *Pagellus erythrinus,* larvae increased as temperature was increased from 16°C to 18°C, but within the range of 18°C to 21°C, growth of larvae was independent of water temperature. Growth of southern flounder larvae until the completion of metamorphosis was similar in water temperatures of 17°C, 21°C and 25°C although survival through metamorphosis was significantly higher at 21°C (van Maaren and Daniels, 2001). In sea bass *Dicentrarchus labrax*, growth was similar for larvae reared at 15°C and 20°C up until 14.5 mm TL after which growth rate was higher at 20°C (Koumoundouros et al., 2001). Steinardsson and Björnsson (1999) reported that the temperature for optimal daily growth rate (% day^{-1}) of Atlantic cod, *Gadus morhua*, larvae increased from 8.6°C for 73µg dry weight larvae, to 13.4°C for 251 µg dry weight larvae. More recently Jordaan and Kling (2003) concluded that the optimal temperature for growth of *G. morhua* was 7.9°C and attributed the lower figure to incubation of eggs at the temperature of each treatment rather than incubating eggs at one treatment and starting temperature treatments at the larval stage as followed by Steinardsson and Björnsson (1999). Yellowtail Kingfish eggs in our study were all incubated at 21°C and larvae were subsequently acclimated to desired treatment temperatures following stocking of the experimental system. Future studies may consider maintaining desired water temperatures during egg incubation to include effects of temperature during the embryonic stage of development as practiced by Jordaan and King (2003).

The increased growth of Yellowtail Kingfish cultured at 25°C that was apparent from 19 DPH may be due to improved functionality of larvae due to development of tissues, organs and structures associated with a major ontogenetic event such as metamorphosis (Kamler, 1992). Previous research (Chen et al., 2006) with Yellowtail Kingfish identified that metamorphosis and the associated development of a fully functional digestive system is completed by approximately 18 DPH. Changes in functionality associated with completion of major organ system differentiation at metamorphosis may allow larvae to become more tolerant to elevated water temperature from this time. Alternatively the divergence of growth of Yellowtail Kingfish from 19 DPH may be attributed to an increased feed availability to the low number of larvae in this treatment as a high level of mortality was recorded at early stages following stocking of larvae at 25°C.

Jaw deformity

The high incidence of malformations displayed by hatchery reared Yellowtail Kingfish continues to be a significant issue confronting commercial producers. Jaw malformation is the most common

abnormality observed in hatchery produced Yellowtail Kingfish fingerlings and can be identified from as early as 4 DPH (Cobcroft et al., 2004). The skull is the first skeletal region to commence development due to the needs of fish larvae for feeding and respiration (Vandewalle et al., 1997). When eggs or larvae are reared at the extremes of their temperature range, abnormalities such as poor articulation of the jaw often occur (Howell and Baynes, 2004). This suggests that the occurrence of jaw deformities in Yellowtail Kingfish larvae may be associated with exposure to adverse abiotic or biotic conditions at an early stage of development. It is recognised that water temperatures outside of the optimal range for a species can affect the incidence of deformities in fish larvae (Georgakopoulou et al., 2010).

Results for Yellowtail Kingfish showed that the highest incidence of jaw deformity occurred in larvae cultured at 25°C and significantly lower incidence occurred for larvae cultured at 21°C until 13 DPH then at 23°C until 28DPH. When temperature regimes were compared as degree days a second order polynomial relationship ($R^2 = 0.70$) showed that higher incidence of jaw deformity occurred at the highest and lowest levels of temperature and that over the range investigated, an optimal temperature occurred at 555 degree days (average temperature = 22.2°C). Similar increases in the incidence of deformity at water temperatures considered to be outside of the optimal range have been reported for other species. A higher incidence of abnormal goldfish, *Carassius auratus*, larvae was found when larvae were reared at 27°C compared to 22°C which is the optimum temperature for rearing eggs and larvae of this species (Wiegand et al., 1988). The incidence of gaping jaws and yolk sac oedema in yolk sac larvae of Atlantic halibut *Hippoglossus hippoglossus* was greater when water temperature exceeded 4°C (Bolla and Holmefjord, 1988; Lein et al., 1997). In *Sparus aurata*, 19°C is optimal for development during the period from fertilization to mouth opening and higher survival until this stage occurred in water temperatures between 16°C and 22°C. Outside of this range mortality and the number of abnormalities increase considerably (Polo et al., 1991). A more recent study of *Sparus aurata*, also showed that 16°C water temperature significantly affected the incidence of gill cover and fin deformities. In this study gill cover deformities were the most severe and at a significantly higher incidence from larvae cultured at 16°C compared to larvae cultured at 16°C until 7-9 DPH then increased to 19°C, or for larvae cultured at a constant temperature of 19°C (Georgakopoulou et al., 2010). Hatching and survival to completion of yolk resorption of Japanese eel, *Anguilla japonica*, larvae was highest at between 22°C and 26°C and higher incidences of abnormalities were observed at lower water temperatures of 20°C and 22°C (Kurokawa et al., 2008).

The incidence of jaw deformities was high in all treatments suggesting that although water temperature may be a contributing factor, there are likely to be other factors involved. The lowest water temperature investigated for Yellowtail Kingfish larvae in this study was 21°C and future investigations could include lower temperature close to the minimum reported for spawning (i.e. 19°C), or less than this, to reveal further effects of the minimum temperature.

3.6 Conclusion

The present study shows that the water temperature regime provided during rearing of Yellowtail Kingfish larvae affected survival, growth and the incidence of jaw deformity. Results indicate that water temperatures of 21°C and 23°C provided better survival of larvae than temperatures regimes that included 25°C although improved survival was observed when temperature was increased to 25°C from 18 DPH. Growth of larvae was similar in all temperature regimes until after 16 DPH and from 19DPH growth was significantly higher at 25°C while growth in all other treatments remained similar until 28 DPH. The highest incidence of jaw deformity was found for larvae cultured at 25°C indicating high water temperature during early larval rearing stage affects jaw deformity for Yellowtail Kingfish. Progressive increasing temperature treatments from 21°C to 23°C or 25°C reduced the incidence of jaw deformity. Results support information from other species that show that elevated water temperature can reduce survival of larvae and ultimately the quality of juveniles produced by hatcheries. It is suggested that benefits of improved survival and lower incidence of jaw deformity of Yellowtail Kingfish may be achieved by delaying elevation of water temperature during larval rearing.

4. Effect of live food feeding protocols on Yellowtail Kingfish *Seriola lalandi* larval performance: survival, growth and jaw malformation

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4. 1 Abstract

Low survival and high deformity of Yellowtail Kingfish, *Seriola lalandi*, fingerlings continue to be problematic for commercial hatchery operators. Two experiments were conducted in 170 L conical tanks to investigate the effect of rotifer, *Brachionus plicatilis*, and *Artemia* nauplii feeding protocols on larval performance. Experiment 1 had eight treatments that included four different constant rotifer densities (10, 20, 30 or 40 rotifers mL^{-1}); and four rotifer densities that were progressively increased (1-10, 5-20, 10-30 and 15-40 rotifers mL^{-1}) from 2 days post hatching (DPH) until 12 DPH. Experiment 2 compared eight treatments in which the density of *Artemia* nauplii was progressively increased following different feeding protocols from 12 DPH until 21 DPH with or without the addition of rotifers after 15 DPH. The control treatment was only fed rotifers. In both experiments, feeding protocols significantly affected the survival of larvae but did not affect mean growth or individual size variation, except in the control treatment in Experiment 2. In Experiment 1, the highest survival (28.2 ± 8.9%, mean ± SD) of larvae was recorded at 20 rotifer mL^{-1} and survival (9.4 ± 8.8%) was significantly reduced at 40 rotifers mL^{-1}. For the treatments in which the density of rotifers were progressively increased, significantly ($P < 0.05$) higher survival (28.9 ± 4.5%) was recorded in the treatment in which food density was increased from 15 to 40 rotifers mL^{-1} compared to all other treatments. A quadratic polynomial function generated with data from all treatments indicated that optimal survival was achieved at an initial density of 21.7 rotifers mL^{-1}. In Experiment 2, the highest larval survival (55.5 ± 14.0%) was achieved when *Artemia* density was progressively increased from 1 to 25 nauplii mL^{-1} over 9 days with rotifer feeding stopped from 15 DPH after a 3 day co-feeding period. The control treatment that was fed only rotifers during this period recorded significantly ($P < 0.05$) lower survival (5.8 ± 1.9%) than all other treatments. Larval survival was not significantly different ($P > 0.05$) among treatments that maintained rotifers at 5 individuals mL^{-1} beyond 15 DPH compared to similar treatments that stopped rotifer feeding from this time. The different rotifer and *Artemia* feeding treatments in the two experiments did not affect the incidence of jaw malformation that ranged from 47.4 ± 6.0% to 87.0 ± 5.5%. These results suggest that rotifer densities between 15 and 30 rotifers mL^{-1} should be maintained from the onset of first feeding of *Seriola lalandi* larvae followed by progressively increasing *Artemia* density from 1 to 25 nauplii mL^{-1} between 12 DPH and 21 DPH. Results indicate that there is no advantage in maintaining rotifers in combination with *Artemia* from 15 DPH following a 3 day co-feeding period. It is suggested that live food densities, in the range investigated in these experiments, do not contribute to deformities observed in Yellowtail Kingfish larvae.

4.2 Introduction

Yellowtail Kingfish *Seriola lalandi*, a member of the Carangidae family, are found in warm temperate and sub-tropical coastal and oceanic waters of the southern hemisphere and northern Pacific adjoining countries including Australia, Japan, New Zealand, South Africa, South America, the Hawaiian Islands and the west coast of USA (Benetti et al., 2005; Gomon et al., 2008). Aquaculture production of *Seriola* spp. began in Japan in 1927 where the bulk of the annual 140 000 – 160 000 t of production is comprised of yellowtail, *Seriola quinqueradiata* and amberjack, *Seriola dumerili* (Nakada, 2000). *S. lalandi*, also known in Japan as goldstriped amberjack, *Seriola aureovittata*, contributes less than 5% of the total production but is regarded as a high quality sashimi fish (Nakada, 2000; Benetti et al., 2005). In Australia, aquaculture of Yellowtail Kingfish commenced in Port Augusta, South Australia in 1998 following successful broodstock spawning and larval rearing. The industry has since expanded with approximately 1,700 t of Yellowtail Kingfish produced during the 2007/2008 financial year (EconSearch, 2009). Apart from Australia and Japan, the aquaculture of *Seriola* spp. is currently being developed in the Mediterranean, USA, Chile, Ecuador, New Zealand and Saudi Arabia. However, low survival and high incidence of deformity continue to hinder the expansion of Yellowtail Kingfish production from hatchery reared fingerlings (Cobcroft et al., 2004; Kolkovski and Sakakura, 2004).

The survival of marine finfish larvae depends on food availability in the early life stages (May, 1974; Yufera and Darias, 2007). Due to poor mobility and low feeding efficiency, only larvae exposed to a high density of live food can survive during the early life stage (May, 1974; Houde and Schekter, 1981; Munk, 1995; Downing and Litvak, 2001) and they have adapted to live on plankton patches in the pelagic environment (Boehlert and Yoklavich, 1984). Under larviculture conditions, high mortality is often associated with the transition from endogenous to exogenous feeding as developing larvae deplete yolk and oil droplet reserves and must quickly make the transition to live prey (Brown and Laland, 2001; Gimenez and Estevez, 2008). Feeding success and consequently survival of marine finfish larvae is often low during this first feeding period because of their poor ability to capture prey (Houde and Schekter, 1980; Hunter, 1981; Doi et al., 1997). Once larvae have experienced first successful ingestion through repeated attempts at prey capture they become accustomed to feeding on moving prey (Stoecker and Govoni, 1984) and as they grow they also develop improved swimming speed that assists prey searching and capture efficiency (Pandey et al., 2008).

Suitable prey density is essential for the survival of marine fish larvae and a shortage of food will cause mortality particularly during the early stages of rearing when feeding ability of larvae has not fully developed (Fukuhara, 1983). Prey searching and feeding success are influenced by the ambient prey density (Yufera and Darias, 2007) with the perception being that the higher the prey density the greater the chance that larvae will encounter and ingest prey resulting in a positive effect on larval survival (Duray et al, 1996; Giménez and Estévez, 2008). Therefore, manipulation of food density during hatchery rearing is a key factor to increase prey encounter and food consumption to improve survival and growth of marine finfish larvae.

As fish larvae grow, their digestive system will develop a capacity to ingest and process larger quantities of food during the first two weeks after hatch (Govoni et al., 1986). Larvae may consume 50 to 300% of their body weight each day compared to between 2 and 10% daily for fish approaching market size (Bryant and Matty, 1980). Fish larvae have a high potential for growth during the early life stages and starvation of larvae at any point has a negative impact upon growth and survival (Jordaan and Brown, 2003; Chen et al., 2007). Since 1998, hatchery methods have been developed for production of Yellowtail Kingfish fingerlings based on methods used for other species. However, this fast growing fish may have a unique requirement for live food provision at various stages. Yellowtail Kingfish is faster growing than most temperate marine finfish species especially during the early stage (Chen et al., 2007). We hypothesise that the fast growth of Yellowtail Kingfish larvae requires a high feeding rate to achieve high survival and growth under intensive rearing conditions.

In conventional larval rearing operations, a wide range of prey densities have been used to increase the encounter rate between larvae and prey (Kiorboe et al., 1985; Drost, 1987). Puvanendran and Brown (1999) found that at least 4 rotifers mL^{-1} are required to obtain a higher incidence of initial feeding and to support good growth and survival of Atlantic cod, *Gadus morhua,* larvae. Gimenez and Estevez (2008) found that a density of 10 rotifers mL^{-1} was the minimum prey density required to feed larvae of common dentex, *Dentex dentex*. The standard density used for gilthead sea bream, *Sparus auratus,* larval rearing is also 10 rotifers mL^{-1} (Parra and Yúfera, 2000) although Zaki et al. (2004) concluded that 16 rotifers mL^{-1} was the optimal density for larval rearing of this species. An average density of 18 rotifers mL^{-1} was maintained until 8 DPH during larval rearing of mutton snapper, *Lutjanus analis* (Watanabe et al., 1998). Duray et al. (1996) reported that the highest rotifer intake and survival rate of grouper, *Epinephelus suillus,* larvae was achieved at 20 rotifers mL^{-1}. In

New Zealand, Yellowtail Kingfish larvae were fed at a density of 20 rotifers mL^{-1} (Hilton et al., 2008).

Typical protocols for feeding *Artemia* to marine fin fish larvae start with introduction of a low density of nauplii in conjunction with rotifers followed by a gradual increase in *Artemia* density as the amount of rotifers added is reduced over several days. The feeding protocol for rearing sand bass, *Paralabrax maculatofasciatus*, maintains prey density at between 7 and 15 rotifers mL^{-1} for the first 2 weeks, followed by feeding with newly hatched *Artemia* at a density of between 2 and 10 nauplii mL^{-1} (Alvarez-Gonzalez et al., 2001). In the larval rearing of fat snook, *Centropomus parallelus*, rotifers are introduced at a density of 10 mL^{-1} at first feeding, followed by a combination of 30 - 40 rotifers mL^{-1} and 0.5 – 4 *Artemia* nauplii mL^{-1} towards the end of the live food phase (Alvarez-Lajonchére et al., 2002). Benetti et al. (2008) utilised a pulse feeding approach to culture cobia, *Rachycentron canadum*, larvae whereby rotifers were added at a density of 5 individuals mL^{-1} every 3 to 4 hours and from 9 DPH *Artemia* were added every 3 to 4 hours at a density between 0.1 and 1.0 nauplii mL^{-1}. Puvanendran and Brown (1999) evaluated *Artemia* densities ranging from 0.25 mL^{-1} to 16 mL^{-1} for culture of Atlantic cod and found significantly higher survival of larvae cultured using *Artemia* densities between 4 and 16 nauplii mL^{-1}. Hilton et al (2008) maintained *Artemia* density between 20 and 30 naupli mL^{-1} from 9 DPH for culture of Yellowtail Kingfish larvae. The feeding protocols developed for these species accommodate the increasing energy and nutritional requirements needed to support growing larvae.

There is a high incidence of jaw deformity in hatchery reared Yellowtail Kingfish and the factors involved have not yet been resolved. Various nutritional factors are implicated in the incidence of deformities of marine fish including phospholipids (Kanazawa et al., 1981; Cahu et al., 2003; Villeneuve et al., 2005; Tocher et al., 2008; Cahu et al., 2009; Kjøsvik et al., 2009), highly unsaturated fatty acids (Villeneuve et al., 2005, 2006; Roo et al., 2009; Izquierdo et al., 2010), Vitamin A (Dedi et al., 1998; Haga et al., 2003; Villeneuve et al., 2006; Fernández et al., 2008; Mazurais et al., 2009, Fernández and Gisbert, 2010; Lewis-McCrea and Lall, 2010), Vitamin D (Darias et al., 2010), Vitamin C (Lewis-McCrea and Lall, 2010), phosphorous (Lewis-McCrea and Lall, 2010), lipid peroxidation (Lall and Lewis-McCrea, 2007; Lewis-McCrea and Lall, 2010) and amino acids (Cahu et al., 2003; Saavedra et al., 2009). A number of environmental factors are also reported to contribute to the incidence of deformities include swimming intensity due to water current velocity (Kihara et al., 2002; Koumoundouros et al., 2001; Sfakianakis et al., 2006), water temperature (Sfakianakis et al., 2006; Georgakopoulou et al., 2010; Katharios et al., 2010), gas

super-saturation (Rosenlund and Haldórsson, 2007) and salinity (Ottesen and Bolla, 1998). In relation to live food, Izqueriedo et al. (2010) suggest that kyphosis in red porgy, *Pagrus pagrus*, may be caused by larvae consuming large amounts of *Artemia* nauplii that distend the abdomen and press upon the cephalic vertebrae.

To improve survival and quality of Yellowtail Kingfish fingerlings, two experiments were conducted to investigate optimal densities of rotifers and *Artemi*a. This study was initiated in response to a suggestion that the accepted live food feeding protocol employed to culture Yellowtail Kingfish larvae was not optimal for this fast growing species. A range of live food densities and feeding protocols were investigated to optimise first feeding success and maintain growth and survival of Yellowtail Kingfish larvae. Larval survival, growth and the incidence of jaw deformity were used as indicators to evaluate the response of larvae to the different rotifer and *Artemia* feeding protocols investigated.

4.3 Materials and methods

Egg transportation and incubation

Yellowtail Kingfish eggs were collected in October 2008 from broodstock maintained under controlled photoperiod and water temperature conditions at the Arno Bay hatchery of Clean Seas Tuna Limited, South Australia. Eggs were transported to the South Australian Aquatic Sciences Centre, Adelaide where they were hatched in 220 l fibreglass incubators at a water temperature between 22°C and 24°C. After hatching, the larvae were stocked into larval rearing tanks at 1 day post hatching (DPH).

Experimental system

The experiments used a system of 36 conical bottom fibreglass tanks each with a working volume of 170 L. Each tank had a white bottom and the vertical walls were coated with a 'marble' pattern adhesive film (Alkor-Venilia GMBH, Am Haag 8, Graefelfing, D-82166, Germany) to minimise 'walling' behaviour of larvae (Cobcroft and Battaglene, 2009). In both experiments water temperature was maintained at 22.2 ± 0.2°C (mean ± SD) by solenoid operated valves to mix ambient, hot and cold seawater delivered in response to a thermostat located in the larval rearing tank. All seawater was filtered to 5µm and UV treated before delivery to the bottom of each tank (i.e. up-flow). Discharged water exited through a 'banjo' screen (300µm or 500µm). A small air stone was located in each tank for additional aeration and mixing. During the experiments, salinity was 37.5 gL^{-1} and the photoperiod was 14 h light:10 h dark. A 50 w halogen light (520 BL-50 pond

light, Nelson Industries Pty Ltd, Knoxfield, Victoria, Australia) mounted at 85 cm above each tank provided a light intensity of 1473 ± 207 lx at the water surface measured using a digital luxmeter (Light Probemeter™ 403125, Extech Instruments Corporation, Waltham, MA 02451, USA).

Experimental design and larval rearing

Two experiments were carried out from October to November, 2008. In Experiment 1, thirty two tanks were stocked with 1 DPH larvae at 60 larvae L^{-1}. Four constant density rotifer feeding treatments and four progressively increasing rotifer density treatments were compared, with four replicates of each treatment. The constant rotifer density treatments were 10 rotifers mL^{-1} (Con10), 20 rotifers mL^{-1} (Con20), 30 rotifers mL^{-1} (Con30) and 40 rotifers mL^{-1} (Con40) (Table 4.1). The progressively increasing rotifer feeding treatments were from 1 to 10 rotifers mL^{-1} (Inc1-10), 5 to 20 rotifers mL^{-1} (Inc 5-20), 10 to 30 rotifers mL^{-1} (Inc10-30) and 15 to 40 rotifers mL^{-1} (Inc15-40). For these treatments, the initial rotifer density started at 2 DPH with the final density reached at 10 DPH and maintained until 12 DPH (Table 4.1). The experiment was terminated at 13 DPH.

Table 4.1.

Live food densities (rotifers mL^{-1}) for different treatments investigated during the rotifer only feeding phase for larval rearing of Yellowtail Kingfish, *Seriola lalandi*, from 2 to 13 days post hatch. (Con = constant density; Inc = progressively increasing density).

Day post hatch	Treatment (rotifers mL^{-1})							
	Con 10	**Con 20**	**Con 30**	**Con 40**	**Inc 1-10**	**Inc 5-20**	**Inc 10-30**	**Inc 15-40**
2	10	20	30	40	1	5	10	15
3	10	20	30	40	1	5	10	15
4	10	20	30	40	1	5	10	15
5	10	20	30	40	3	10	15	20
6	10	20	30	40	3	10	15	20
7	10	20	30	40	3	10	15	20
8	10	20	30	40	5	15	20	30
9	10	20	30	40	5	15	20	30
10	10	20	30	40	10	20	30	40
11	10	20	30	40	10	20	30	40
12	10	20	30	40	10	20	30	40
13	0	0	0	0	0	0	0	0

In Experiment 2, larvae were initially cultured in a recirculating system of twelve 1,700 L tanks. At 10 DPH larvae were transferred into thirty six 170 l tanks at an initial stocking density of 40 larvae L^{-1} and maintained with rotifers (20 rotifers mL^{-1}) until the *Artemia* feeding experiment started at 12 DPH. In sixteen of these tanks, four different *Artemia* feeding treatments were trialled with each treatment replicated in four tanks. At 12 DPH, *Artemia* nauplii were introduced at densities of 0.6, 1.0, 1.4 or and 1.8 nauplii mL^{-1} then progressively increased to the final *Artemia* densities of 15 (AR15), 25 (AR25), 35 (AR35) or 45 mL^{-1} (AR45) respectively at 20 DPH (Table 4.2). In each of these treatments rotifer densities were 20 mL^{-1} at the start (12 DPH) of the trial period, decreased to 10 $^{-1}$ at 13 DPH and were then maintained at 5 mL^{-1} from 14 to 20 DPH. In another sixteen tanks, four treatments (A15, A25, A35 and A45) were trialled that matched the first four but each had rotifers discontinued after 15 DPH In the remaining four tanks, a rotifer only treatment (R) was included in which rotifers were kept at 20 mL^{-1} until 17 DPH when the rotifer density was increased to 30 mL^{-1} (Table 4.2). The experiment was terminated at 21 DPH.

In both experiments, fish were fed three times daily at 0900, 1300 and 1700 h to maintain targeted food densities with amounts added determined from residual counts taken before each feeding time. Rotifers were nutritionally enriched with Rotiselco Alg ™ (INVE Aquaculture NV, Hoogveld 91, b-9200 Dendermonde, Belgium) following manufacturers recommendations. *Artemia* (INVE Thailand Ltd., 79/1, Moo 1, Nakhon Sawan-Phitsanulok Rd., Tambon Nonglum, Amphoe Wachirabarami, Pichit 66220, Thailand) were decapsulated, incubated and nutritionally enriched with DC DHA Selco ™ (INVE Thailand Ltd) following manufacturers recommendations. Concentrated microalgae (80% *Nannochloropsis* sp., Nanno 3600™ and 20% *Isochrysis* sp., Isochrysis 1800™, Reed Mariculture Inc., 871 East Hamilton Ave, Suite D, Campbell, CA 95008 USA) was added to larval rearing tanks twice a day, and live *Nannochloropsis oculata* and *Isochrysis sp* (Tahitian strain T. Iso), in the same ratio, was harvested from 400 L bag cultures and added once each day between the applications of microalgal concentrate.

Fish sampling and growth measurement

In Experiment 1, larvae were randomly sampled from each tank on 0, 4, 8 and 13 DPH, and on 12, 17 and 21 DPH in Experiment 2. Standard length was measured, as a mean of 10 larvae per tank before 21 DPH, and total length to the nearest 0.05 mm was measured on 21 DPH. Larval growth was determined by calculating the absolute growth rate (AGR) as mm day^{-1} and specific growth rate (SGR) as % day^{-1} (Hopkins, 1992). AGR was calculated as $AGR = (L_f - L_i)/\Delta t$, and SGR was determined as $SGR = 100 \times (\ln L_f - \ln L_i)/\Delta t$, where L_f is the larval length (mm) at the end of experiment and L_i at the beginning; Δt: is time interval (d). Coefficients of variation (CV) of larval lengths between treatments were calculated from standard deviation and the mean, i.e., CV (%) = 100 × SD/Mean. On the final day of the trial Aqui-S® (Aqui-S New Zealand Ltd, Lower Hutt, New Zealand) was added to each tank at 28.6 mgL^{-1} to anaesthetise larvae before sampling and harvesting to avoid fish flaring the operculum during fixation. To determine final survival (%), all remaining larvae were harvested from each tank at the end of both experiments and preserved in 10% neutrally buffered formalin until counting.

Table 4.2.

Live food densities (prey mL^{-1} *; Rots* = rotifers; *Arts* = *Artemia* nauplii) for different treatments investigated during the transition from rotifers to *Artemia* feeding from 12 to 21 days post hatch during larval rearing of Yellowtail Kingfish, *Seriola lalandi*. (R = Rotifers; A = *Artemia* only from 15 DPH; AR = *Artemia* and rotifers until 21 DPH).

Day post hatch	**Treatment (prey mL^{-1})**																	
	R		**A15**		**AR15**		**A25**		**AR25**		**A35**		**AR35**		**A45**		**AR45**	
	Rots	*Arts*	*Rots*	*Arts*	*Rots*	*Arts*	*Rots*	*Arts*	*Rots*	*Arts*	*Rots*	*Arts*	*Rots*	*Arts*	*Rots*	*Arts*	*Rots*	*Arts*
12	20.	0.0	20.0	0.6	20.0	0.6	20.0	1.0	20.0	1.0	20.0	1.4	20.0	1.4	20.0	1.8	20.0	1.8
13	20.0	0.0	10.0	0.9	10.0	0.9	10.0	1.5	10.0	1.5	10.0	2.1	10.0	2.1	10.0	2.7	10.0	2.7
14	20.0	0.0	5.0	1.4	5.0	1.4	5.0	2.4	5.0	2.4	5.0	3.4	5.0	3.4	5.0	4.3	5.0	4.3
15	20.0	0.0	0.0	2.1	5.0	2.1	0.0	3.5	5.0	3.5	0.0	4.9	5.0	4.9	0.0	6.3	5.0	6.3
16	20.0	0.0	0.0	3.6	5.0	3.6	0.0	6.0	5.0	6.0	0.0	8.4	5.0	8.4	0.0	10.8	5.0	10.9
17	30.0	0.0	0.0	5.1	5.0	5.1	0.0	8.6	5.0	8.6	0.0	12.0	5.0	12.0	0.0	15.4	5.0	15.4
18	30.0	0.0	0.0	6.9	5.0	6.9	0.0	11.5	5.0	11.5	0.0	16.1	5.0	16.1	0.0	20.7	5.0	20.7
19	30.0	0.0	0.0	9.0	5.0	9.0	0.0	15.0	5.0	15.0	0.0	21.0	5.0	21.0	0.0	27.0	5.0	27.0
20	30.0	0.0	0.0	15.0	5.0	15.0	0.0	25.0	5.0	25.0	0.0	35.0	5.0	35.0	0.0	45.0	5.0	45.0
21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Jaw malformation assessment

At the end of each experiment, 100 anaesthetised larvae were randomly collected from each tank and preserved in 10% neutral buffered formalin. Jaw malformation was assessed under a stereo microscope (Leica MZ6, Leica Microsystems GmbH, Wetzlar, Germany) and each larvae was rated on a scale of 0 to 3 according to the jaw malformation index (Cobcroft et al., 2004) modified for Yellowtail Kingfish larvae. A score of 0 indicated normal jaw formation while a score of 0.5 indicated a very minor malformation 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 score was 1 (minor), 2 or 3 (major) which would be considered a malformation of commercial significance resulting in fingerlings being culled following quality control protocols.

Statistical analysis

Statistical analyses were performed with PASW Statistics 18 (PASW Statistics, Rel. 18.0.2. 2009 Chicago: SPSS Inc.). One-way ANOVA was used to test effects of live food feeding protocols on larval survival, growth and jaw malformation. Duncan post hoc analysis was used to determine differences between means when significant treatment effects were detected. The level of significant difference was set at $P < 0.05$ unless otherwise stated.

4.4 Results

Survival

Experiment 1: Rotifer only feeding period (2 -13 DPH)

Rotifer feeding treatments significantly affected the survival of Yellowtail Kingfish larvae during the first feeding period from 2 to 13 DPH. Highest survival (28.2 ± 8.9%, mean ± SD) was recorded for larvae fed a constant density of 20 rotifers mL^{-1} which was significantly ($P < 0.05$) higher than survival of larvae constantly fed rotifers at a density of 10 individuals mL^{-1} (13.1 ± 9.4%) or 40 individuals mL^{-1} (9.4 ± 8.8%). Survival of larvae fed a constant density of 30 rotifers mL^{-1} (22.8 ± 10.5%) was also significantly ($P < 0.05$) higher than survival of larvae constantly fed rotifers at a density of 40 individuals mL^{-1}. Larval survival was not significantly different ($P > 0.05$) between constant food densities of 20 and 30 rotifers mL^{-1}or between constant food densities of 10 and 30 rotifers mL^{-1} (Fig. 4.1).

In treatments in which the density of rotifers was progressively increased, significantly ($P < 0.05$) higher survival (28.9 ± 4.5%) occurred when food density was increased from 15 to 40

rotifers mL^{-1} compared to treatments in which food density was increased from 1 to 10 rotifers mL^{-1} (4.2 ± 3.0%) or from 5 to 20 rotifers mL^{-1} (12.0 ± 7.5%). Survival of larvae in the treatment in which food density was increased from 10 to 30 rotifers mL^{-1} (18.1 ± 6.2%) was also significantly ($P < 0.05$) greater than the survival in the treatment in which food density was increased from 1 to 10 rotifers mL^{-1}. There was no significant ($P > 0.05$) difference in the survival of larvae between the treatments in which food density was increased from 1 to 10 rotifer mL^{-1} or from 5 to 20 rotifers mL^{-1}, or between the treatments in which food density was increased from 5 to 20 rotifer mL^{-1} or from 10 to 30 rotifer mL^{-1}.

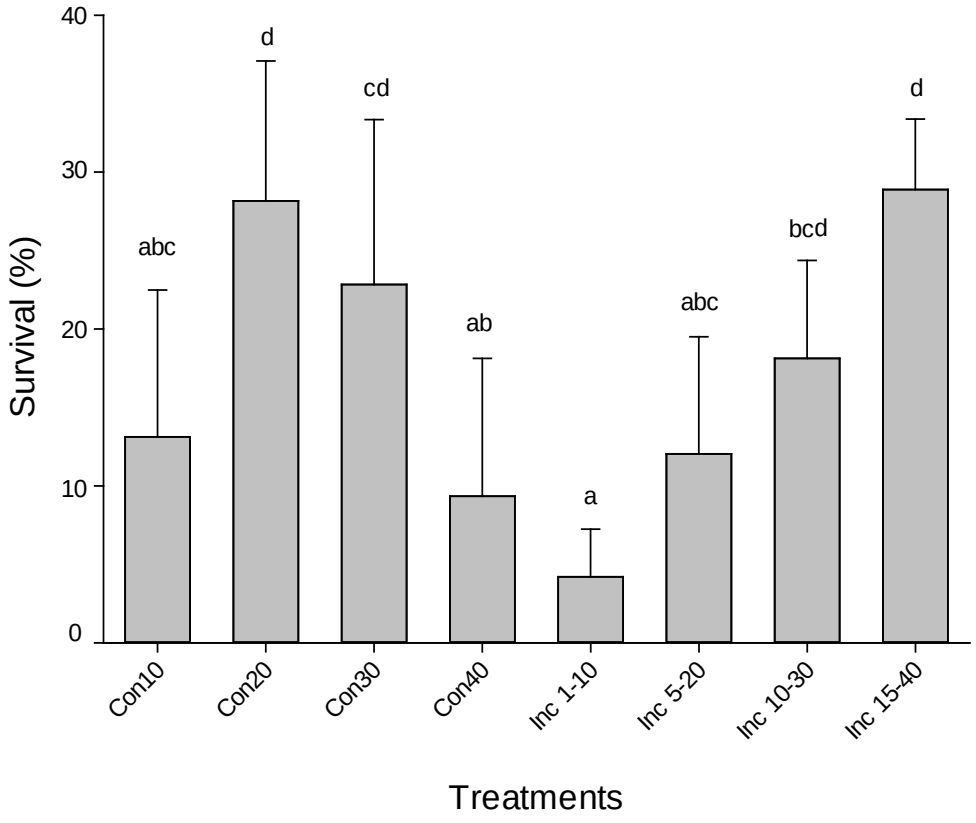

Fig. 4.1. Mean survival (% ± SD, $n = 4$) at 13 DPH for Yellowtail Kingfish larvae fed with constant rotifer density or progressively increasing rotifer density treatments from 2 DPH. Con10 = 10 rotifers mL^{-1}; Con20 = 20 rotifers mL^{-1}; Con30 = 30 rotifers mL^{-1}; Con40 = 40 rotifers mL^{-1}; Inc1-10 = density increased from 1 to 10 rotifers mL^{-1}; Inc5-20 = density increased from 5 to 20 rotifers mL^{-1}; Inc10-30 = density increased from 10 to 30 rotifers mL^{-1}; and Inc15-40 = density increased from 15 to 40 rotifers mL^{-1}. Treatments that share a common superscript are not significantly different ($P < 0.05$) in multiple comparisons.

Combining data from all rotifer feeding treatments to generate a quadratic polynomial function showed that an initial food density of 21.7 rotifers mL^{-1} corresponded with the optimum survival of 27.8% (Fig. 4.2).

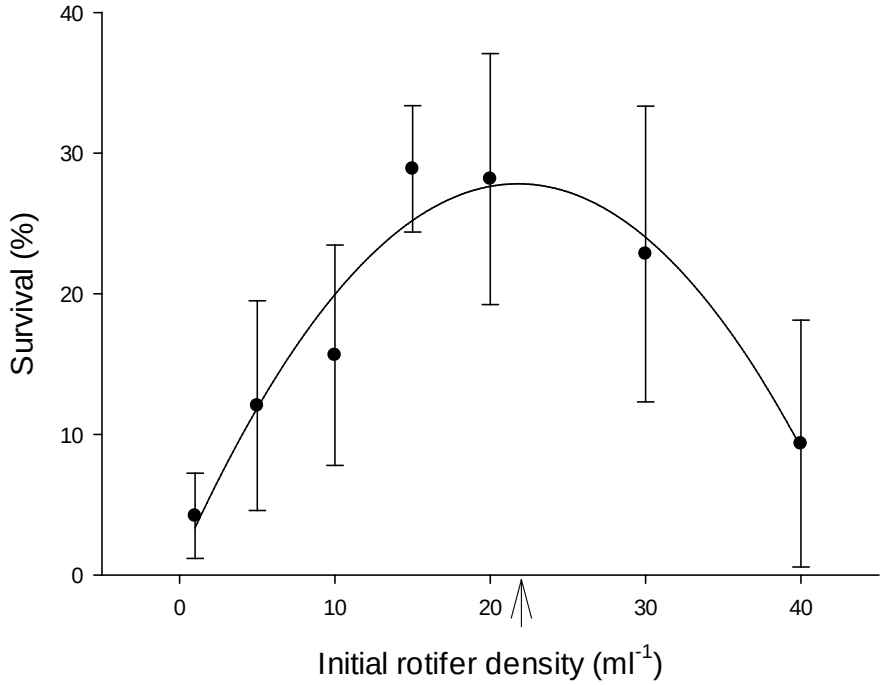

Fig. 4.2. Relationship between survival rate (%) of Yellowtail Kingfish larvae until 12 DPH and initial rotifer density (rotifers mL^{-1}) described with a quadratic polynomial curve. Survival (%) = 0.0095 + 0.0247RD – $0.0006RD^2$ (RD = Rotifer density (rotifers mL^{-1}); *df* = 2, 6; *F* = 29.55; r^2 = 0.90; *P* = 0.004). Arrow indicates the predicted maximum survival (21.7 rotifers mL^{-1}). Treatments that share a common superscript are not significantly different ($P < 0.05$).

Experiment 2: Transition from rotifer feeding to *Artemia* feeding (12 - 21 DPH)

The survival (5.8 ± 1.9%) of larvae in treatment R (fed only rotifers) was significantly ($P < 0.05$) lower than the survival in all the other treatments (Fig. 4.3). The highest survival (55.5 ± 14.0%) of Yellowtail Kingfish larvae was observed in treatment A25 which recorded significantly ($P < 0.05$) higher survival than treatments of A45, AR45 and R. Treatment A35 recorded significantly ($P < 0.05$) higher survival (51.5 ± 11.4%) than treatments of AR45 and R (Fig. 4.3). There was no significant ($P > 0.05$) difference for survival between any similar treatments in which rotifers were maintained at 5 individuals mL^{-1} until 21 DPH compared to treatments in which no rotifers were fed after 15 DPH.

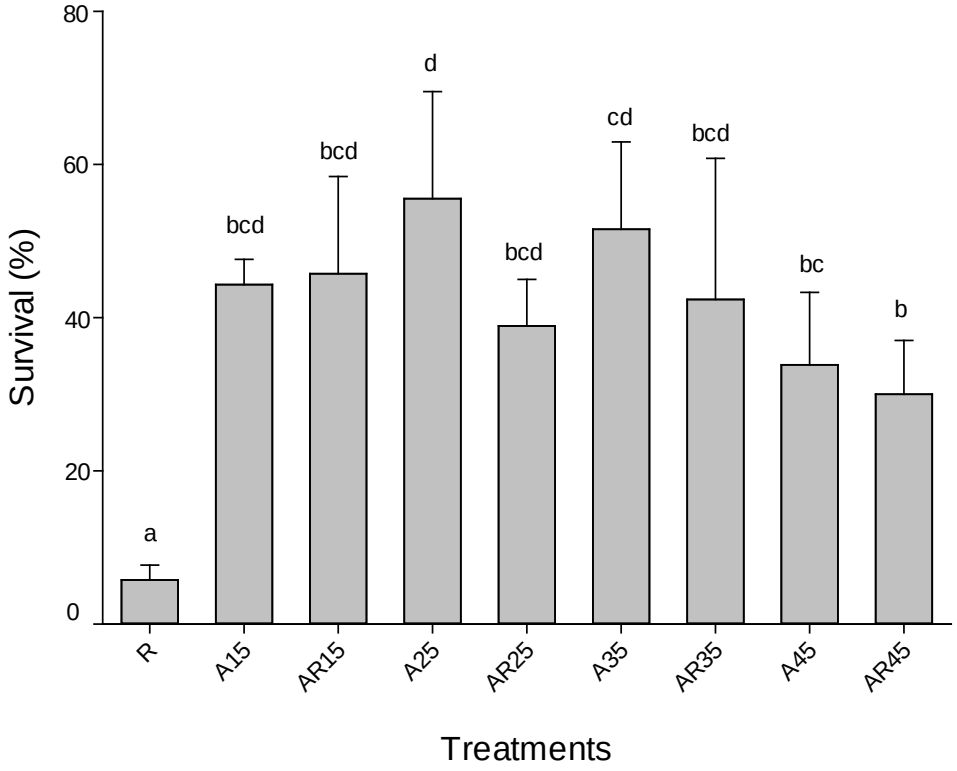

Fig. 4.3. Mean survival (% ± SD, *n* = 4) at 21 DPH for Yellowtail Kingfish larvae fed different rotifer and *Artemia* density treatments during the transition from rotifers to *Artemia* feeding between 12 and 21 DPH. (R = Rotifers; A = *Artemia* only from 15 DPH; AR = *Artemia* and rotifers until 21 DPH). Treatments that share a common superscript are not significantly different ($P < 0.05$) in multiple comparisons.

Growth

Experiment 1: Rotifer only feeding period (2 -13 DPH)

No significant ($P > 0.05$) differences were detected in final standard length of larvae among constant rotifer density treatments or progressively increasing rotifer density treatments at the end of Experiment 1 (Fig. 4.4). Similarly, for AGR, SGR and individual size variation (CV) of larvae, no significant ($P > 0.05$) difference was found among any of the treatments (Table 4.3).

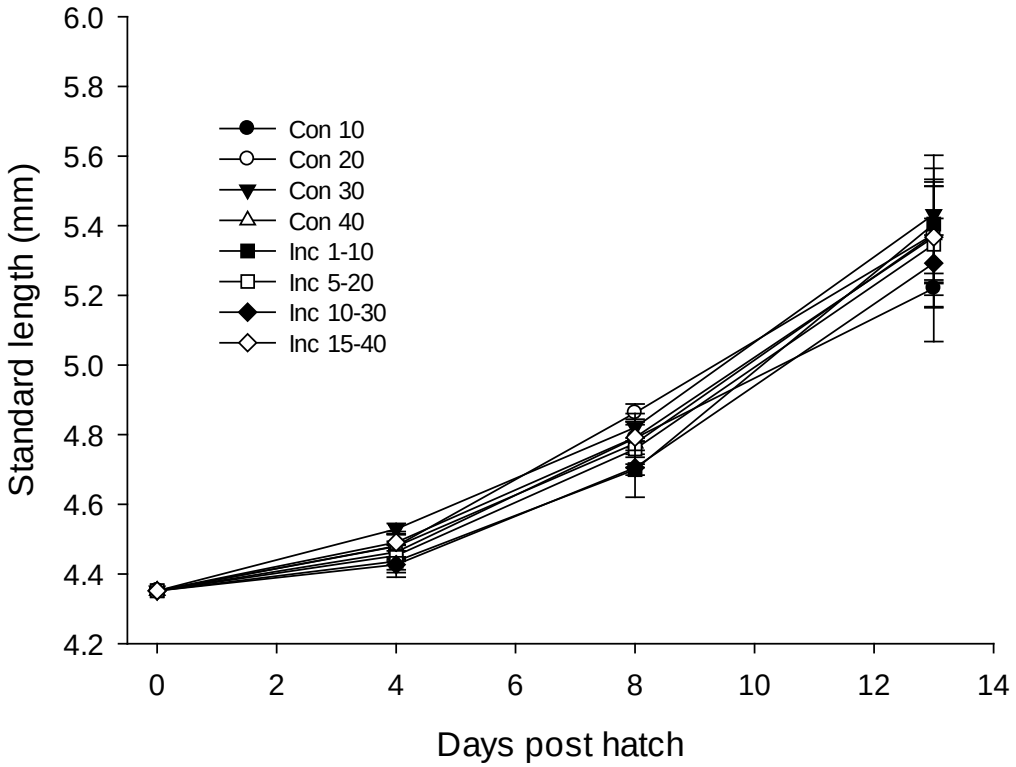

Fig. 4.4. Growth (standard length, mm ± SD, *n* = 4) of Yellowtail Kingfish larvae fed with constant rotifer densities and progressively increasing rotifer densities from 2 DPH until 13 DPH. Con10 = 10 rotifers mL^{-1}; Con20 = 20 rotifers mL^{-1}; Con30 = 30 rotifers mL^{-1}; Con40 = 40 rotifers mL^{-1}; Inc1-10 = density increased from 1 to 10 rotifers mL^{-1}; Inc5-20 = density increased from 5 to 20 rotifers mL^{-1}; Inc10-30 = density increased from 10 to 30 rotifers mL^{-1}; and Inc15-40 = density increased from 15 to 40 rotifers mL^{-1}.

Table 4.3.
Mean absolute growth rate (AGR; mm d^{-1} ± SD, n = 4), mean specific growth rate (SGR; % d^{-1} ± SD, n = 4) and mean individual size variation (CV; % ± SD, n = 4) of Yellowtail Kingfish larvae cultured following different rotifer density treatments during the rotifer only feeding phase from 2 DPH until 13 DPH. (Con = constant density; Inc = progressively increasing density). There was no significant difference ($P < 0.05$) between treatments.

Treatment (rotifers mL^{-1})	**AGR (mm d^{-1})**	**SGR (% d^{-1})**	**CV (% ± SD)**
Con 10	0.067 ± 0.010	1.40 ± 0.20	5.51 ± 0.52
Con 20	0.079 ± 0.007	1.63 ± 0.12	4.96 ± 1.17
Con 30	0.083 ± 0.018	1.70 ± 0.33	5.01 ± 1.18
Con 40	0.079 ± 0.008	1.62 ± 0.15	4.99 ± 0.25
Inc 1-10	0.081 ± 0.011	1.67 ± 0.20	5.54 ± 1.18
Inc 5-20	0.077 ± 0.019	1.58 ± 0.35	5.47 ± 1.13
Inc 10-30	0.072 ± 0.010	1.50 ± 0.19	4.43 ± 0.56
Inc 15-40	0.078 ± 0.013	1.61 ± 0.24	5.65 ± 1.02

Experiment 2: Transition from rotifer feeding to *Artemia* feeding (12 - 21 DPH)

In Experiment 2, no significant ($P > 0.05$) differences in final standard length were detected between any of the feeding protocols that included *Artemia*, but the standard length of larvae in the rotifer only treatment was significantly ($P < 0.05$) lower than that in all other treatments (Fig. 4.5). No significant ($P > 0.05$) difference was found between AGR, SGR and CV of larvae for any of the treatments except larvae in the rotifer only treatment that were significantly lower ($P < 0.05$) for each of these measures (Table 4.4).

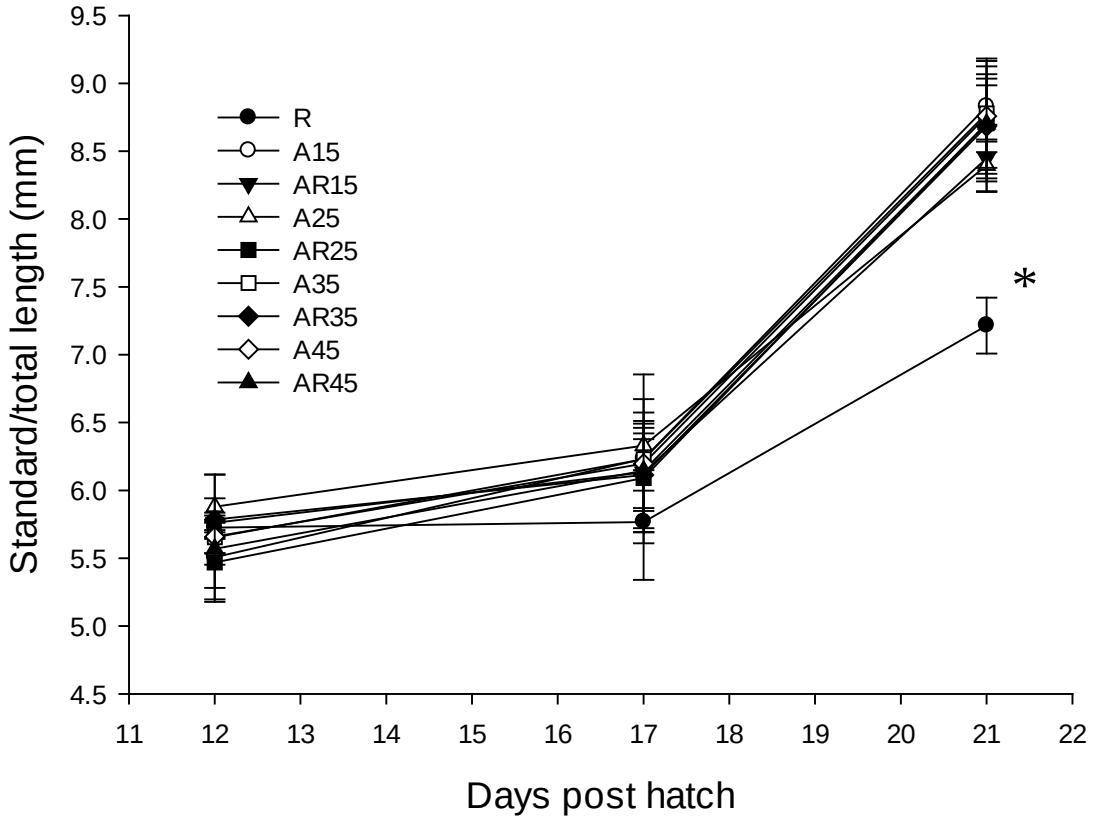

Fig. 4.5. Growth (mm ± SD, *n* = 4; 12 DPH and 17 DPH = standard length, 21 DPH = total length) of Yellowtail Kingfish larvae fed with different rotifer and *Artemia* density treatments during the transition from rotifers only, to *Artemia* only feeding between 12 DPH and 21 DPH. (R = Rotifers; A = *Artemia* only from 15 DPH; AR = *Artemia* and rotifers until 21 DPH). * denotes a significant difference ($P < 0.05$) from the other treatments.

Table 4.4.

Mean absolute growth rate (AGR; mm d^{-1} ± SD, *n* = 4), mean specific growth rate (SGR; % d^{-1} ± SD, *n* = 4) and mean individual size variation (CV; % ± SD, *n* = 4) of Yellowtail Kingfish larvae cultured following different live food density (prey mL^{-1}) treatments during the transition from rotifers to *Artemia* feeding from 12 DPH until 21 DPH. (R = Rotifers; A = *Artemia* only from 15 DPH; AR = *Artemia* and rotifers until 21 DPH). Treatments that share a common superscript are not significantly different ($P < 0.05$) in multiple comparisons.

Treatment (prey mL^{-1})	AGR (mm d^{-1})	SGR (% d^{-1})	CV (% ± SD)
R	0.17 ± 0.03 ^{a}	2.62 ± 0.40 ^{a}	14.42 ± 1.52 ^{a}
A15	0.36 ± 0.05 ^{b}	5.08 ± 0.73 ^{b}	19.16 ± 2.72 ^{b}
AR15	0.30 ± 0.02 ^{bc}	4.34 ± 0.24 ^{bc}	18.84 ± 3.45 ^{b}
A25	0.29 ± 0.02 ^{c}	4.16 ± 0.26 ^{c}	19.83 ± 2.64 ^{b}
AR25	0.35 ± 0.04 ^{bc}	4.96 ± 0.49 ^{bc}	17.29 ± 2.35 ^{ab}
A35	0.35 ± 0.03 ^{bc}	4.87 ± 0.36 ^{bc}	17.79 ± 1.47 ^{ab}
AR35	0.33 ± 0.06 ^{bc}	4.62 ± 0.74 ^{bc}	17.99 ± 2.44 ^{ab}
A45	0.34 ± 0.04 ^{bc}	4.84 ± 0.48 ^{bc}	18.61 ± 1.46 ^{b}
AR45	0.34 ± 0.05 ^{bc}	4.86 ± 0.63 ^{bc}	17.02 ± 1.88 ^{ab}

Effect of live food feeding protocols on the incidence of jaw malformation

By the end of Experiment 1, the proportion of fish with jaw malformation was high in all feeding treatments. The incidence of jaw deformity was not significantly ($P > 0.05$) affected by the feeding treatments investigated (Fig. 4.6A and 4.6B) ranging from 50.0 ± 0.0% in the Inc15-40 treatment to 65.3 ± 5.0% in the Con 40 treatment. At the end of Experiment 2 the proportion of larvae with severe jaw malformation was significantly ($P < 0.05$) affected by the rotifer and *Artemia* feeding treatment (Fig. 4.7A and 4.7B). The proportion of jaw deformity was lowest (49.4 ± 6.0%) in larvae fed rotifers only and was highest in the AR15 treatment (87.0 ± 5.5%).

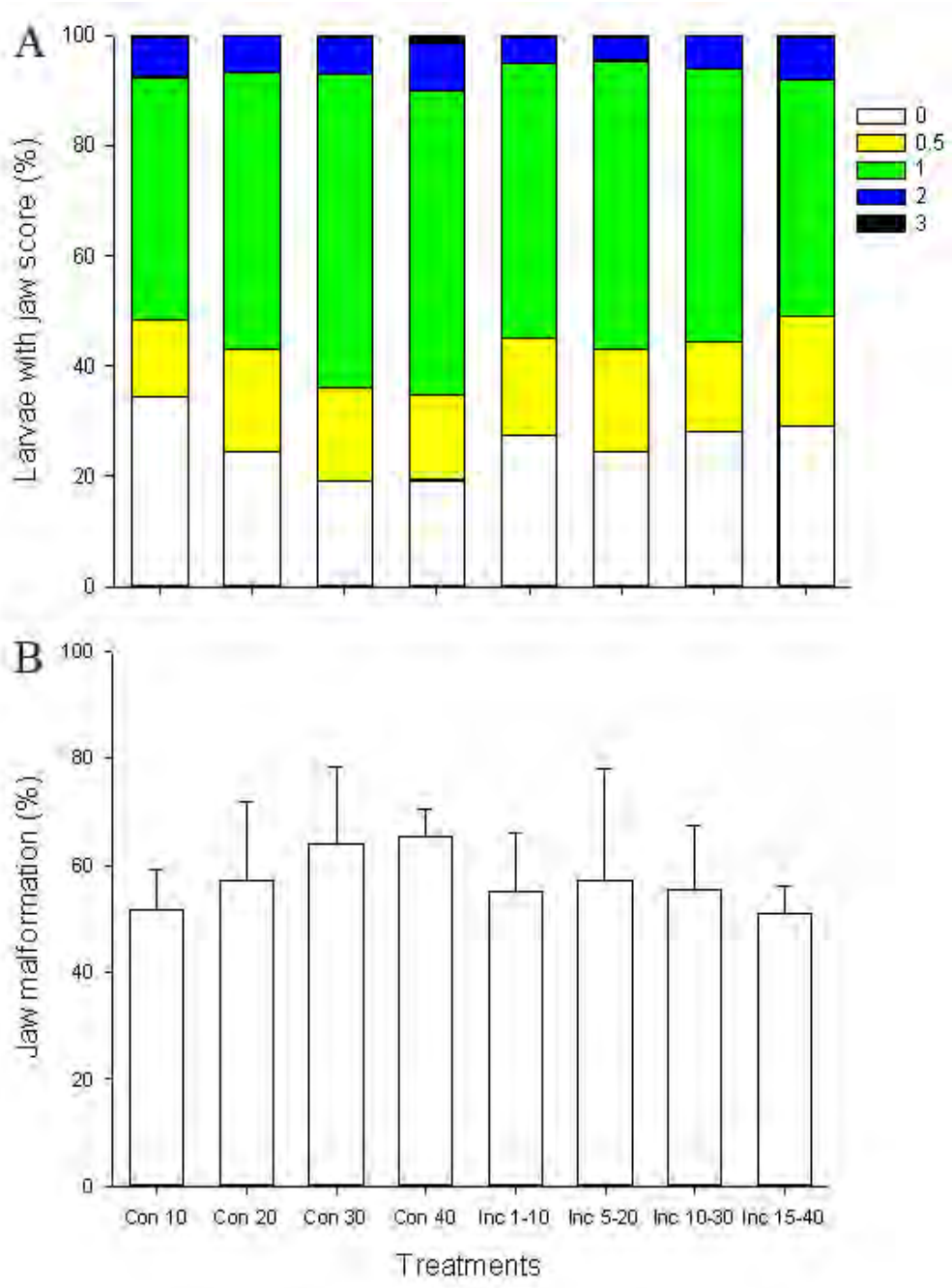

Fig. 4.6. Mean incidence of jaw malformations (% ± SD, *n* = 4) for 13 DPH Yellowtail Kingfish larvae fed with constant and progressively increasing rotifer density treatments from 2 DPH until 13 DPH. (A) All jaw deformity scores. (B) Total of commercially significant jaw deformity scores (i.e. > 0.5).

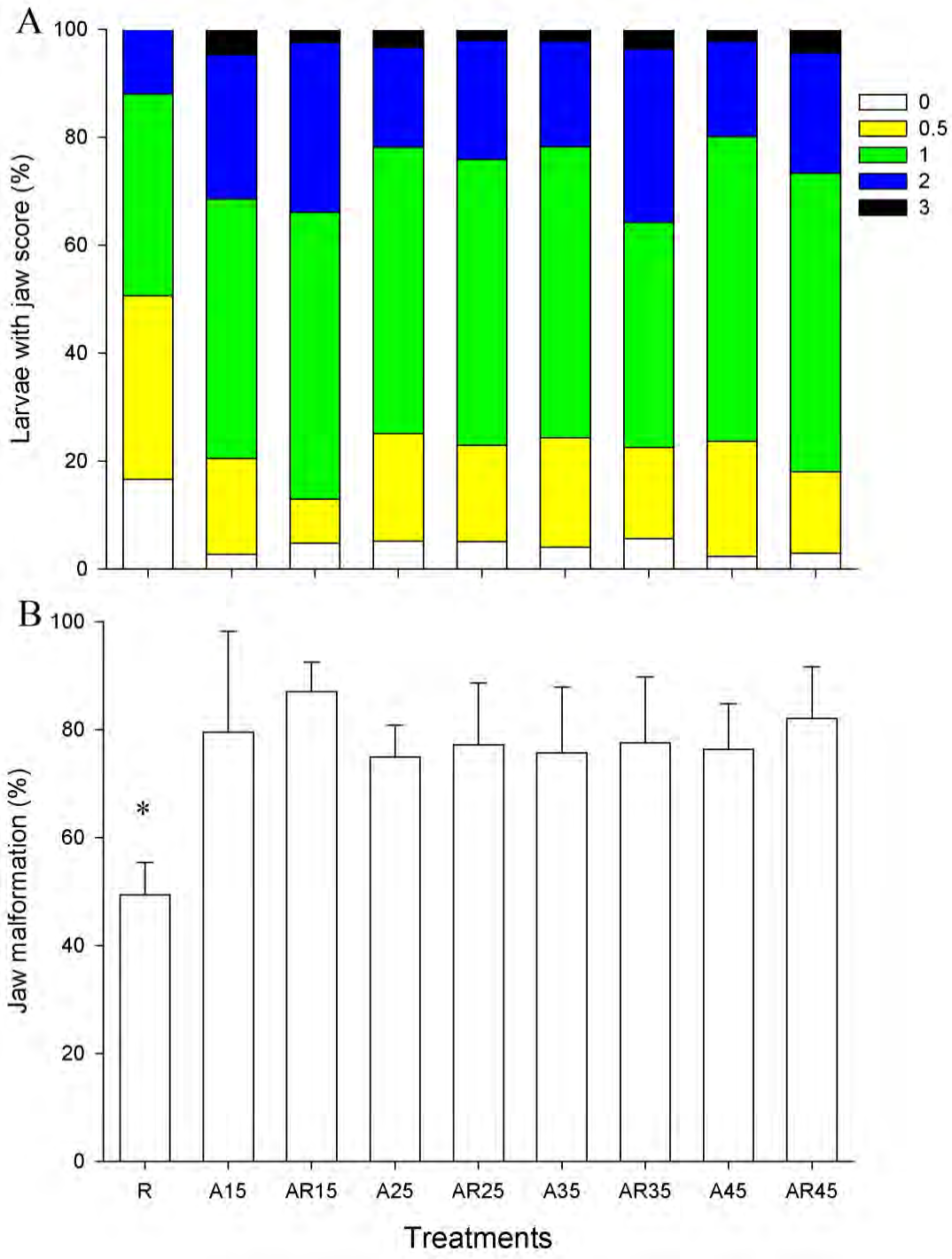

Fig. 4.7. Mean incidence of jaw malformations (% ± SD, *n* = 4) for 21 DPH Yellowtail Kingfish larvae fed with different rotifer and *Artemia* density treatments during the transition from rotifers only, to *Artemia* only feeding from 12 DPH until 21 DPH. (A) All jaw deformity scores. (B) Total of commercially significant jaw deformity scores (i.e. > 0.5). (R = Rotifers; A = *Artemia* only from 15 DPH; AR = *Artemia* and rotifers until 21 DPH).

4.5 Discussion

Effect of rotifer feeding protocols on Yellowtail Kingfish larval rearing

In this study, the results of larval survival suggest that the optimal range of prey density to promote successful first feeding and optimise survival of Yellowtail Kingfish larvae was between 15 and 30 rotifers mL^{-1}. At the end of the rotifer only feeding period, no significant difference in survival of Yellowtail Kingfish larvae was recorded between treatments in which food densities of 20 or 30 rotifers mL^{-1} were maintained although the best survival (28.2 ± 8.9 %) was achieved in the treatment in which larvae were fed at 20 rotifers mL^{-1}. In the progressively increasing rotifer density treatments, survival of larvae was highest (28.9 ± 4.5 %) when the initial rotifer density was 15 individuals mL^{-1} at 2 DPH then progressively increased to 40 rotifers mL^{-1} at 12 DPH. A quadratic function using survival data from all treatments suggested that the optimal initial rotifer density was 21.7 mL^{-1}. This is within the range of rotifer density (i.e. 10 – 30 rotifers mL^{-1}) considered optimal for first feeding larvae of Pacific northern bluefin tuna, *Thunnus thynnus* (Sawada et al., 2000) and is similar (i.e. 20 rotifers mL^{-1}) to that used during larval rearing of Yellowtail Kingfish in New Zealand (Hilton et al., 2008). A rotifer density of 20 individuals mL^{-1} also provided better prey intake by 7 DPH, grouper larvae compared to larvae cultured at 5 or 10 rotifers mL^{-1} and survival at 14 DPH was 13.5% for larvae cultured at 20 rotifers mL^{-1} compared to 1.5% and 1.8% for larvae cultured at 5 and 10 rotifers mL^{-1} respectively (Duray et al., 1996). Daily consumption of rotifers by gilthead seabream larvae was also significantly higher when rotifer density was 12 or 16 individuals mL^{-1} compared to 4 or 8 individuals mL^{-1} and the survival of larvae fed at the two higher densities was approximately double that of larvae fed at the two lower rotifer densities (Zaki et al., 2004).

In this study there was a decline in survival of Yellowtail Kingfish larvae cultured using initial rotifers densities less than, or in excess of, 15 - 30 individuals mL^{-1}. Studies on other marine fish species suggest that although a high density of rotifers is beneficial during early stages of larval development, this advantage may become less significant as larvae learn to search and feed effectively and their mouths become larger (Blaxter, 1986; Doi et al., 1997; Parra and Yúfera, 2000; Olsen et al., 2004). Fukuhara (1983) showed that swimming speed of black porgy, *Acanthopagrus schegeli*, larvae was highest at low prey density but there was no increase in swimming speed when prey density exceeded 5 individuals mL^{-1}. There was no significant increase in intake of prey by gilthead seabream larvae cultured at densities of 12 or 16 rotifers mL^{-1} (Zaki et al., 2004).

In this study the highest prey density of 40 rotifers mL^{-1} did not improve survival of Yellowtail Kingfish larvae. During the rotifer feeding period, survival was lowest when 40 rotifers mL^{-1} were added from the start of first feeding (i.e. 2 DPH), although high survival was achieved when rotifer density was progressively increased to achieve this density at 10 DPH. Sawada et al. (2000) concluded that rotifer consumption by 4 DPH Pacific northern bluefin tuna larvae was lower at a density of 40 rotifers mL^{-1} than at 10, 20 or 30 rotifers mL^{-1} and implicated poor water quality in the highest feed density tanks as a possible cause of reduced feeding. Although high prey density favours capture success through increasing encounter rates and ingestion, absorption efficiency usually decreases at high density (Klumpp and von Westernhagen, 1986; Olsen et al., 2004). A high ingestion rate is inversely related to gut evacuation rate such that the degree of digestion of prey at high concentrations is reduced due to a shorter retention time in the gut (Olsen et al., 2004). Werner and Blaxter (1980) reported that the rate of evacuation of *Artemia* nauplii from the digestive tract of herring, *Clupea harengus*, was fastest at densities of 3.0 and 30 nauplii mL^{-1} and little apparent digestion occurred to the extent that some nauplii were alive when defecated by larvae. At high food densities the residence time of food in the rearing tanks is greater resulting in a decline in nutritional quality before consumption (Olsen et al., 2004). Skjermo and Vadstein (1999) suggest that live feeds may be the most important source of opportunistic pathogens during first feeding of marine fish larvae and input of these bacteria into larval rearing tanks will be greater at high rotifer densities. These studies, together with the results presented, suggest that a density of 40 rotifers mL^{-1} is too high during the early stage of Yellowtail Kingfish larval rearing, but may be advantageous towards the end of the rotifer feeding period when larvae are more developed and have greater capacity for food intake and digestion.

In contrast to survival, neither larval growth or size variation were affected by the feeding protocols investigated for the first 20 days of Yellowtail Kingfish larval rearing with the exception of the poor growth observed in the treatment that was fed with rotifers only from 12 to 20 DPH. Similar results were reported by Duray et al. (1996) who found that the growth of grouper larvae fed at 5, 10 or 20 rotifers mL^{-1} was similar at 14 DPH. Contrary to this result, Parra and Yúfera (2000) reported a slower growth rate for gilthead seabream larvae cultured at 10 rotifers mL^{-1} in comparison to larvae cultured using 0.1 and 1.0 rotifer mL^{-1} while for the same species, Zaki et al. (2004) reported significantly better growth when

fed at rotifer densities of 12 and 16 individuals mL^{-1} compared to larvae cultured using rotifer densities of 4 and 8 individuals mL^{-1}. Dowd and Houde (1980) suggest that the functional response of fish growth in relation to prey density will only be detectable when the prey density is under a threshold level. A possible reason why prey density did not impact the growth of Yellowtail Kingfish larvae was that the prey densities used in this study were much higher than the threshold food density below which a difference in growth can be observed. Usually, the lowest live prey density used in fish hatcheries is higher than the feeding level which may promote a growth difference (Van der Wal and Nell, 1986). This scenario may explain why the different live food densities investigated in this study did not result in significant variations in growth of Yellowtail Kingfish larvae.

Effect of feeding protocols during the transition of Yellowtail Kingfish larvae from rotifer to Artemia feeding

As larval fish grow the size of prey should increase to meet the energy gain for each feeding effort (Munk, 1992). The choice of prey type, size and density at different developmental stages is pivotal for larval rearing success (Margulies, 1993). For fast growing fish species, co-feeding of rotifers and *Artemia* nauplii should be introduced earlier than for slow growing species. In fast growing species such as cobia, rotifers are fed to larvae at between 3 and 5 individuals mL^{-1} from 3 to 9 DPH and *Artemia* at between 0.1 and 1.0 nauplii $mL^{-1}$are provided from 7 DPH until weaning is completed between 20 and 22 DPH (Benetti et al., 2008). Alternatively, for slower growing species such as red seabream, *Pagrus major*, *Artemia* nauplii do not completely replace rotifers until 29 DPH (Mobin et al., 2001) and for Atlantic cod, cultured at between 10 and 12^{o}C, rotifers are maintained until larvae are 20 to 40 DPH (Brown et al., 2003). Yellowtail Kingfish is a fast growing species as suggested by the rapid development of its digestive system and large mouth gape size after hatch (Chen et al., 2006, 2007), and the high density of *Artemia* (i.e. between 20 and 30 nauplii mL^{-1} from 9 DPH) used for larval rearing of this species (Hilton et al., 2008). Results of our study show that survival rates between 42.4% and 55.5% were achieved when the density of *Artemia* fed to Yellowtail Kingfish larvae was progressively increased from between 0.6 and 1.4 nauplii mL^{-1} at 12 DPH to between 15 and 35 nauplii mL^{-1} at 20 DPH. In addition, survival and growth of Yellowtail Kingfish larvae was not improved by continuing to add rotifers beyond a 3 days period of co-feeding larvae with rotifers and *Artemia*. Further, survival and growth was significantly reduced when rotifers were the sole feed type provided to larvae after 12 DPH. This suggests that Yellowtail Kingfish larvae require increasing amounts of *Artemia*

to sustain fast growth and do not benefit from prolonged co-feeding of rotifers together with *Artemia*.

Among the *Artemia* feeding treatments the lowest survival occurred in the highest food density treatment that achieved 45 nauplii mL^{-1} from 20 DPH. Mobin et al. (2001) investigated feeding of red seabream larvae and compared the use of a conventional rotifer and *Artemia* feeding protocol used by hatcheries in the western part of Japan (i.e. rotifers = 3 individuals mL^{-1} at 3 DPH increasing to 20 individuals mL^{-1} at 29 DPH; *Artemia* = 0.1 nauplii mL^{-1} from 18 DPH increasing to 1.1 nauplii mL^{-1} at 29 DPH) with a protocol that used live food densities that were five times greater (i.e. rotifers = 15 individuals mL^{-1} at 3 DPH increasing to 100 individuals mL^{-1} at 29 DPH; *Artemia* = 0.5 nauplii mL^{-1} from 18 DPH increasing to 5.5 nauplii mL^{-1} at 29 DPH). The higher density feeding treatment produced increased growth of larvae and juveniles but a significantly lower survival was recorded as the higher feeding densities provoked pathological alterations in the digestive system of larvae, leading to high levels of mortality. Increased mortality of Atlantic cod larvae was observed following addition of *Artemia* at densities greater than 4 nauplii mL^{-1} and especially in the highest prey density treatment of 16 nauplii mL^{-1} with short term exposure of larvae to high concentrations of metabolites associated with high amounts of *Artemia* nauplii implicated in larval mortality (Puvanendran and Brown, 1999). It is suggested that an *Artemia* density of 45 nauplii mL^{-1} is excessive for Yellowtail Kingfish larvae and the lower survival observed may be attributed to factors such as adverse affects of high food intake on gut function and exposure of larvae to adverse water quality associated with high food densities investigated.

Effect of live food feeding protocols on the incidence of jaw malformation in Yellowtail Kingfish larvae

Deformities are a significant issue affecting the quality of Yellowtail Kingfish fingerlings available for stocking sea cages and currently no definitive cause has been associated with the deformities observed. This problem is not restricted to Yellowtail Kingfish as it is estimated that 30% of marine fish fingerlings produced in commercial hatcheries exhibit some form of deformity (Boglione et al., 2001). The effect of rotifer and *Artemia* feeding treatments on the incidence of jaw malformation recorded at 21 DPH showed that lowest rate of jaw malformation was found in the treatment in which larvae were fed with rotifers only. However, it is suggested that the low jaw malformation rate observed in larvae from this

treatment is likely to be because these larvae were smaller (7.2 ± 0.2 mm TL) than the mean length of larvae from all other treatments (8.7 ± 0.2 mm TL) and did not reach a size sufficient to display malformation as the incidence jaw malformation is size dependent (Cobcroft et al., 2004). Alternatively a high proportion of the small number of surviving larvae are likely to have been those that were least compromised resulting in less expression of deformities while deformed larvae are likely to have succumbed to mortality in this treatment. Consequently, feeding Yellowtail Kingfish larvae with rotifers only until 21 DPH is not recommended as a strategy to reduce jaw malformation during larval rearing of Yellowtail Kingfish.

The observation that there was a high incidence of jaw deformity recorded for all feeding treatments suggests that factors other than live food density is contributing to the occurrence of jaw deformity during larval rearing of Yellowtail Kingfish. A higher incidence of deformities is reported for fingerlings reared in intensive hatcheries compared to semi-intensive systems or wild caught fingerlings (Boglione et al., 2001; Izquierdo et al., 2010; Roo et al., 2010) and a range of biotic and abiotic factors have been implicated in various types of deformities recorded for hatchery reared fingerlings (Cahu et al., 2003). It is possible that some of the nutritional factors implicated as affecting the incidence of deformities may also be affected by live food density. However the results presented suggest that live food density within the range investigated do not affect the incidence of deformities in hatchery reared Yellowtail Kingfish.

4.6 Conclusion

Live food density affected survival of Yellowtail Kingfish larvae but not growth and size variation. Survival rates were similar in the feeding protocols with constant densities between 20 and 30 rotifers mL^{-1} although the highest survival was achieved using a rotifer density of 20 individuals mL^{-1}. In treatments in which the food density was progressively increased, the regime starting with15 rotifer mL^{-1} at 2 DPH and ending with 40 rotifer mL^{-1} at 12 DPH resulted in the highest larval survival. A quadratic polynomial function generated with data from all treatments indicated that optimal survival was achieved at an initial density of 21.7 rotifers mL^{-1}. This suggests that in a commercial hatchery the use of a relatively high density between 15 and 30 rotifers mL^{-1} from the onset of first feeding should be followed. The *Artemia* feeding treatments tested indicate the density of *Artemia* fed to Yellowtail Kingfish larvae can be progressively increased from between 0.6 and 1.4 nauplii mL^{-1} at 12

DPH to between 15 and 35 nauplii mL^{-1} at 20 DPH prior to the start of weaning. Results presented suggest that for Yellowtail Kingfish larvae, live food density is more critical to survival than to growth during the early life stage. These studies support the suggestion that Yellowtail Kingfish is a fast growing species and the higher survival achieved at these food densities reflect the need of this species for a high feed intake to maintain this rate of growth. Findings of these experiments also suggest that continued addition of rotifers together with *Artemia* nauplii beyond 15 DPH after a 3-day co-feeding period does not provide any advantage to survival or growth for culture of Yellowtail Kingfish larvae. Apart from in the rotifer only feeding treatment, the incidence of deformities recorded showed no difference between feed density treatments suggesting that other factors are determining the quality of Yellowtail Kingfish fingerlings. These results should contribute to the improved productivity of commercial Yellowtail Kingfish hatcheries.

5. Effect of age of Yellowtail Kingfish (*Seriola lalandi*) larvae on success of weaning from live food to a formulated diet.

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5.1 Abstract

Weaning is a critical stage in the process of commercial hatchery production of marine finfish fingerlings. However, selecting the right time for weaning is mostly based upon the operators' experience. Previous work has shown that the digestive system of Yellowtail Kingfish larvae cultured at 24^{o}C matures at around 15 days post hatch (DPH) when gastric glands form in the stomach accompanied by the gradual disappearance of acidophilic supranuclear vacuoles (SNVs) in the posterior intestine, indicating protein digestion has switched from extracellular to intracellular digestion. This development allows larvae to digest and assimilate more complex protein found in formulated diets from this time. In this study, we investigated the effect of the age of Yellowtail Kingfish, *Seriola lalandi*, larvae at the start of feeding microparticulate diets (weaning) on subsequent rates of survival, growth and jaw malformation. Five experimental treatments were compared that started addition of a formulated microparticulate diet from 10 (W10), 13 (W13), 16 (W16), 19 (W19) and 22 (W22) DPH. In each treatment larvae were cultured at water temperature 24.4 ± 0.2 °C and the microparticulate diet was introduced progressively during a 5 day co-feeding period during which live food densities were maintained. This was followed by a 5 day weaning period during which the ration of live food was progressively reduced to zero as the amount of artificial diet added was increased. The data indicate that survival and growth was significantly lower for Yellowtail Kingfish larvae that were offered an artificial diet from either 10 DPH or 13 DPH as compared to larvae that were offered it from 16 DPH, 19 DPH or 22 DPH. There was no significant difference ($P > 0.05$) in survival or growth of larvae that started the weaning process at 16 DPH, 19 DPH or 22 DPH. The proportion of fish with jaw malformation at 33 DPH was not significantly affected by when the larvae were offered an artificial diet. These results indicate that feeding of microparticulate diets can be started from when Yellowtail Kingfish larvae have developed a functional stomach between 15 DPH and 18 DPH. The appearance of gastric glands in the stomach is suggested to be an accurate indicator of the appropriate time to commence weaning of Yellowtail Kingfish larvae and should help to develop the optimal weaning strategy to follow during hatchery production of this species.

Keywords: Yellowtail Kingfish; *Seriola lalandi*; Growth; Larval rearing; Survival; Co-feeding; Weaning; Jaw malformation.

5.2 Introduction

Weaning of marine finfish larvae involves the replacement of live food organisms (i.e. rotifers and *Artemia*) with formulated diets. This is a critical step for commercial production of marine finfish fingerlings as live food production cannot continue to maintain the increasing nutritional demands needed to support exponentially increasing growth and development of large numbers of fingerlings destined for on-growing to market size using formulated feeds (Rosenlund et al., 1997). The weaning process needs to follow a procedure that allows developing larvae to make the transition from live foods to formulated diets while minimising the high mortality and possible adverse effects on latter growth and survival that can be associated with this transition if not undertaken in manner appropriate to the species. Ideally the weaning protocol used should consider the ontogenetic development of the digestive system of the larvae and juveniles of the species (Kolkovski, 2001).

There are a number of advantages that are provided by completing the change from live foods to manufactured diets during hatchery production of marine fish. These include reduction in the costs and labour associated with production and preparation of live foods (Lee et al., 1996; Callan et al., 2003; Stoss et al., 2004; Hamre, 2006), reducing the variability in nutrition provided by cultured live foods (Callan et al., 2003), decreasing the opportunity for transfer of microbial loads known to be associated with rotifers and *Artemia* nauplii (Hart and Purser, 1996; Vadstein et al., 2004; Cutts et al., 2007), removal of operational tasks allowing improved process control in the hatchery; and further minimising the potential for human error in the production process. Consequently it is advantageous to achieve successful weaning of larvae at the earliest time that will allow high survival and optimal quality of fingerlings.

First feeding marine fish larvae do not possess a fully functional digestive system. In particular the stomach is not differentiated at this time (Cahu and Zambonino Infante, 2001) and digestion occurs within the intestine that is alkaline with trypsin like enzymes accounting for proteolytic activity (Walford and Lam, 1993). The larval phase typically ends when the stomach and pyloric caeca are developed (Kolkovski, 2001). This coincides with metamorphosis and at this time the digestive system of marine fish larvae becomes fully functional after the formation of gastric glands within the stomach that secrete pepsinogen (the precursor of pepsin) and hydrochloric acid to allow a more efficient acidic digestion (Gawlicka et al., 2001; Darias et al., 2007). At this time there is a gradual disappearance of

intestinal acidophilic SNVs indicating that protein digestion becomes mainly extracellular instead of intracellular as larvae switch from the less efficient alkaline digestion to the more efficient acid digestion of the adult (Govoni et al., 1986; Darias et al., 2007). Generally, weaning is conducted when larvae have completed metamorphosis (Stoss et al., 2004) as their digestive system becomes fully functional (Govoni et al., 1986; Segner et al., 1994) allowing them to start digesting and assimilating the more complex proteins from fish in the wild, or from microparticulate diets in the hatchery (Dabrowski, 1984; Douglas et al., 1999; Kvale et al., 2007).

In this paper "co-feeding" is defined as a period during which both live and artificial foods are provided to larvae before the amount of live food starts to be reduced (i.e. weaning proper). Co-feeding can start from the commencement of exogenous feeding at an early larval age (Rosenlund et al., 1997). "Weaning" is defined as the period during which the amount of live food is reduced while the amount of manufactured diet is increased until it constitutes all of the food offered. "Early weaning" is considered to describe attempts to wean larvae before they have completed metamorphosis and have developed a functional stomach with gastric digestion.

Currently weaning of marine fish larvae is best achieved by co-feeding live foods in combination with manufactured diets (Stoss et al., 2004) for a period of several days before weaning proper commences. It is generally accepted that co-feeding can improve growth and survival of marine fish larvae (Rosenlund et al., 1997) and can reduce the amount of live food required (Stoss et al., 2004). This is attributed to formulated microparticulate diets providing larvae with additional nutrition to that provided by live foods and providing a period of time for larvae to become conditioned to consuming microparticulate diets so that they more readily ingest this food type as live food is withdrawn during weaning (Rosenlund et al., 1997; Cahu and Zambonino Infante, 2001; Kolkovski, 2001). For greenback flounder, *Rhombosolea tapirina*, cultured at 15-17^{o}C it is suggested that co-feeding should start from 23 days post hatching (DPH) following complete differentiation of the stomach but prior to metamorphosis (Hart and Purser, 1996). Growth and survival of halibut, *Hippoglossus hippoglossus*, was significantly better for co-fed fish compared to others maintained on *Artemia*, while sea bass, *Dicentrarchus labrax*, and sea bream, *Sparus auratus*, showed higher mean final body weight in comparison to fish fed only *Artemia* prior to weaning (Rosenlund et al., 1997). For barramundi, *Lates calcarifer*, survival was significantly

improved when co-feeding of microparticulate diet and *Artemia* occurred from when larvae were 5 mm standard length (SL) coinciding with initiation of stomach differentiation (Curnow et al., 2006). Final weight of juvenile co-fed tongue sole, *Cynoglossus semilaevis*, was significantly greater than for fish that were fed only live food prior to weaning (Chang et al., 2006). Growth of cobia, *Rachycentron canadum*, larvae cultured at 30ºC was significantly greater when co-feeding of microparticulate diets was started from 8 DPH compared to starting feeding from 13 DPH and 18 DPH (Nhu et al., 2010). These beneficial effects of co-feeding support the accepted premise that co-feeding improves growth and survival of marine fish larvae ultimately leading to improved weaning success.

Achieving successful early weaning can potentially lower production costs considerably by reducing the live food culture effort (Baskerville-Bridges and Kling, 2000b; Callan et al., 2003), but this must be considered against possible reduction in growth and survival if weaning onto formulated diets is conducted too early (Stoss et al., 2004; Kvale et al., 2009). Early weaning has led to slow growth, mass mortality (Baskerville-Bridges and Kling, 2000a; Hamlin and Kling, 2001) and poor fish quality as characterised by skeletal deformities (Segner and Witt, 1990; Person Le Ruyet et al., 1993; Cahu and Zambonino Infante, 2001). Although acceptable survival is possible with early weaning of halibut, subsequent growth rates during on-growing are far less than optimal and fish also exhibited incomplete metamorphosis, skeletal defects and problems with eye migration (Stoss et al., 2004). Premature introduction of microparticulate diet inhibited growth of barramundi larvae and prevented maturation leading to low survival at 28 DPH despite larvae being observed to ingest microparticulate diet particles (Curnow et al., 2006). Southern flounder, *Paralichthys lethostigma*, larvae were weaned onto a microparticulate diet by 17 DPH but growth was significantly better in live food fed control fish (Faulk and Holt, 2009).

These results suggest that early ingestion of manufactured diets formulated with complex ingredients cannot be effectively digested by pre-metamorphosed larvae with a digestive system that is not fully functional. In particular the ability of larvae to digest proteins is less effective using their alkaline digestive system compared with when gastric digestion becomes functional around the time of metamorphosis. This increased digestive capacity coincides with an increasing demand by juveniles to digest protein needed to support accelerating growth and development that occurs from this time. Previous research (Chen et al., 2006a) revealed that in Yellowtail Kingfish, *Seriola lalandi,* larvae cultured at 24°C the

gastric glands developed in the stomach at around 15 DPH and the fundic region of stomach and pyloric caecae form around 18 DPH. Acidophilic SNVs disappeared gradually during this stage, indicating that the stomach becomes functional and can denature more complex proteins. It is expected that at this time, larvae may improve their ability to digest complex diets with the help of pepsin and it is hypothesised that this transitional period, when Yellowtail Kingfish larvae gain a functional stomach, would be the right time to commence weaning.

In this study we investigated the effect on survival, growth and jaw malformation of commencing co-feeding and weaning at a range of times related to the development of digestive system of Yellowtail Kingfish larvae, especially the appearance of gastric glands in stomach. An objective of this study was to determine the earliest time that the co-feeding and weaning protocol could be commenced so as to establish a cost-effective weaning strategy for commercial production of Yellowtail Kingfish fingerlings.

5.3 Material and methods

Egg incubation and larval rearing until 10 DPH

Yellowtail Kingfish eggs were collected in December 2009 from broodstock maintained under artificial photothermal conditions at the hatchery of Clean Seas Tuna Limited, Arno Bay, South Australia. Eggs were transported to the South Australian Aquatic Sciences Centre, Adelaide, South Australia where they were hatched in 220 L fibreglass incubators at water temperature between 22.8°C and 23.5°C. At 2 DPH larvae were stocked into 7 x 1700 L larval rearing tanks supplied with recirculated seawater. Larvae were fed rotifers, *Brachionus plicatilis*, at densities ranging between 10 and 20 individuals mL^{-1} until 10 DPH. Water temperature was maintained at 24.7 ± 0.1°C (mean ± SD), dissolved oxygen between 6.1 and 7.3 mg L^{-1}, pH between 7.95 and 8.11; and salinity was 37.5 g L^{-1}. "Green water" conditions were maintained by addition of microalgal paste (*Nannochloropsis* sp., Nanno 3600™, Reed Mariculture Inc., 871 East Hamilton Ave, Suite D, Campbell, CA 95008 USA) to larval rearing tanks three times each day. At 10 DPH, aggregations of larvae were scooped from the larval rearing tanks using clear bowls (3 L) and transferred into a 20 L bucket. This was mixed to evenly distribute the larvae, and 3 x 50 mL sub-samples were taken and counted to estimate the total number of larvae in each bucket before stocking into the experimental

tanks. A tally of the number of larvae stocked into each tank was kept until the target number required was reached.

Experimental system

The experiment was conducted in 25 conical bottom fibreglass tanks each with a working volume of 170 L. The vertical walls of each tank were coated with a 'marble' pattern adhesive film (Alkor-Venilia GMBH, Am Haag 8, Graefelfing, D-82166, Germany) to minimise 'walling' behaviour of larvae (Cobcroft and Battaglene, 2009). The water temperature was maintained at 24.4 ± 0.2 °C by a building management system controlling solenoid operated valves to mix ambient, hot and cold seawater delivered in response to a thermostat located in the larval rearing tank. All seawater was filtered to 5 µm and UV treated before delivery to the bottom of each tank (i.e. up-flow). Water exchange rate was at 0.7 L min^{-1} at the beginning of each experiment and increased to 1.2 L min^{-1} by the end of each experiment. Discharged water exited through a 'banjo' screen (300 µm or 500 µm) at the side of each tank. A small air stone was located in each tank for additional aeration and mixing. During the experiment salinity was 37.5 g L^{-1}, mean dissolved oxygen was 7.47 ± 0.02, mean pH was 8.05 ± 0.01 and the photoperiod was 14 h light:10 h dark. A 50 w halogen light (520 BL-50 pond light, Nelson Industries Pty Ltd, Knoxfield, Victoria, Australia) mounted at 85 cm above each tank provided a light intensity of 999 ± 121 lx at the water surface measured using a digital luxmeter (Light Probemeter™ 403125, Extech Instruments Corporation, Waltham, MA 02451, USA).

Experimental design and larval rearing procedure

The experiment included 5 co-feeding and weaning treatments (Fig. 5.1) that were each replicated in 5 randomly allocated experimental tanks. The larval rearing protocol followed that used by industry for Yellowtail Kingfish culture in South Australia at the time the experiment was conducted. Each treatment extended for 10 days and comprised a 5 day co-feeding period during which microparticulate diet type and live food additions followed the standard larval rearing protocol. Following this a 5 day weaning period commenced during which the amount of live food was progressively reduced to zero while the amount of microparticulate diets was increased. Different co-feeding and weaning treatments commenced at 3 day intervals with the first treatment W10 starting from 10 DPH and ending at 19 DPH, followed by treatment W13 starting from 13 DPH and ending at 22 DPH, W16

starting from 16 DPH and ending at 25 DPH, W 19 starting from 19 DPH and ending at 26 DPH, and W22 starting from 22 DPH (W22) and ending at 33 DPH.

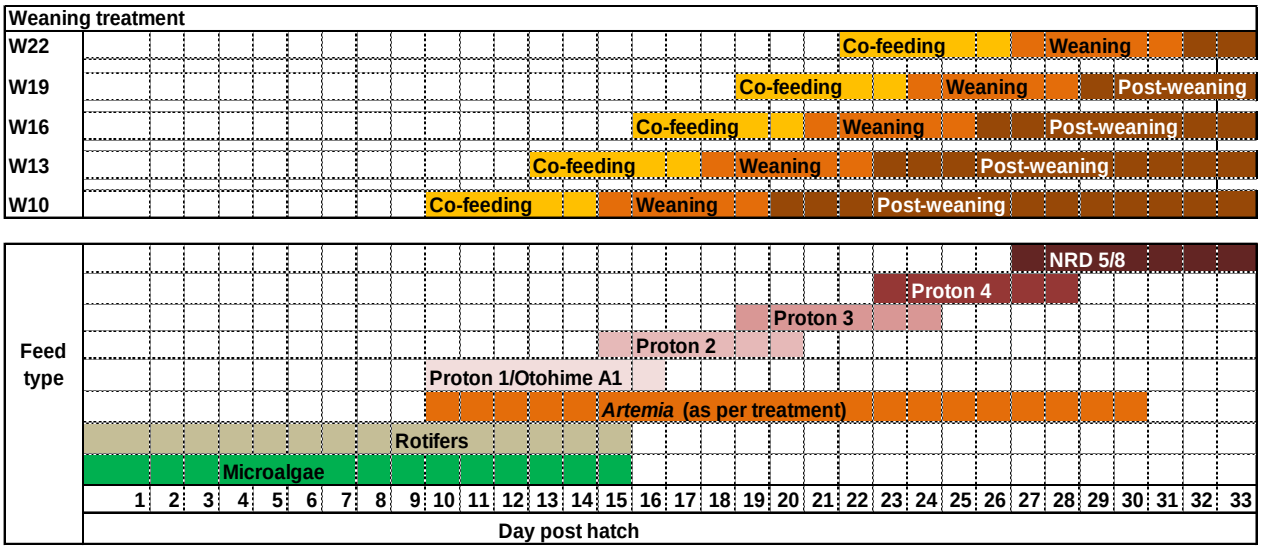

Fig. 5.1. Different co-feeding and weaning treatments investigated and the feed types used for culture of Yellowtail Kingfish larvae from 10 DPH until 33 DPH.

Live food was added 3 times daily at 0900, 1300 and 1700 h to maintain targeted densities. Rotifers were nutritionally enriched with Rotiselco Alg ™ (INVE Aquaculture NV, Hoogveld 91, b-9200 Dendermonde, Belgium) following manufacturers recommendations for dosage and environmental parameters during enrichment. *Artemia* nauplii were first introduced at 1 nauplii mL^{-1} then progressively increased to a final density of 15 nauplii mL^{-1}. *Artemia* cysts (INVE Thailand Ltd., 79/1, Moo 1, Nakhon Sawan-Phitsanulok Rd., Tambon Nonglum, Amphoe Wachirabarami, Pichit 66220, Thailand) were decapsulated, incubated and nutritionally enriched with DC DHA Selco ™ (INVE Thailand Ltd) following manufacturers recommendations for dosage and environmental parameters during enrichment. Residual concentration of live food was checked prior to each feeding as the basis to add live food to the targeted levels. Microalgal paste (80% *Nannochloropsis* sp., Nanno 3600™, and 20% *Isochrysis* sp., Isochrysis 1800™, both from Reed Mariculture Inc., 871 East Hamilton Ave, Suite D, Campbell, CA 95008 USA) was added to larval rearing tanks twice a day, and live *Nannochloropsis oculata* and *Isochrysis sp* (Tahitian strain T. Iso), in the same ratio, was harvested from 400 L bag cultures and added once each day between the applications of microalgal paste. The commercial microparticulate diets, with a range of particle sizes, used over the duration of the experiment were: Otohime A1 (~250 µm, Marubeni Nisshin Feed Co., Ltd., Sakura Muromachi bldg., 4-5-1, Nihonbashi Muro-

machi, Chuo-ku, Tokyo, 103-0022 Japan), Proton® 1, 2, 3, 4 (80-200 µm, 150-300 µm and 200-400 µm, 300-500 µm respectively, INVE Aquaculture NV, Hoogveld 91, b-9200 Dendermonde, Belgium) and NRD 5/8 (500-800 µm, INVE Aquaculture NV). Small amounts of microparticulate diets were distributed by hand to each tank at approximately hourly intervals from 9.00 am until 7.00 pm each day. Outlet screens were cleaned and tank bottoms were siphoned daily to remove dead larvae, uneaten foods and faeces.

Fish sampling and growth measurement

To monitor the growth, larvae (n = 10) were randomly sampled from each tank on 0, 1, 2, 4 and 8 DPH before they were transferred into the experimental tanks and at 12, 16, 20, 24, 28 and 33 DPH after transfer. Before 21 DPH standard length (SL) was measured to the nearest 0.05 mm from the tip of the upper jaw to the end of the notochord of 10 larvae per tank, and afterwards total length (TL) was measured from the tip of the jaw to the end of the caudal fin tip. After measurement the 10 larvae were used to assess dry weight. Larvae from each tank were pooled and then rinsed with 0.5 M ammonium formate solution to remove external salt, transferred into pre-weight foil cells, dried at 90°C for 72 h in a drying oven and the pooled sample weighed to the nearest 0.5 mg on a Mettler AE 240 microbalance. From these data fish growth was determined by measuring the absolute growth rate (AGR) as mm d^{-1} and specific growth rate (SGR) as % d^{-1} (Hopkins, 1992). AGR was calculated as: $AGR = (L_f - L_i)/\Delta t$, and SGR was determined as: $SGR = 100 \times (\text{Ln } L_f - \text{Ln } L_i)/\Delta t$, where L_f is the fish length (mm) at the end of experiment and L_i at the beginning; Δt: is time interval (d). Coefficients of variation (CV, %) of fish lengths between treatments were calculated from standard deviation and the mean for each treatment at each sampling time (i.e. CV = 100 x SD/mean). On the final day of the trial Aqui-S® (Aqui-S New Zealand Ltd, Lower Hutt, New Zealand) was added to each tank at 28.6 mg L^{-1} to anaesthetise larvae to avoid fish flaring the operculum during fixation. Larvae required for measurement and deformity assessment were removed from each tank and all remaining larvae were harvested and preserved in 10% neutrally buffered formalin to determine final survival at the end of both experiments.

Histological observation of stomach, gastric glands

To monitor development of the digestive system ten larvae (n = 10) from each of five replicated tanks from each treatment were randomly sampled on 10, 12, 15, 18, and 20 DPH. Larvae were anaesthetized using Aqui-S® then fixed in 10% neutral buffered formalin. The

fixed larvae were embedded in paraffin blocks and sectioned in serial sagittal sections (5 µm thick) using a Leica RM 2135 rotary microtome. Haematoxylin-eosin (HE) staining was used to prepare slides for general histological observations of the development of the digestive tract (Chen et al., 2006a). The slides with the sections were mounted using DePex for permanent transparent preparation. The stained sections were observed using a light microscope (Leitz Ortholux II, Ernst Leitz GmbH, Wetzlar, Germany) and images were taken with a Canon digital photomicrographic attachment.

Jaw malformation assessment

Jaw malformation was assessed under a Leica, MZ6 stereo microscope (Leica Microsystems GmbH, Wetzlar, Germany) using the method by Cobcroft et al. (2004) and Cobcroft and Battaglene (2009). At the end of each experiment 100 anaesthetised larvae were randomly harvested from each tank and preserved in 10% neutral buffered formalin at about 10 times formalin to fish tissue volume. Larvae were anaesthetised by 28.6 mg L^{-1} Aqui-S® before preservation in formalin, which is a critical step to avoid fish flaring the operculum during fixation. For assessment, the appearance of the jaws of the fixed fish was rated on a scale of 0 to 3 according to the jaw malformation index (Cobcroft et al., 2004) modified for Yellowtail Kingfish larvae. A score of 0 indicated normal jaw formation while a score of 0.5 indicated a very minor malformation that was unlikely to impair larval performance and that would not be considered a commercially significant malformation. Larvae were defined as malformed when the jaw score was 1 (minor), 2 or 3 (major) which would be considered a malformation of commercial significance resulting in fingerlings being culled following quality control protocols used at the end of the nursery phase prior to transfer of fingerlings to on-growing in sea cages.

Statistical analysis

Statistical analyses were performed with PASW Statistics (PASW Statistics, Rel. 18.0.2. 2009 Chicago: SPSS Inc.). Separate one-way analysis of variance (ANOVA) was used to test effects of weaning treatments on larval survival, growth and jaw malformation. Tukey HSD post hoc analysis was used to determine differences between means when significant treatment effects were detected. All results were presented in mean ± SD and the level of significant difference was set at $P < 0.05$ unless otherwise stated.

5.4 Results

Survival

Survival rates in this experiment were low compared to results achieved in previous experiments ranging from 0.16 ± 0.22 % (W10) to 3.30 ± 0.85 % (W22). This was primarily attributed to an inappropriate counting procedure used during stocking of larvae that induced high mortality in all experimental tanks during the following 2 days. Regardless, survival at 33 DPH was significantly affected by the co-feeding and weaning schedules (Fig. 5.2). The highest survival rate of Yellowtail Kingfish larvae was observed in the treatment where co-feeding and the weaning protocol started on 22 DPH. Survival of larvae in this treatment was significantly higher ($P < 0.05$) than survival in the W10 and W13 (1.70 ± 0.60 %) treatments, but was not significantly higher ($P > 0.05$) than survival in the W16 (2.30 ± 0.69 %) or W19 (2.51 ± 83 %) treatments. Survival of larvae in the W10 treatment was significantly lower ($P < 0.05$) than in all other treatments.

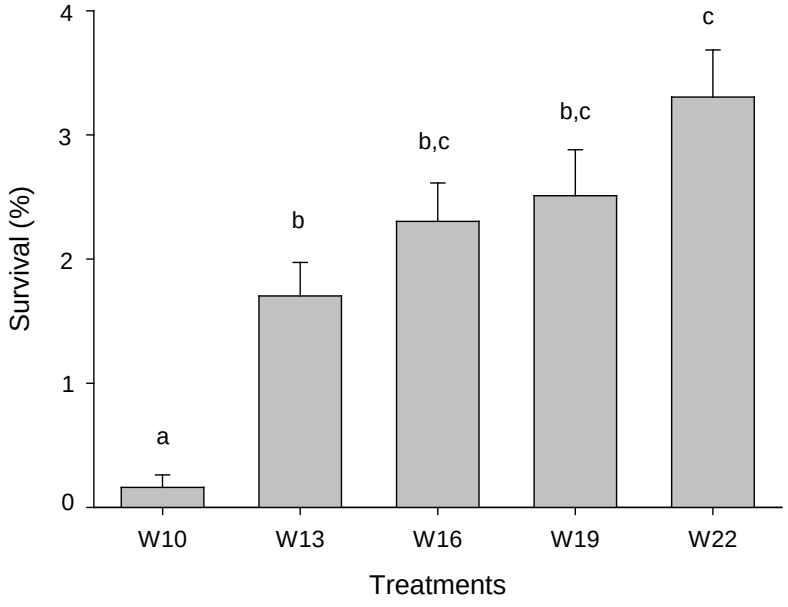

Fig. 5.2. Comparison of survival (mean ± SD, $n = 5$ replicates per treatment) until 33 DPH for Yellowtail Kingfish larvae reared under different co-feeding and weaning treatments. Treatments that share a common superscript are not significantly different ($P > 0.05$).

Growth

Growth of larvae to 33 DPH was significantly affected by the co-feeding and weaning treatments investigated (Fig. 5.3). Both body length (SL or TL) and dry weight (mg) of Yellowtail Kingfish larvae in treatments where co-feeding and weaning started on 16, 19 and 22 DPH were significantly ($P < 0.05$) longer and heavier than larvae in treatments where co-feeding and weaning started on 10 or 13 DPH. Using age and length data compiled from the best performing treatments (W16, W19 and W22) the following age and length relationship was generated:

$$\text{Length (mm)} = 2.5701e^{0.065x} \ (R^2 = 0.97, \text{x = DPH})$$

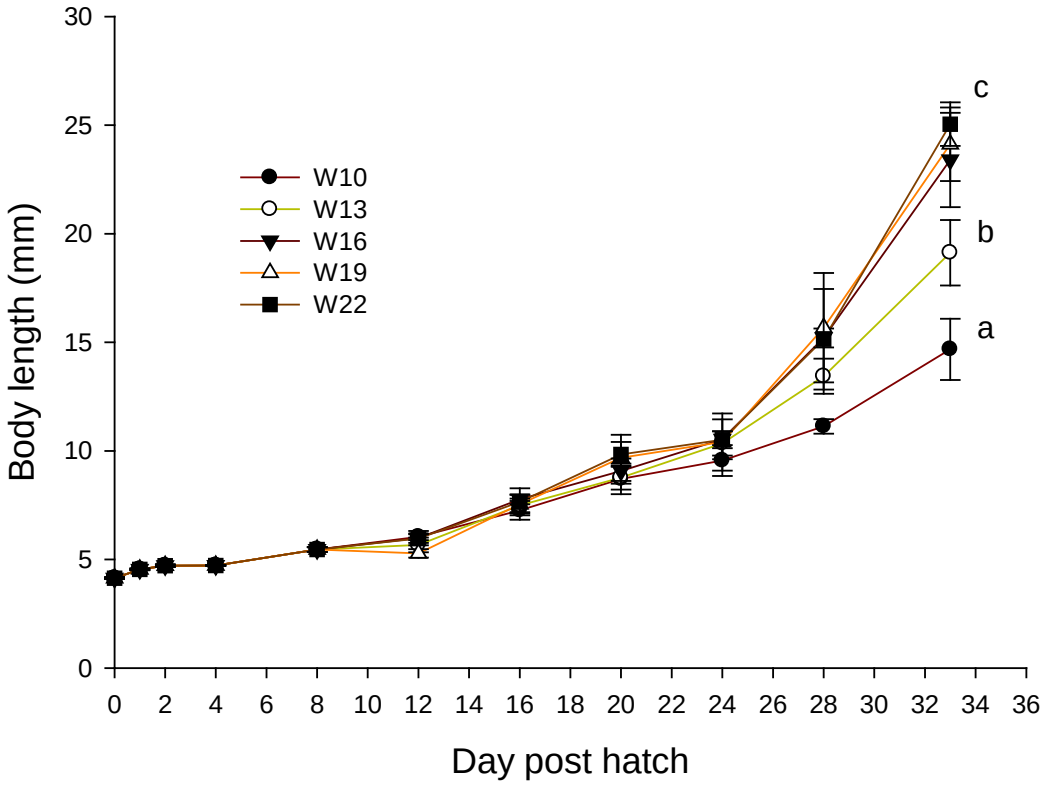

Fig. 5.3. Comparison of growth (SL before 21 DPH and TL after 21 DPH, mean ± SD, *n* = 5 replicates per treatment) of Yellowtail Kingfish larvae reared following different co-feeding and weaning treatments until 33 DPH. Values that share a common superscript are not significantly different ($P > 0.05$).

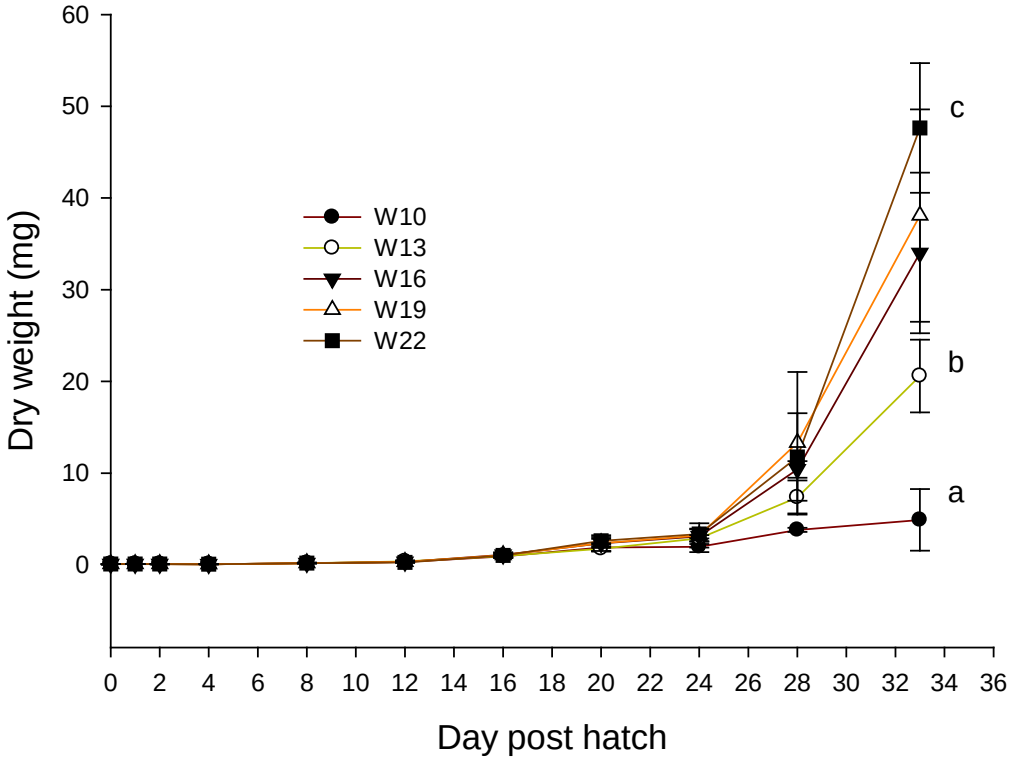

Fig. 5.4. Comparison of dry weight (mean ± SD, *n* = 5 replicates per treatment) of Yellowtail Kingfish larvae reared under different co-feeding and weaning treatments until 33 DPH. Values that share a common superscript are not significantly different (*P* > 0.05).

The AGR and SGR of larvae in treatment W10 were significantly lower (P < 0.05) than in the rest of the treatments, whilst there was no significant difference (P > 0.05) for AGR or SGR of larvae in treatments W13, W16, W19 and W22 (Table 5.1). The CV of larvae in the W10 and W13 treatments was significantly higher (P < 0.05) than those from the W22 treatment (Table 5.1) while the CV of larvae from the W16, W19 and W22 treatments were not significantly different (P > 0.05).

Table 5.1.

Absolute growth rate (AGR, mean ± SD, *n* = 5), specific growth rate (SGR, mean ± SD, *n* = 5) and individual size variation (CV, mean ± SD, *n* = 5) of Yellowtail Kingfish larvae reared under different co-feeding and weaning treatments up to 33 DPH. * = significantly different ($P < 0.05$).

Treatment	AGR (mm d^{-1})	SGR (% d^{-1})	CV (%)
W10	0.32 ± 0.04*	3.83 ± 0.29*	22.0 ± 1.9[a]
W13	0.45 ± 0.05	4.63 ± 0.23	21.5 ± 5.1[a]
W16	0.58 ± 0.07	5.24 ± 0.28	16.2 ± 3.1[ab]
W19	0.61 ± 0.05	5.33 ± 0.21	15.1 ± 1.6[ab]
W22	0.63 ± 0.03	5.45 ± 0.12	13.5 ± 2.4[b]

Development of the digestive system

In this study of Yellowtail Kingfish larvae cultured at 24.4 ± 0.2 °C, gastric glands were typically first observed at 15 DPH in larvae from all treatments. The gastric glands were composed of single type secretory cells beneath the epithelium between the cardiac and pyloric regions (Fig. 5.5). The gastric glands increased in numbers and clusters of gastric glands were found in the cardiac and fundic regions (Fig. 5.6). Meanwhile, rudimentary pyloric caeca were found on the anterior midgut (Fig. 5.6). By 18 DPH, the stomach had divided into cardiac, fundic and pyloric regions. As larvae grew, the fundic region was elongated and formed the largest portion of the stomach. The length and age relationship generated using data from larvae that were sampled from the three best performing treatments estimates that the length of larvae at this time (i.e. 18 DPH) was approximately 8.3 mm SL (Table 5.2). The number of SNVs in the hindgut decreased, whereas the number of gastric glands increased at 18 DPH, as observed in a previous study (Chen et al., 2006a).

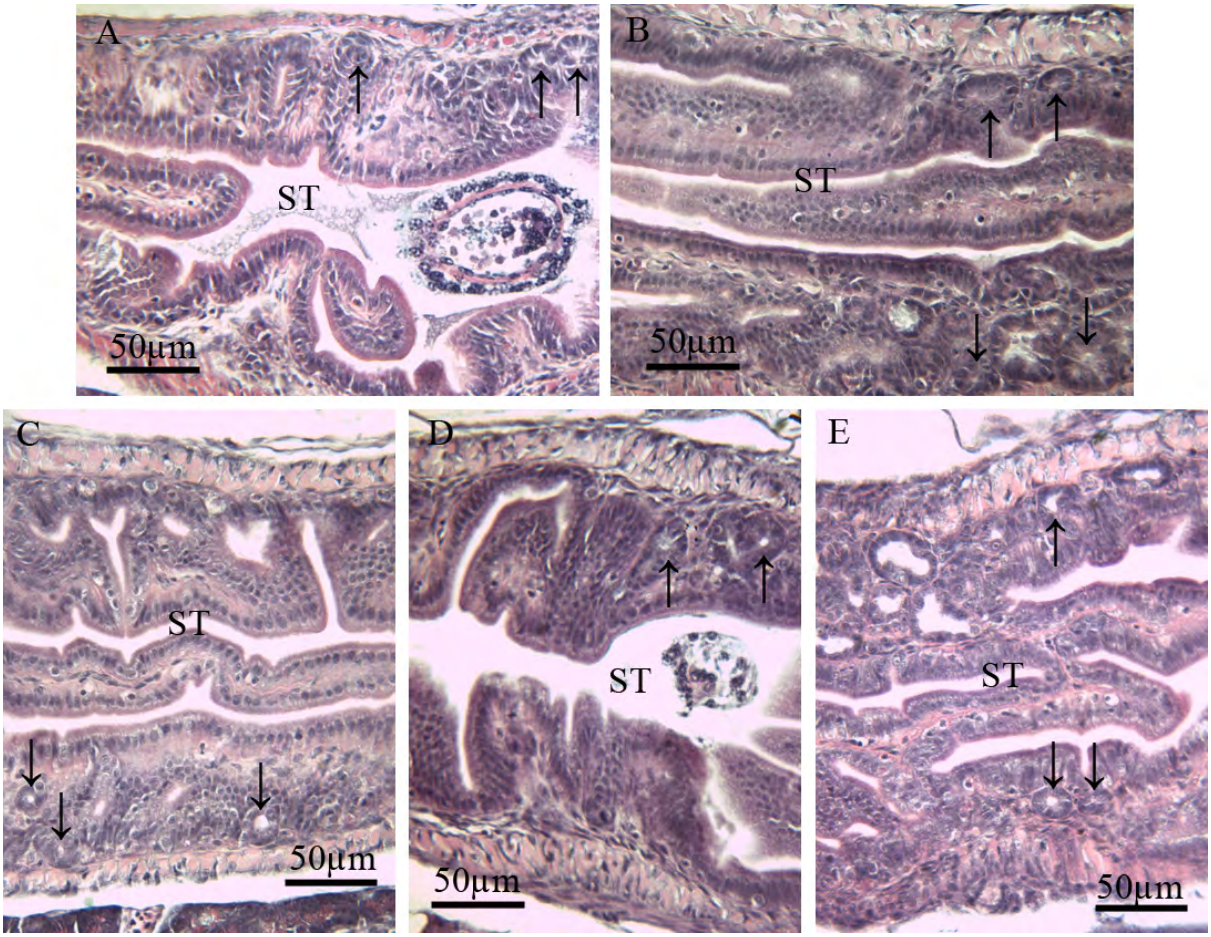

Fig. 5.5. Sagittal section of the stomach of 15 DPH Yellowtail Kingfish larvae. Note the gastric glands (arrows) in the stomach. A) A larva from treatment W10 that started co-feeding then weaning from 10 DPH. B) A larva from treatment W13 that started co-feeding then weaning from 13 DPH. C) A larva from treatment W16 that started co-feeding then weaning from 16 DPH. D) A larva from treatment W 19 that started co-feeding then weaning from 19 DPH. E). A larva from treatment W22 that started co-feeding then weaning from 22 DPH. ST = stomach.

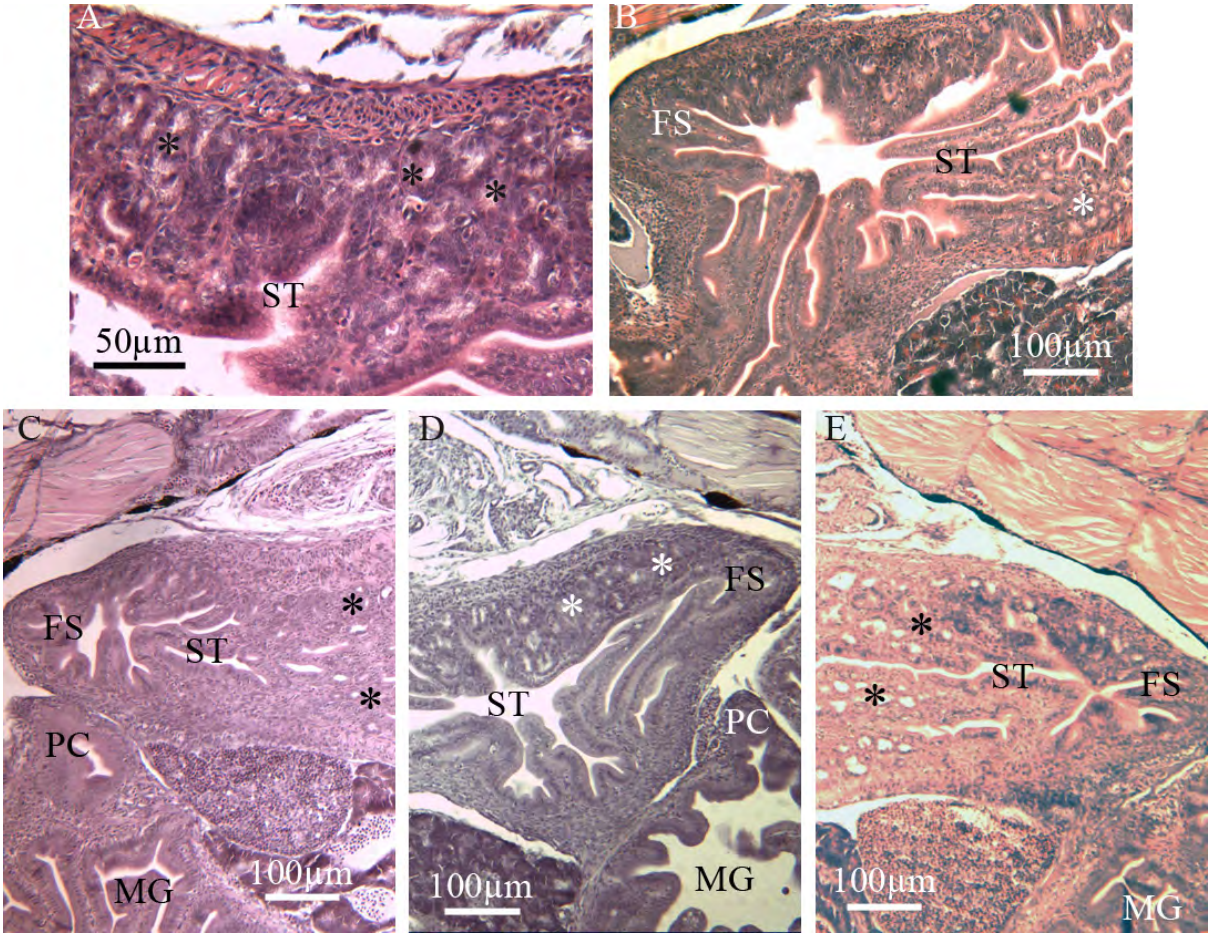

Fig. 5.6. Sagittal section of the stomach of Yellowtail Kingfish larvae on 18 DPH. Note the cluster of gastric glands (*) in the stomach. A) A larva from treatment W10 that started co-feeding then weaning from 10 DPH. B) A larva from treatment W13 that started co-feeding then weaning from 13 DPH. C) A larva from treatment W16 that started co-feeding then weaning from 16 DPH. D) A larva from treatment W19 that started co-feeding then weaning from 19 DPH. E). A larva from treatment W22 that started co-feeding then weaning from 22 DPH. FS = fundic stomach; MG = midgut; PC = pyloric caeca; ST = stomach.

Table 5.2.

Estimated length (SL or TL, mm) and age (DPH) of Yellowtail Kingfish larvae at the start of co-feeding and weaning; and at the end of weaning, for each treatment.

	Length (mm) and age (DPH) of larvae at the		
Treatment	**start of co-feeding**	**start of weaning**	**end of weaning**
W10	4.9 (10)	6.8 (15)	8.3 (18)
W13	6.0 (13)	8.3 (18)	10.1 (21)
W16	7.3 (16)	10.1 (21)	12.2 (24)
W19	8.8 (19)	12.2 (24)	14.9 (27)
W22	10.7 (22)	14.9 (27)	18.1 (30)

Jaw malformation

The proportion of fish with jaw malformation at 33 DPH was not significantly ($P > 0.05$) affected by any of the co-feeding and weaning treatments (Fig. 5.7).

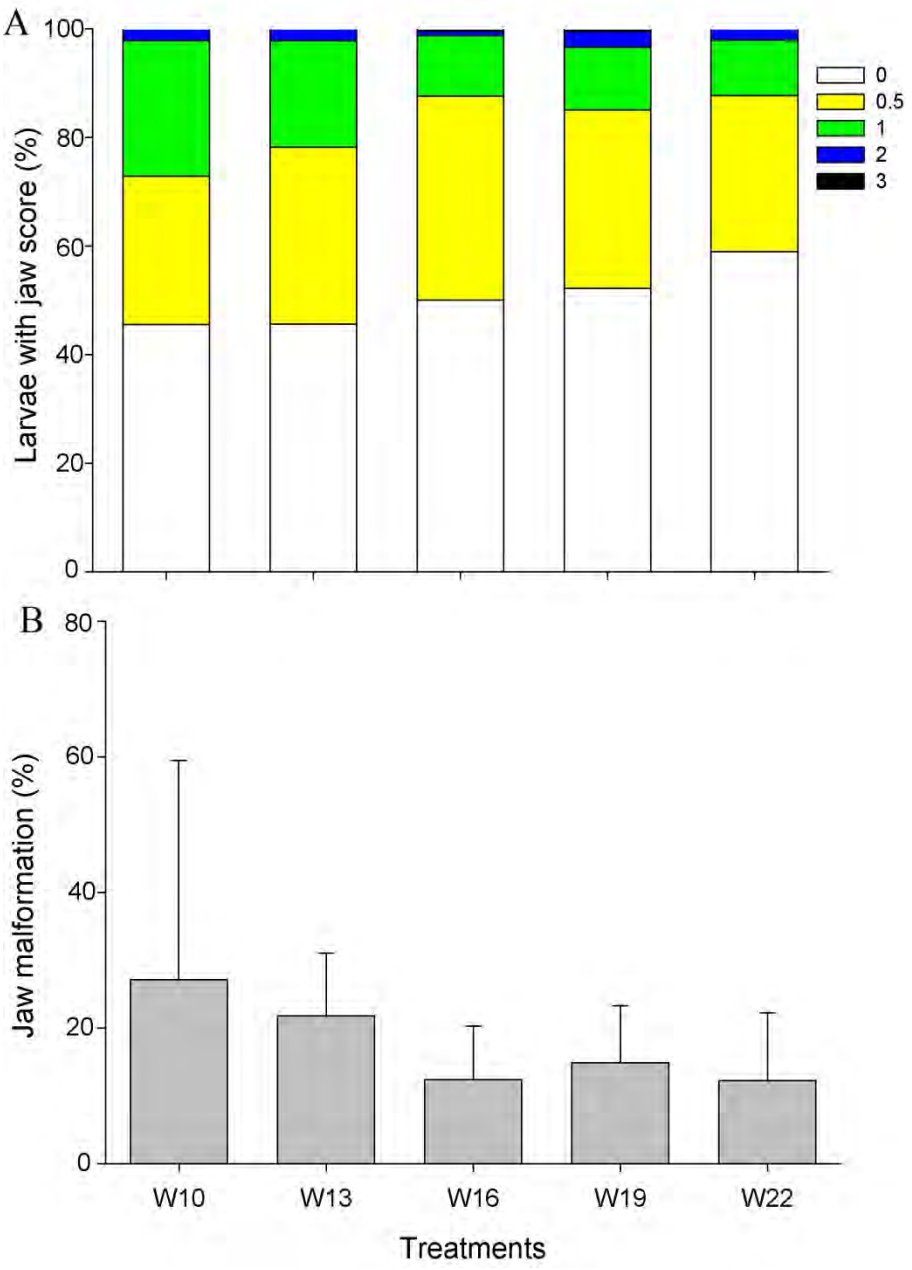

Fig. 5.7. Comparison of the proportion (mean % of 50 larvae per tank ± SD, *n* = 5 tanks per treatment) of Yellowtail Kingfish with jaw malformations on 33 DPH following different co-feeding and weaning treatments. (A) Larvae with jaw score of 0, 0.5, 1, 2 and 3, (B) Percentage of larvae with a jaw score >1, which is considered to be of commercial significance.

5.5 Discussion

For intensive culture of marine fish it is necessary for larvae to successfully make the change from the consumption of live food to a formulated diet to maintain the ever increasing demands for food intake and nutrition of large numbers of fish that are capable of growing at an exponential rate. High mortality can occur during the weaning process causing severe economic losses for hatcheries. Ideally, an appropriate weaning strategy will not only reduce the loss of production by avoiding mass mortality during this process, but will also enhance the growth and quality of fingerlings. In general, the earlier that fish larvae are able to effectively digest and assimilate microparticulate diets, the earlier and easier it is to wean fish onto these diets (Watanabe and Kiron, 1994). Early introduction of a microparticulate diet also has the potential to reduce the quantity of *Artemia* required, considerably lowering costs for cysts and reducing the labour required to maintain and prepare these cultures. This is particularly important for Yellowtail Kingfish that consume large amounts of food and have a fast growth rate.

For developing marine fish larvae the appearance of gastric glands actively secreting pepsinogen and hydrochloric acid are characteristic of a functional stomach (Segner et al., 1994; Huang et al., 1998; Douglas et al., 1999; Gawlicka et al., 2001) allowing metamorphosed juvenile fish to carry out an adult type of digestion (Baglole et al., 1997; Hamlin et al., 2000). In addition, the fundic stomach and pyloric caeca that develop during the same period provide a high capacity to store ingested food allowing efficient regulation of the passage of ingesta through the gastrointestinal tract to further improve the efficiency of digestion (Kvale et al., 2007; Kvale et al., 2009). Pepsinogen and hydrochloric acid activity were detected from 20 DPH in winter flounder, *Pleuronectes americanus,* (Douglas et al., 1999) after the formation of gastric glands implying the fish had acquired the adult digestive capacity from this time. The expression of genes for pepsinogen and the gastric proton pump that secretes hydrochloric acid were detected from 30 DPH in red porgy, *Pagrus pagrus*, (Darias et al., 2007). Faulk et al. (2007) detected the presence of gastric glands in the stomach of cobia larvae from between 8 and 12 DPH along with the gradual disappearance of intestinal acidophilic SNVs and an increase in pancreatic enzymes activity suggesting that larvae were able to digest and assimilate compound diets from this time. A later weaning study on cobia larvae confirmed this and showed that co-feeding of larvae from 8 DPH with microdiets achieved a significant positive effect on growth (Nhu et al., 2010). These studies provide support for the hypothesis that the morphological developmental of

the digestive tract is a good indicator of the appropriate time to commence co-feeding and weaning of Yellowtail Kingfish larvae.

Chen et al., (2006b) demonstrated that trypsin activity associated with intracellular protein digestion in the intestine of Yellowtail Kingfish larvae decreased at this time indicating a change to extracellular protein digestion within a functional (i.e. secreting) stomach. Previous research (Chen et al., 2006a) on Yellowtail Kingfish larvae cultured at 24°C has also shown that gastric glands developed in stomach around 15 DPH with the fundic region of stomach and pyloric caeca forming from 18 DPH, coinciding with the disappearance of acidophilic SNVs during this stage. The presence of SNVs in posterior intestine indicates intracellular digestion of protein macromolecules by pinocytotic absorption. Generally the disappearance of SNVs occurs simultaneous with the appearance of gastric glands and a subsequent increase in pepsin activity in the stomach (Govoni et al., 1986; Walford and Lam, 1993). In this study, gastric glands were firstly observed in the stomach for all the treatments on 15 DPH and increased in number as Yellowtail Kingfish larvae grew. Fundic region of stomach and pyloric caeca developed on 18 DPH and increased in size, whereas acidophilic SNVs decreased in number. These results not only confirm the previous observations on the ontogeny of the digestive tract of larval Yellowtail Kingfish, but also indicate that the function of the digestive tract depends on fish age (or size) rather than the time of onset of weaning.

In the current study, co-feeding and weaning of Yellowtail Kingfish larvae was successful from as early as 16 DPH without affecting the survival and growth as compared to the treatments that started at 19 DPH and 22 DPH. In all treatments a five day co-feeding period to habituate larvae to microparticulate diets preceded a five day weaning period during which the amount of *Artemia* was progressively decreased until only microparticulate diet was provided. Faulk et al (2007) suggested that larval development of cobia was more closely relate with length (SL) than age except for the first few days after hatching. Length of Yellowtail Kingfish larvae on each day can be estimated using the length and age relationship generated from larvae grown at 24.4 ± 0.2^{o}C and sampled periodically during this investigation. In the best performing treatments (i.e. W16, W19 and W22), larvae were between 10.1 mm and 14.9 mm TL when weaning proper started and were between 12.2 mm and 18.1 mm when weaning ended (Table 5.3). When the time of weaning proper is considered it is apparent that, for these treatments, the period of *Artemia* removal occurred

coincident with, or following, the development of a functional stomach in developing Yellowtail Kingfish larvae as gastric glands appeared and the stomach became fully functional in larvae during this period of development.

In the current study the early introduction of the microparticulate diet to Yellowtail Kingfish larvae at 10 DPH (W10) and 13 DPH (W13) resulted in significantly poorer growth and reduced survival compared to treatments that introduced the microparticulate diet from 16 DPH to 22 DPH. In addition, larvae from these treatments had significantly higher individual size variation (CV) than larvae that were offered microparticulate diets from 22 DPH (W22). Larvae that were offered microparticulate diet from 10 DPH or 13 DPH were estimated to be 6.8 mm and 8.3mm (SL) respectively at the start of weaning proper and 8.3 and 10.1 mm respectively at the end of the weaning period (Table 5.3). Histological observations suggest that at 10 DPH or 13 DPH, larvae in these treatments had not completed development of a functional stomach. These treatments can considered to represent early weaning treatments in which the reduction in *Artemia* was started before metamorphosis of the digestive system was complete. Similar results have been reported for early weaning of larvae of other marine fish species. Attempts to wean turbot *Scophthalmus maximus* larvae have proven difficult and have been only been successful from a time after the stomach has become functional with gastric glands formed and pepsin digestion has become functional during metamorphosis between 20 and 35 DPH (Segner et al., 1993; Segner et al., 1994). Starting weaning from 23 DPH prior to metamorphosis but following complete differentiation of the stomach from 20 DPH was successful with greenback flounder (Hart and Purser, 1996). It is suggested that the lack of gastric glands and presumably a functional stomach in larval yellowtail flounder up to and including day 29 may prevent weaning onto a commercial diet during the early larval stage (Baglole et al., 1997). The weaning of summer flounder *Paralichthys dentatus* larvae to artificial diets is best done as the fish approach and undergo metamorphosis around 35 DPH, and treatment with thyroid hormone does not improve the weaning of summer flounder before that stage (Bengtson et al., 2000). In addition, studies on other species indicate that early weaning generally produces inferior results when compared to maintaining live food until the stomach has differentiated and become fully functional. Survival of haddock, *Melanogrammus aeglefinus*, larvae maintained with live food was 37.9% up to 42 DPH compared with survival of between 2.5% and 6.3% for larvae exposed to early microparticulate diet weaning regimes from 14 until 35 DPH (Hamlin and Kling, 2001). Early weaning of barramundi larvae showed that the premature introduction

of microparticulate diet, even when co-feeding with live food, inhibited growth and this was attributed to the inability of larvae to efficiently digest the microparticulate diet before the stomach had begun to differentiate at 5 mm SL (Curnow et al., 2006). Early weaning with a microparticulate diet from 10 DPH resulted in reduced mean growth of turbot larvae compared to larvae fed live food organisms, a result frequently reported in other weaning trials with turbot and other marine fish species (Refs?). This information from other species, combined with histological observations and survival and growth recorded in the current study, suggests that weaning Yellowtail Kingfish earlier than 16 DPH (7.3 mm SL) cultured at 24^{o}C will result in poorer performance compared to starting weaning after the development of a fully functional stomach.

The ability of larvae to effectively utilise formulated diets is suggested to be increased by peptic digestion at an acidic pH allowing better assimilation of more complex protein from compound diets (Dabrowski, 1984; Govoni et al., 1986). This may also explain why fish larvae can ingest but not digest formulated diets from an early stage, evidenced by larvae having been observed to die with their digestive tract full of particles of formulated diets (Cahu and Zambonino Infante, 2001). Sea bass larvae weaned as early as 10 to 15 DPH were observed to suffer from malnutrition despite the fact that their intestinal tract was always full of the formulated diets (Cahu and Zambonino Infante, 1994). Alternatively, turbot larvae were successfully weaned with a formulated diet from 24 DPH before the foregut started to turn acidic on 28 DPH. This suggests that peptic protein digestion is not necessary for the successful utilisation of formulated diet, although the efficiency of digestion of formulated diets is probably increased by peptic digestion at an acidic pH (Hoehne-Reitan et al., 2001). It is suggested that microparticulate diets can slightly delay the onset of the maturation process of gut enterocytes, and inadequate diet can prevent it, leading to death of larvae (Cahu and Zambonino Infante, 2001). In addition, the lower availability of proteins from formulated diets compared with live food is considered to be an important reason for the low performance of formulated diets (Kvale et al., 2007; Kvale et al., 2009).

In this study, comparison of the incidence of jaw deformity showed that there was no significant effect of co-feeding and weaning treatments on Yellowtail Kingfish assessed at 33 DPH. This suggests that either the co-feeding and weaning treatment has not influenced the incidence of deformity, or alternatively, the higher variability of jaw deformity recorded between replicate tanks in the lower performing larvae offered microparticulate diet from

either 10 DPH or 13 DPH, has not allowed the effect of these treatments to be discriminated. In many fish species early weaning usually leads to poor survival and growth and inferior quality larvae with an increased risk of skeletal deformities (Baskerville-Bridges and Kling, 2000a; Cahu and Zambonino Infante, 2001; Hamlin and Kling, 2001). Early weaning preceded by a short co-feeding period between 17 and 23 DPH resulted in a high incidence of spinal deformities of southern flounder compared to fish that had been weaned later following a longer co-feeding period until 29 DPH (Faulk and Holt, 2009). Although full replacement of *Artemia* from first feeding is possible for sea bass, early weaning onto manufactured diets risks a lower survival rate, growth retardation and lower juvenile quality, including greater size variability and skeletal abnormalities (Person Le Ruyet et al., 1993). A similar increase in size variability between larvae from early weaning treatments was recorded for Yellowtail Kingfish in this study suggesting that inadequate procedures for introduction of microparticulate diets to developing larvae may influence the quality of fingerlings produced from intensive rearing procedures.

5.6 Conclusion

The results of the current study indicate that Yellowtail Kingfish larvae can be changed from a live food to an artificial diet as early as 16 DPH (7.3 mm SL), starting with five days of co-feeding followed by a five day weaning period, without significantly reducing survival, growth and increasing the incidence of jaw deformity. Histological observations confirm that from this time the transition from live food to microparticulate diets coincides with the time when the digestive tract of Yellowtail Kingfish larvae differentiates and becomes fully functional as confirmed by the appearance of gastric glands in the stomach and the disappearance of acidophilic SNVs in the intestine. It is suggested that the introduction of microparticulate diets before 16 DPH may result in significantly reduced growth, reduced survival and greater size variation attributed to introduction of more complex diets before the stomach in developing Yellowtail Kingfish larvae has become fully functional.

6. Further Development

The approach used in this series of trials has provided significant improvements of our understanding of YTK larval development and some insights as to the culture conditions that will favour high survival, low malformation rates, faster growth and more efficient and consistent hatchery production. Despite these advancements, there are so many more questions that need to be addressed. These include:

- The impact of water temperature during egg incubation and hatching on future larval survival and malformation rates
- The impact of photoperiod and light intensity on walling behaviour and the incidence of jaw malformation, and role of light as a cue for swimbladder inflation behaviours
- Further questions can be asked about live feeds, a comparison of live algae vs concentrated algal paste for green water or for feeding rotifers, the effectiveness of different nutritional enrichment products for live feeds, the benefits of disinfection of live feeds before they are introduced to the larval rearing tanks
- The effects of different approaches for creating hydrodynamic circulation in the rearing tanks, the potential benefit of increased structure/floating covers on larval distribution, the importance of oil skimming on swimbladder inflation.

There is no doubt that poor survival rates and high rates of larval malformation continue to constrain the efficiency and economics of YTK aquaculture. These issues must be addressed to create a financially sustainable industry based on this high quality product. The collaborative approach used in this project has brought many benefits and rewards, the continuation of this arrangement will help to solve these issues sooner.

7. Conclusion

This component of the overall project has shown that:

- To reduce mortality rates and the incidence of jaw malformation water temperatures need to be less than 23^{o}C for several days post-hatch, but after 18 DPH the temperature can be increased to 25^{o}C. The larvae appear to develop a greater tolerance to the higher water temperatures over time, and the warmer temperatures seem to improve larval growth.
- Rotifer densities of 20-25 mL^{-1} from 2 DPH were best for larval survival (up to 28%) when larvae were stocked at 60 L^{-1} (compared to the commercial stocking rate of ~100 L^{-1}). A regime whereby rotifer density was increased from 15 mL^{-1} at 2 DPH, to 40 mL^{-1} at 10 DPH, also resulted in a high survival rate (29%) of YTK larvae. The incidence and extent of jaw malformations was high (50-65%) and not significantly affected by the different rotifer density treatments. A high constant rotifer density of 40 mL^{-1} proved to be deleterious to larval survival.
- Constant *Artemia* densities between 15 and 35 mL^{-1} from 12 DPH resulted in high survival rates (up to 56%) but with no differences between the densities tested. As was found with rotifers, a high *Artemia* density (45 mL^{-1}) was deleterious to larval survival. There was no benefit of persisting with a low density (5 mL^{-1}) of rotifers out to 20 DPH. No *Artemia* density treatment evaluated in this project made a substantial change to the overall high incidence of jaw malformation (77-95%).
- The best weaning outcomes (3.3% survival) were observed when microparticulate diet was introduced after 22 DPH. This was several days after the larvae were observed ingesting the diet, and structural and functional developments of the gut have occurred. Later weaning times also tended to lower the incidence of jaw malformations of commercial significance (to as low as 10%). Attempts to undertake early weaning of YTK larvae in this system are likely to have deleterious consequences for production performance.
- R&D must continue, both on-farm and in specialist facilities, to resolve the current situation whereby YTK larval survival and juvenile quality are too low.
- Information from this project on the optimal water temperature, live feed densities and feeding durations, and weaning strategy has been incorporated into the Standard Operating Procedures (SOPs) used by CST YTK hatcheries.

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Appendix 1 – Intellectual property

Some intellectual property was derived from this project. This IP is in the details of changes made to a number of existing, and the creation of several new SOPs for use in the CST YTK hatcheries. These SOPs are commercial-in-confidence to CST.

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Improvements in Yellowtail Kingfish Larval Survival and Juvenile Quality

Appendix D – Darwin Aquaculture Centre Component

Dr Glenn Schipp and Dr Jérôme Bosmans

Project No. 2009/749.30

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

DEPARTMENT OF
PRIMARY INDUSTRY, FISHERIES AND MINES

project was conducted by Northern Territory Government, Department of Primary Industry, Fisheries and Mines

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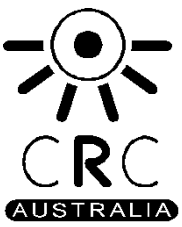

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Abbreviations

CST	Clean Seas Tuna Ltd
CT	contact time (for ozonation; calculated as exposure in ppm x time in mins)
DAC	Darwin Aquaculture Centre
DPH	days post-hatch
ppm	parts per million
ppt	parts per thousand
SOP	standard operating procedure
TAFI/IMAS	Tasmanian Aquaculture and Fisheries Institute / Institute for Marine and Antarctic Studies
YTK	Yellowtail Kingfish

Non-Technical Summary

2009/749.30 – Improvements in Yellowtail Kingfish larval survival and juvenile quality (DAC component)

INVESTIGATORS: Glenn Schipp and Jérôme Bosmans

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NON TECHNICAL SUMMARY:

Changes to Northern Territory legislation were made and new aquatic biosecurity policies and procedures were developed to permit the importation of Yellowtail Kingfish (YTK) eggs from interstate hatcheries. Following this, three separate batches of YTK eggs were received by the Darwin Aquaculture Centre hatchery in 2009.

Improvements in egg transportation practices were made in successive shipments. The best quality eggs were received from Challenger CIT, and whether this was due to broodstock factors, the shorter transport duration , the lower number of eggs/bag, the cooler water temperature and/or the lower dissolved oxygen concentration compared to the SA shipments cannot be determined.

As with egg transportation, the outcome of each successive egg incubation improved with each batch of eggs. Providing sufficient aeration to prevent the eggs from settling on the bottom of the tank, but not so much that it could cause mechanical damage to the eggs, developing larvae and hatched larvae, proved to be critical.

Survival of larvae after hatch was poor, with high rates (about 95%) of mortality from hatch through to 9 days post-hatch. Whether this mortality was caused by shortcomings during the egg transportation and incubation phases, or due to inappropriate biotic (live food prey type, density, or nutritional quality) and/or abiotic (water quality, light regime) parameters during larval rearing is not known.

The DAC feeding regime of live rotifers (10-40 rots mL^{-1}) from 2 DPH to at least 14 DPH, *Artemia* nauplii (at up to 1 mL^{-1}) from 7 to 20 DPH, and co-feeding with microparticulate feeds from 15 DPH to weaning by 20 DPH, appeared to be successful for at least some

of the larvae. Thus, despite the numerous difficulties experienced during the project, several thousand larvae from runs 2 and 3 were still alive at 21 and 26 DPH, respectively. The surviving larvae, however, showed high rates of jaw (>30%), spinal (about 40%) and swim bladder malformations (detailed data are in the IMAS sub-project report). The high mortality rate and incidences of jaw and swim bladder malformation are consistent with the experiences of other YTK hatcheries in Australia and New Zealand.

The challenges encountered during the first 3 runs with YTK have not provided sufficient numbers of larvae to fully evaluate the potential of the DAC intensive larval rearing system through this project. There were substantial improvements in progress made during the 3 runs, and additional opportunities to work with YTK eggs and larvae in the system would progress our understanding of the causes of mortality and deformities, and consequently provide rearing conditions that optimize larval survival and juvenile quality.

KEYWORDS: Yellowtail Kingfish, aquaculture, larval rearing, live feeds, swim bladder, jaw malformation

1. Background

This sub-project describes the work done by the Darwin Aquaculture Centre (DAC) in association with the CST hatcheries at Arno Bay and Port Augusta and project collaborators at TAFI (now IMAS). Separate reports are provided by CST and IMAS for their respective components of the project and will be cross-referenced as appropriate.

The work described in this report was carried out in the 2008 and 2009 YTK spawning seasons, therefore the procedures described herein reflect the YTK and DAC larval rearing practices at that time. As a result of this project, and the others carried out by our CRC collaborators, many of the YTK hatchery practices and procedures will since have been modified as necessary. In this way, the hatchery manuals and SOPS developed by CST and DAC are very much 'living documents'.

Survival rates of YTK from hatched larvae to weaned juveniles is very low (~5-10%) in comparison to many other marine species produced in larger more mature industries, for example the sea bass and seabream sectors in Europe. Mortality in the hatchery stage can be classified into a number of categories, the most common of which are deaths in the tank and culls due to deformities. Of particular note, several skeletal malformations particularly of the spine, jaws and opercula, have been reported in Australian and New Zealand marine hatcheries, although few are well documented (Cobcroft *et al*., 2004), with a classification system completed for YTK jaw malformations in a recent CRC project (Battaglene and Cobcroft, 2008).

A number of biotic and abiotic factors have been shown to be associated with higher levels of mortalities and deformities at the hatchery stage in other species, and it seems likely that some of these factors are also likely to be important for YTK larval rearing success.

The DAC hatchery has developed a very reliable intensive larval rearing system for marine finfish (Bosmans et al., 2005; Curnow et al., 2006). This system operates with the continuous addition of live feeds (rotifers) supplied from an intensive production system; and continuous maintenance of "green water". Both components of the system use algal paste. This innovative system has never been used for larval rearing of Yellowtail Kingfish larvae. Early weaning was also investigated using co-feeding with microdiets and a simple semi-automated feeding device as DAC has considerable experience with this approach used for commercial barramundi culture.

In this way, the DAC component specifically addressed Objective 2 of the project:

- To assess the suitability of some novel larval and juvenile rearing techniques for YTK: recirculating intensive larval rearing system with semi-automatic feeding, artificial light, algal paste, small rotifer, high prey density and early weaning.

1.1 Need

The costs of producing juvenile YTK which are fit (i.e. no or low level of deformity) to be stocked at sea for growout need to be reduced. Currently, the low survival rates and high deformity rates of YTK larvae and juveniles is constraining the growth and profitability of this industry in price-sensitive markets. This project aims to determine the factors that contribute most to the low survival and high deformity rates, and to seek industry-relevant ways in which to address them.

1.2 Objectives

1. To identify improvements to be made to commercial scale YTK larval and juvenile rearing systems and procedures resulting in higher survival (> 25% by end 2010), better growth, reduced levels (<5% by end 2010) and severity of malformations and more cost efficient juvenile production.
2. To assess the suitability of some novel larval and juvenile rearing techniques for YTK: recirculating intensive larval rearing system with semi-automatic feeding, artificial light, algal paste, small rotifer, high prey density and early weaning.
3. To test a range of key biotic and abiotic factors and rearing strategies on YTK larvae and juveniles in replicated tanks and identify optimal regimes for adoption in commercial scale hatcheries.

2. Methods and Results

In order for this component of the project to proceed two key barriers had to be overcome; they were:

1. Legislation had to be enacted by the Northern Territory Government to allow translocation of YTK eggs to the Darwin Aquaculture Centre
2. Protocols for egg transport, health testing and biosecurity had been agreed and implemented.

This report will not go into the detail of how each of these activities was achieved, but the appropriate legislation and protocols were created in order for the experimental work to progress (Appendix 3 and Appendix 4).

2.1 Hatchery Runs

A total of 3 batches of YTK eggs were sent to the Darwin Aquaculture Centre during the course of this project, the first two batches were sent from CST's Arno Bay hatchery, the third was sent from Challenger Institute of Technology (CIT), Fremantle, Western Australia (Table 1). Whilst the experienced DAC hatchery staff had planned and anticipated for many of the biotic and abiotic culture requirements of the YTK eggs, the actual egg incubation and larval rearing activity was still a learning process. Some of the lessons were learned by the hatchery staff in how to send the eggs such that they would arrive in better condition, other lessons were learned by the hatchery staff in knowing how to better handle the eggs and larvae during the incubation and rearing phases, respectively. This occurred as a result of each run, and as such the procedures and protocols used by the researchers 'evolved' over successive runs. As a result, the poor outcomes of hatchery run 1 were subsequently improved upon during hatchery runs 2 and 3.

Table 1. Egg batches received by Darwin Aquaculture Centre during this project.

	Hatchery run		
	1	**2**	**3**
Date	14th Jan 2009	26th Jan 2009	28th Jul 2009
Source Hatchery	Arno Bay	Arno Bay	Challenger
Number of eggs	960,000	1,450,000	400,000
Transport Duration (h)	24	15	12
Egg diameter (µm)	1,377	1,428	1,429
Oil droplet diameter (µm)	303	345	345
Fertilisation (%)	73.6	83.6	100
Development stage	Early embryo	late embryonic, blastula stages and some live larvae	neurula to very early embryo stages

During the egg and larval rearing phases of the YTK hatchery runs a number of water quality parameters were monitored including temperature (mercury thermometer), pH (TPS pH meter), dissolved oxygen concentration (Oxyguard oxygen meter), salinity (refractometer) and the concentrations of ammonia and nitrite (HACH testing kit, colour wheel method).

2.2 Egg Transport

The YTK eggs sent from Arno Bay arrived in six insulated boxes each holding approximately 160,000 eggs. Five sealed bags were immediately transferred into two 1,000 L incubation tanks for temperature acclimatization and the sixth bag was opened to check water parameters, development and fertilisation rate and bacterial analysis; before refilled with oxygen, resealing and transfer to an incubation tank (Table 2).

Table 2. Water parameters in transport bags received at Darwin Aquaculture Centre. DO = dissolved oxygen.

Parameter	Run 1	comment	Run 2	comment	Run 3	comment
Temperature (^oC)	21.5		21.8		17.7	reduced
Salinity (g L^{-1})	37.0		37.0		38.0	
DO (mg L^{-1})	30.5	very high	21.6	very high	7.2	good
DO saturation (%)	416	very high	303	very high	108	good
pH	7.05	Low	7.54	reduced	7.52	reduced
N-NH_3 (mg L^{-1})	13.2	very high	9.6	very high	0.6	slightly elevated
Un-ionised NH_3 (mg L^{-1})	0.06	Low	0.11	elevated	0.008	low

On all occasions the sealed bags were left to acclimate to the temperature in the incubation tanks for one hour after which they were opened. For both batches of eggs that arrived from CST the water was cloudy, and the first batch had a strong 'fishy' smell but this was not as pronounced in the second batch. In comparison, the water quality parameters in the 3rd batch of eggs (provided by Challenger CIT), were much less extreme on arrival and the water appeared clean and without odour.

The eggs in each batch were pooled in a 150 μm hand net and thoroughly rinsed in UV treated seawater. The eggs were then ozonated at a CT of 0.86 – 0.99 and rinsed again with the same UV treated seawater supplied to the hatching tanks. The rinsed and disinfected eggs were stocked into two 250 L or 1,000 conical hatching tanks.

2.3 Egg Incubation

Water quality conditions during each of the runs are shown in Table 3.

Run 1: During incubation, aeration was provided by a central 250 μm screen and 3 air stones half way from the bottom of the tank. The aeration provided by this set up was gentle and not sufficient to keep the eggs in suspension as the eggs were observed to quickly settle on the bottom of the tank.

Run 2: The intention in this run was to provide a greater degree of water movement than was produced in run 1, so this time aeration was supplied around the central 250 μm screen but with one additional air stone and two open 4 mm air lines located on the bottom of the tank. This system generated a strong level of aeration sufficient to keep the eggs in suspension at all times.

Run 3: Water supply for the incubation tanks in this run was flow through and was sand filtered, then passed through a temperature control unit followed by a UV unit, biofilter and protein skimmer. The new water came in from the bottom of the tank providing an upwelling effect, with an exchange rate of 100% h^{-1}. During incubation, water was very lightly aerated in the centre of the tank. In this system, the eggs remained suspended in the water column even when aeration was turned off.

Table 3. Parameters for larval incubation of Yellowtail Kingfish eggs incubation; and following hatching at Darwin Aquaculture Centre. DO = dissolved oxygen.

Parameter	Run 1	Run 2	Run 3
Ozone dose (CT)	0.88	0.96	0.86
Water temperature (°C)	24.5	24.2	21.5
Salinity (g L^{-1})	33.0	33.0	39
DO (mg L^{-1})	8.0	6.6	7.0
DO saturation (%)	110	96	100
Ammonia - unionised (NH_3; mg L^{-1})	0.03	0.01	0.03
Water flow (% h^{-1})	25 - 30	25 - 30	100
Hatch rate (%)	Very low	21.0	78.5

2.4 Hatching Outcomes

Hatching outcomes from each of the runs are shown in Table 4.

Run 1: After overnight incubation some of the eggs were found to have hatched, with more hatching but in a desynchronised manner during the day. It was observed that a significant proportion of eggs had stopped their development at late embryonic stage and most of the newly hatched larvae were dead and curled.

Run 2: The eggs started hatching early the following morning and kept hatching throughout that day. On the morning of the second day, the hatched larvae were separated from the non viable eggs, egg shells and un-hatched viable eggs, and the level of aeration was reduced. Later that afternoon 175,000 live larvae were transferred to one 6,000 L recirculated larval tank using gentle scooping with an 8 L basin. The larvae were left in water at all times and the water quality between the incubation tanks and the larval tank were identical.

Run 3: The eggs started twitching the following morning, and the first larvae were observed at 1:00 pm. The next day, the hatched larvae were separated from the egg

shell and un-hatched viable eggs by dumping water from the bottom of the hatching tank and by using skimmers. Larvae were relocated into a 6000L larval rearing tank by hand using jugs and small hand basins ensuring the larvae were in water at all times. The water quality parameters of the larval rearing tank were as close as possible to the incubation tank prior to larval relocation.

Table 4. Characteristics of Yellowtail Kingfish larvae following hatching at Darwin Aquaculture Centre.

Parameter	Run 2	Run 3
Larvae length (mm) at hatch	4.31	4.77
Viable larvae @ 1DPH	175,000	342,500
Viable larvae @ 1DPH (%)	12.0	78.5
Larvae length (mm) at 1DPH	4.4	5.01

2.5 Larval Rearing

The larval rearing conditions used in runs 2 and 3 were generally the same, but with some minor variations as described below.

Biotic and abiotic conditions

At ~1.5 DPH, YTK larvae were stocked into one 6,000L larval rearing tank (run 2 stocked at 29 larvae L^{-1}; run 3 at 56 larvae L^{-1}). Concentrated *Nannochloropsis sp* (Instant Algae®, Nanno 3600™) and *Isochrysis* strain *T-Iso* (Instant Algae®, Isochrysis 1800™; both from Reed Mariculture Inc., 520 East McGlincy Lane, #1 Campbell, CA 95008, USA) were used to maintain 'green water' at a density of 1 - 5 x 10^5 cells mL^{-1} from 2 DPH until 14 DPH (run 2) or 20 DPH (run 3).

Rotifers (SS strain) were cultured intensively in two 1,000 L recirculated systems continuously supplied with DHA and EPA enriched microalgae concentrate (Super fresh Chlorella V12, Pacific Trading Company Co. Ltd., 810 0074, New Life Ohiri Building, 1 – 3 Otemon 3 – Chome, Fukuoka, Japan). From 2 DPH until 20 DPH, rotifers were continuously fed to YTK larvae at a density of 20 - 40 rotifers mL^{-1} (run 2), or 10-40 mL^{-1} (run 3)(Figure 1).

Artemia were given once a day at a maximum density of 1 nauplii mL^{-1} from 10 DPH until 14 DPH (run 2), or 2 - 3 times a day at a maximum density of 3.0 nauplii mL^{-1} from 10 DPH until 20 DPH (run 3).

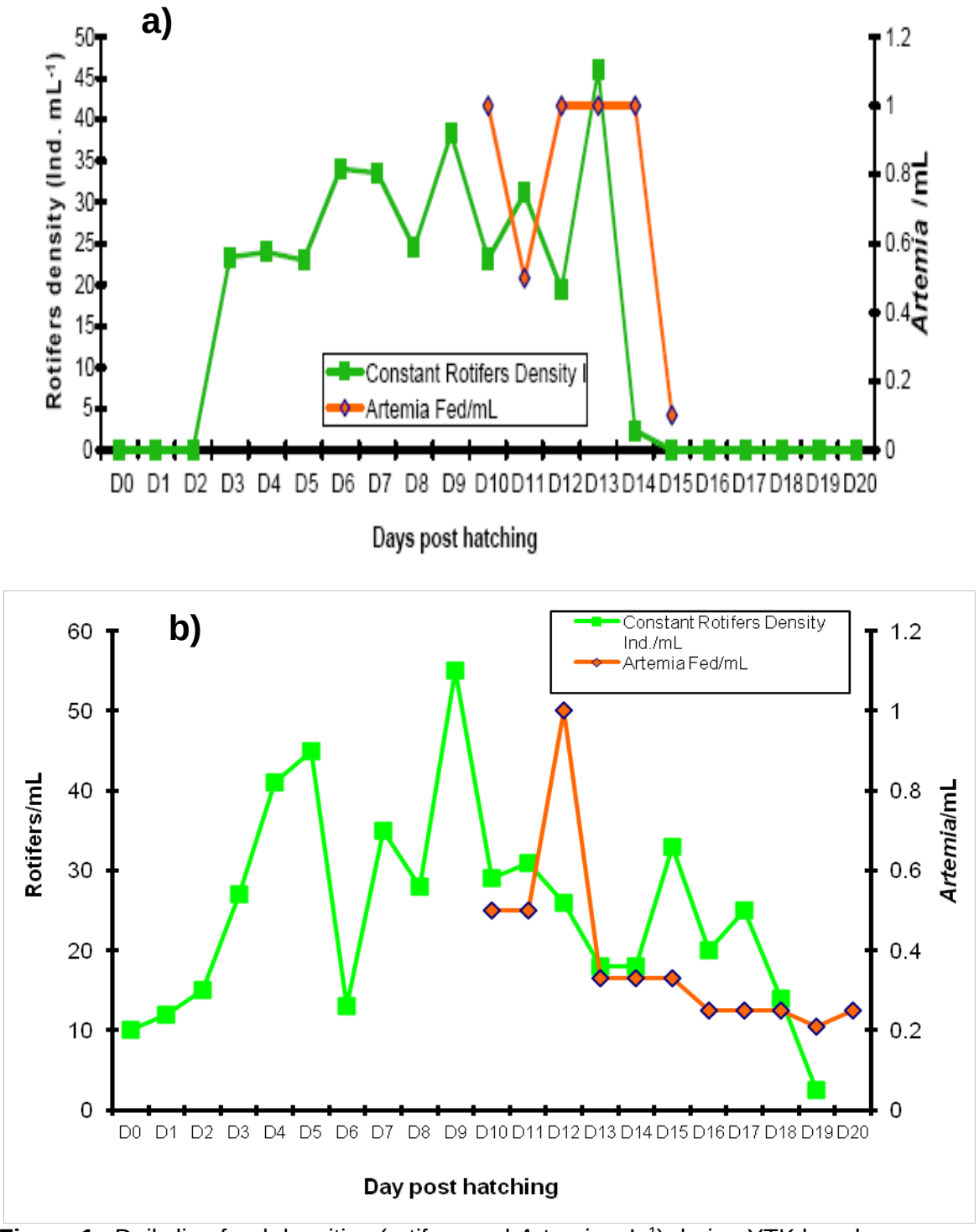

Figure 1. Daily live food densities (rotifers and *Artemia* mL^{-1}) during YTK larval (a) Run 2 and (b) Run 3 at DAC.

Co-feeding of a manufactured microparticulate diet with the live feeds commenced at 7 DPH (run 2) or 8 DPH (run 3) with Otohime A (150-250 µm) (Marubeni Nisshin Feed Co., Japan) and then Otohime B1 (300-500 µm). Observations of larvae under a microscope indicated that most larvae were ingesting the micro-diet at between 14 DPH and 16 DPH and the larvae were considered to be weaned by 20 DPH.

The 6000L larval rearing tank operated as an upwelling system using a recirculating water supply. Sea water for the system was sand filtered, run through a temperature control unit followed by a UV unit, biofilter and protein skimmer. The photoperiod used was 12 hours light and 12 hours dark. Water quality parameters were managed within the ranges shown in Table 5.

Table 5. Water quality characteristics in larval rearing tanks at DAC.

	Run 2	**Run 3**
Water temperature	25.0 - 26.0	21.6 - 24.2
pH	7.6 - 7.9	7.35 – 8.25
Dissolved oxygen mg L^{-1}	5.3 - 8.4	5.6 – 8.5
Salinity g L^{-1}	33 – 34	35 – 40
water exchange rate 2-14 DPH	12-20	17 – 19
Water exchange rate 15 DPH – end	20 -25	20 - 58
Ammonia, nitrite and total dissolved gases	negligible	negligible

The larval rearing tank was not vacuumed during the first week to prevent the loss of non free-swimming larvae. Daily siphoning started at 8 DPH after the first day of co-feeding with artificial food.

Run 2 was terminated at Day 20 and it was considered that at that stage, most of the remaining larvae were weaned. Run 3 continued to 26 DPH.

2.6 Larval Performance

Larval length was determined by observations of 20 larvae each day from 1 DPH until 15 DPH using an Olympus dissecting microscope (SZAD60), a graduated lens and the appropriate conversion factor for the microscope. The percentage of larvae with inflated swim bladders was also recorded on these days. When high mortality was observed samples of moribund larvae were sent to Berrimah Veterinary Laboratory for investigation of pathology. Routine samples of larvae were also taken and sent to TAFI/IMAS for biochemistry and histological assessment of malformations.

2.6.1 Run 2

YTK larvae grew to approximately 10mm (Figure 2a) during the three week larval rearing trial. Swim bladder inflation rate was inconsistent (ranging between 25 to 100% from 3 DPH to 10 DPH), and was 85% at 10 DPH. It was observed that pigmentation of "normal" feeding larvae was black and stressed larvae tended to be transparent or grey. Apart from these transparent larvae, most larvae had full stomach from 3 DPH onwards.

Larval survival (Figure 2b) was estimated by taking into account the cryptic mortality over the first week. A significant period of high mortality was observed between 7 DPH and 8 DPH. The mortality observed during the remainder of the larval rearing period was more accurately assessed than earlier estimates because the dead larvae that were vacuumed from the tank each day were enumerated.

The trial was terminated at Day 20 and it was considered that at that stage, most of the remaining larvae (just under 3,000) were weaned. Initial assessment of a sample of these fish confirmed that a significant proportion were malformed, with a high incidence of jaw (44%) and spinal (>30%) malformations. Details of the types and extents of the jaw and spinal malformations are provided in the IMAS component of the final report. Significant notochord (spinal) malformations were observed from Day 1, supporting the other evidence such as low hatching rate, that the batch was compromised before larval rearing commenced.

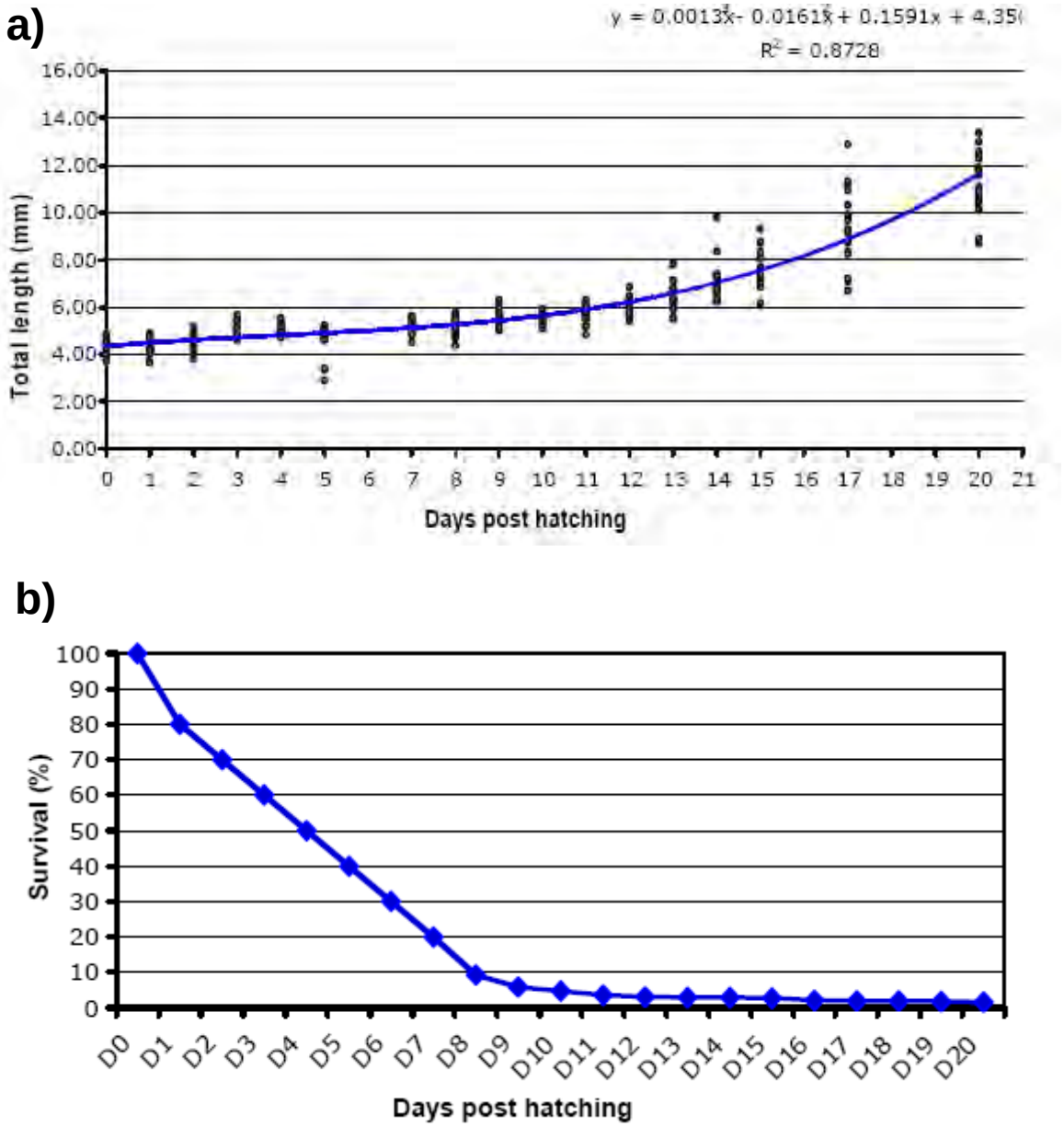

Figure 2. Growth (total length; mm) and estimated survival (%) of Yellowtail Kingfish larvae cultured in a recirculating seawater larval rearing system at the Darwin Aquaculture Centre. Run 2.

2.6.2 Run 3

Over the 26 day trial period at DAC, the YTK larvae in run 3 grew an average of 6.2mm, with lengths ranging between 4.45mm at hatch and 17.29mm on 26 DPH (Figure 3a). There was a 15% loss in larvae to 4 DPH, followed by a loss of about 70% between 4 and 9 DPH (Figure 3b). This period coincides with the depletion of the yolk sac in the larvae and is most likely to be related to their weaning onto live feeds. The YTK did not seem to wean onto the rotifers as well as was hoped. Observations made using the microscope suggested that the larvae fed on the rotifers during the first few days, but then seemed to go off them. The continuous feeding of rotifers may have also increased the levels of bacteria in the larval tank affecting larval survival. After 9 DPH, the rate of larval mortality was low.

As was observed in run 2, the colour of larvae in run 3 ranged from transparent to dark with silver sides by 26 DPH. The dark coloured larvae were larger and had fuller stomachs when compared with the transparent larvae. The dark larvae were also observed to harass the transparent larvae which appeared to be weak and dying. Comments recorded on 15 DPH and 16 DPH suggest larvae displayed a golden colour when fed on artificial feed.

The rate of swim bladder inflation varied greatly between sampling days and ranged between 5% on 5 DPH and 80% on 15 DPH. There was not a consistent increase in the inflation rate of swim bladders in the YTK larvae with time. Again, as with run 2 the surviving juveniles showed a significant proportion were malformed, with a high incidence of jaw and spinal deformities. Details of the types and extents of the jaw and spinal malformations are provided in the IMAS component of the final report.

The use of the upwelling system may have affected early survival of larvae with increased organic matter suspended in the water column (i.e. compared to larval runs with other species the tank water appeared dirty). Consequently, the upwelling system was removed from the tanks on15 DPH so the suspended material could settle and be fully vacuumed out. In future runs this may need to be done earlier or on a more regular basis.

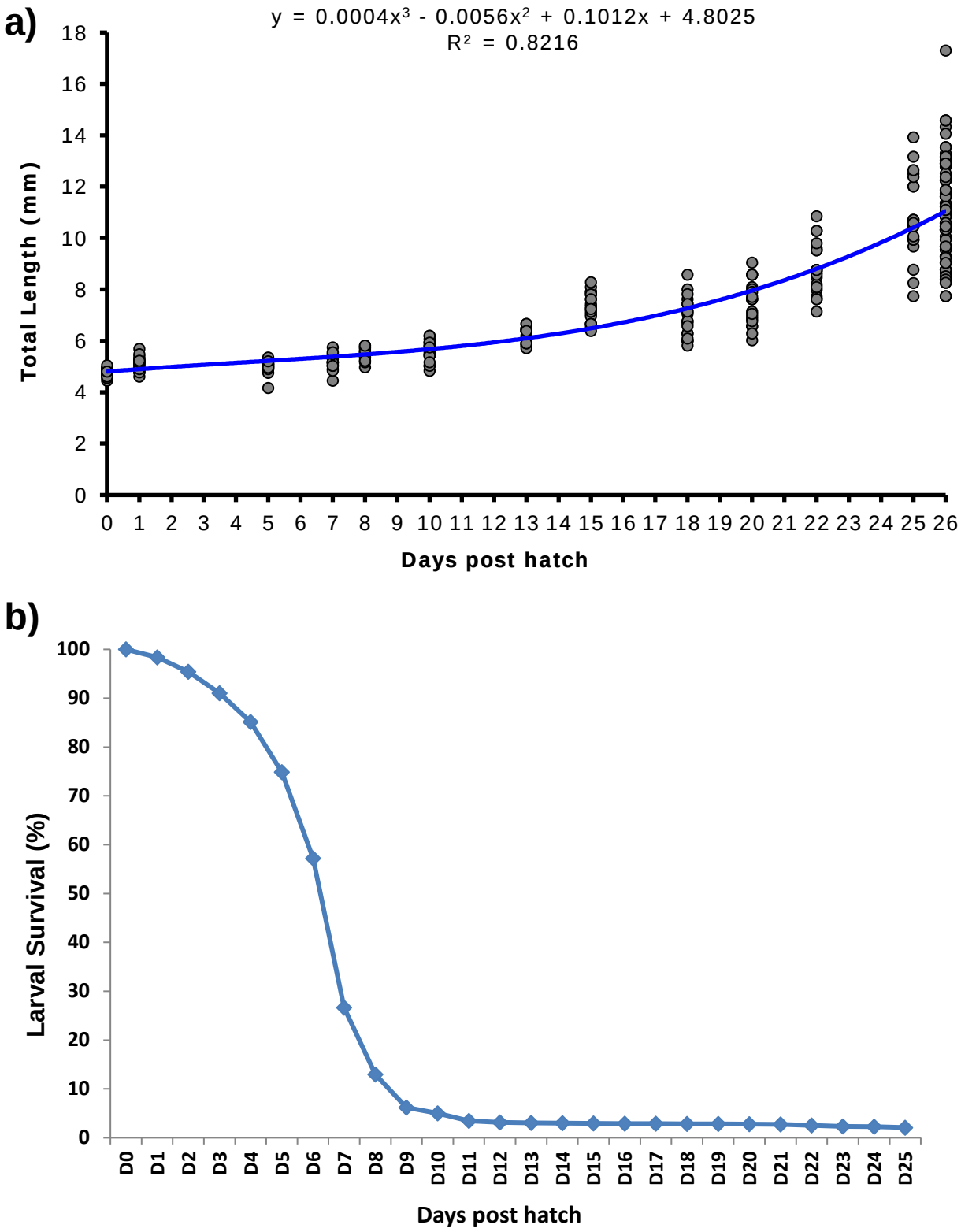

Figure 3. Growth (total length; mm) and estimated survival (%) of Yellowtail Kingfish larvae cultured in a recirculating seawater larval rearing system at the Darwin Aquaculture Centre. Run 3.

3. Discussion

The main aim of this trial was to evaluate the use of a recirculated water intensive larval rearing system, that has proven to be reliable in producing large numbers of high quality barramundi juveniles, for production of Yellowtail Kingfish larvae. This goal was partially achieved but was confounded by difficulties encountered with transportation of eggs from Arno Bay to Darwin that resulted in reduced quality and viability of eggs and newly hatched larvae, which in turn affected the numbers of larvae that could be used to test in the system.

3.1 Egg Transportation

Initial issues with egg transportation were mostly addressed by the 3rd run. The first batch of eggs was clearly the worst, and a number of contributory factors were identified:

1. The polystyrene boxes each had four 1 cm openings punched in the sides. This will likely compromise the main role of the box as an insulator, and water temperature inside each bag could have fluctuated during the transportation period. Some airfreight agencies insist that these boxes are vented to minimize the chances of box failure due to differential pressures during flights. A temperature logging device (sealed inside two layers of thin plastic) in the water of each box would provide additional information about whether this might have been an issue during transportation.
2. The water quality in each egg transport bag sent from CST was poor on arrival at DAC. Some parameters were high (dissolved oxygen and total ammonia), while others were low (pH and ionized ammonia). The high total ammonia level in the bags can probably be explained by the significant number of dead eggs on arrival (close to 20%). The best quality eggs were received from Challenger CIT, and whether this was due to the lower number of eggs/bag, the cooler water temperature, the lower DO and/or as a consequence of the shorter transport duration, is hard to determine.
3. The egg quality in some of the batches might have been poor. It is difficult to estimate the extent to which this may have been the case, as there were too many other factors potentially involved. The desynchronised hatching of eggs and the observed difference in stages of egg development upon arrival at Darwin could indicate that some batches of eggs were from different spawning events.

3.2 Egg Incubation

There were two obvious issues with egg incubation during this project. The first was water temperature. CST hatchery staff recommend a water temperature of 21 - 22ºC for incubation of YTK eggs. It was not possible to achieve this water temperature in the DAC facility because the incoming hatchery water supply was 30ºC and the chillers in the recirculation system did not allow the incubation tanks to operate at less than 24.4ºC. How much impact this factor had on egg development, hatching and subsequent larval survival and growth is not known.

The second issue was the ability to provide enough water movement to keep the incubating eggs in suspension whilst not being over-vigorous as to cause mechanical damage to the eggs/larvae. It is highly likely that settling of the YTK eggs on the bottom of the hatching tank in run 1 adversely impacted their proper development and contributed to high mortality of eggs and newly hatched larvae. Whether this is due to low dissolved oxygen or mechanical damage to the egg is not known. It was observed that the density of YTK eggs increased during egg development, and thus the advanced developing eggs tended to settle quicker and need progressively more vigorous water movement to keep them in suspension prior to hatching. Although improved hatching rates were recorded in runs 2 and 3, they were still well below the rates achieved for eggs of other species. This is an issue that needs to be addressed further.

As well as these main issues, some other minor issues were identified:

- Painting the incubation tanks black would increase the visibility of the eggs and larvae
- Ensure any temperature increases are gradual, not done quickly as was the case in this trial

3.3 Larval Rearing

It is difficult to separate the effects caused by the issues affecting egg transport and incubation (described above), from the effects of several factors impinging the subsequent larval rearing phase. The outcomes of two larval rearing runs do not provide enough data on which to make any firm conclusions in this regard.

Mortality rates of larvae were very high from immediately after hatching. As explained above, this could have been caused by the damage done to the newly hatched larvae by the strong aeration, resuspension of previously settled eggs, the poor quality of the transported eggs or the larval rearing conditions *per se*. In general hatchery

management, high mortality rates observed before full resorption of the oil droplet and the yolk sac are usually due to poor larval quality; whereas, high mortality rates experienced after resorption are generally related to poor feeding, due to either the quantity and/or the nutritional quality of the live feeds.

Larvae with live food in the gut were found from 3 DPH onwards. The feeding larvae were generally dark in colour, whereas those that weren't feeding were clear in colour. Despite feeding on live prey, it is unclear why the larvae did not growing significantly the first 10 - 12 days of the larval rearing phase. The persistent ongoing mortality rate from 8 DPH might suggest that the feeding protocol was inappropriate. The small size of the YTK larvae may affect their ability to prey on rotifers and it might be that the inclusion of copepods in this protocol may be of benefit (Schipp et al., 1999). Despite all of these issues, it was clear that some larvae successfully made the transition from rotifers to *Artemia* to microparticulate diet by 15-20 DPH. Regardless of the many possible ways in which the live feed regime could be improved, it is clear that DAC was able to wean YTK larvae by 20 DPH, and perhaps some potential to wean larvae as soon as the end of the rotifer feeding period.

During the first two weeks of rearing many larvae displayed what was considered to be abnormal swimming behaviour (i.e. fast swimming at surface or a vertical (head up) swimming position). It is suggested that this behaviour may be related to a lack of swim bladder inflation, or a swim bladder abnormality. Sampling larvae to monitor swim bladder inflation is difficult, as it requires a significant number of larvae from the entire water column, this constraint leads to the inconsistent results recorded for swim bladder inflation rate. Swim bladder inflation and its effect on the larval survival is an area that requires further investigation. During these trials probably not enough attention was paid to surface skimming as most focus was on keeping the larvae in suspension. Clearly, the problems with poor swim bladder inflation need to be overcome, and the solution is probably multi-factorial.

The absence of any infectious agents in samples provided for pathology investigations tend to support the hypothesis that the abnormal swimming behaviour was caused by a physical malformation.

Some other suggestions were made based on the experiences to date:

- Using two larval rearing tanks would enable some comparison of systems on the same batch of larvae. For example, use the upwelling system in one tank and not in the other to assess the impact on bacteria counts and water quality (suspended materials). The use of two tanks would also enable trials on feeding

such as the use of batch rotifer feeding while still using the algae paste, rinsing and enriching the rotifers for water quality control.

- Modification of the surface skimmers and incorporate added cleaning of skimmers with paper towel.

4. Further Development

The constraints of the project (only 3 batches of eggs, all needing to be transported for > 12h, and the high egg incubation temperature due to the source of the water supply and limitations of the cooling capability of the recirculation system at the DAC facility), and a great many consequential and subsequent factors that could impact on larval survival and quality, it easy to identify topics that need further research attention. They include:

- Egg transport conditions – minimizing issues with water quality, settling and mechanical damage
- Egg incubation – characterizing the trade-off between water circulation to minimize egg settling but avoid mechanical damage. Critical to reducing hatch and immediate post-hatching mortality.
- Feeding protocols – live prey species, densities and durations. Critical to reducing post yolk sac and oil droplet resorption mortality.
- In this regard, use of copepod nauplii in the larval feeding regime during the first feeding days should be investigated and compared with the growth and mortality rates of larvae fed on the current live food regime.
- Observations to date suggest that further investigation of co-feeding during the rotifer period with micro-diets such as Otohime or Gemma Micro have the potential to reduce the use of *Artemia* during YTK larval rearing.
- Swim bladder inflation – a more thorough sampling and histological study would be needed to determine when and how this occurs. This would allow hatchery staff to better anticipate the needs of the larvae at this time and to provide the optimal conditions that allow swim bladder inflation to happen successfully. Critical to reducing weaning mortality and culling due to malformation.
- Jaw deformities - determining when and how this occurs, allowing hatchery staff to better anticipate the needs of the larvae at this time and to provide the conditions that prevent it from happening. Critical to reducing weaning mortality and culling due to malformation.

5. Conclusions

Despite the constraints of this project there were some useful indications and observations. The main conclusions are that:

- This larval rearing of Yellowtail Kingfish was a first at the DAC and it was certainly a steep learning curve.
- The poor hatching rate and the high early mortality prevented a proper evaluation of the intensive recirculated larval rearing system.
- There was a strong indication that early weaning of YTK larvae can be achieved with limited use of *Artemia*.
- Egg incubation and larval rearing for YTK appear to be somewhat atypical for marine finfish. This appears to be a species with novel and specific requirements in this phase.
- Thus, the difficulties experienced over several years by CST, SARDI and New Zealand hatcheries in reducing the high mortality and malformation rates is not due to poor hatchery processes and practices.
- Concerted and collaborative R&D efforts will resolve these bottlenecks to the efficient production of high quality juveniles for growout.

6. References

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Appendix 1 – Intellectual property

There was no new intellectual property arising from this project.

Appendix 2 – Staff

Darwin Aquaculture Centre
Jerome Bosmans
Glenn Schipp
Chad Mumme
Damon Gore

CST
Mike Thomson
Morten Deichmann
Adrian McIntyre
Travis Dymmott
Alex Czypionka
Konrad Czypionka

IMAS
Stephen Battaglene
Jennifer Cobcroft

Appendix 3 – NT legislation to permit importation of YTK eggs

[Extract from]

NORTHERN TERRITORY OF AUSTRALIA
FISHERIES REGULATIONS
As in force at 21 September 2011

Division 3 Exotic fish, noxious fish and import permits

26 Permit to import fish or aquatic life

(1) The Director may, in accordance with this Division, grant to an applicant a permit to import live fish or live aquatic life into the Territory.

(2) The Director shall not grant a permit to import fish or aquatic life:

(a) unless that species of fish or aquatic life:

(i) is indigenous to the Territory;

(ii) is in pursuance of section 303EB of the Environment Protection and Biodiversity Conservation Act 1999 of the Commonwealth taken to be suitable for live import; or

(iii) **is specified in Schedule 7**; and

(b) if, in his or her opinion:

(i) there may be a risk of disease to people, fish or aquatic life by the importation;

(ii) there may be a risk of variation of the genetic composition or genetic material of Territory fish or aquatic life by the importation;

(iii) the fish or aquatic life to be imported may adversely affect the diversity of genetic material within the Territory; or

(iv) the fish or aquatic life may present an unacceptable risk of detriment to other fish or aquatic life in the Territory

Schedule 7 Species of fish or aquatic life for which permit to import may be granted

regulation 26(2)(a)(iii)

Common name	Scientific name
Golden perch	*Macquaria ambigua*
Silver perch	*Bidyanus bidyanus*
Southern bluefin tuna	*Thunnus maccoyii*
Yellowtail Kingfish	*Seriola lalandi*

Appendix 4 – Importation conditions for YTK eggs

IMPORTATION OF YELLOWTAIL KINGFISH EGGS FROM SOUTH AUSTRALIA: GUIDELINES & PROCEDURES FOR BIOSECURITY AND DISEASE TESTING

Draft Fisheries Policy Document
(Word document: YTK Egg Imports from SA_26-08-08)

Document Control

Version	Date	Author / Reviewer	Location J:/HPVData/Aquahealth/Fisheries Policies & Procedures/	Comments
1	26-08-08	J. Humphrey	IMPORTATIONS/Finfish – Living/Yellowtail kingfish from SA/Policy Douments/Curent Version/YTK Egg Imports from SA – July 08.doc	New version with document control

IMPORTATION, OF YELLOWTAIL KINGFISH EGGS FROM SOUTH AUSTRALIA: GUIDELINES & PROCEDURES FOR BIOSECURITY AND DISEASE TESTING

Purpose

To give clear guidelines and procedures to be adhered to by individuals or organisations importing yellowtail kingfish eggs from South Australia into the Northern Territory for aquaculture purposes.

To describe biosecurity measures to be implemented to minimise the risk of introduction and spread of any disease agents accompanying the imported eggs, together with sampling and testing to be undertaken to evaluate the health status of fish derived from imported eggs and measures to be undertaken to kill and disinfect the imported larvae or juvenile fish in the event of a significant disease occurrence.

Policy Statement

The Director shall implement or cause to be implemented an effective system of health certification and biosecurity to protect Northern Territory aquatic resources from incursions of disease.

Definitions

As per Fisheries Act and subordinate legislation.

References

N.T. Fisheries Act s 9

Scope

This policy applies to all individuals or organisations wishing to import eggs of Yellowtail Kingfish from South Australia into the Northern Territory.

Distribution

Internal distribution to Director of Fisheries, Deputy Director of Fisheries, Director Aquatic Resource Management, Manager; Aquaculture and Fisheries Licensing staff

Maintained on Policy file by Manager, Aquaculture.

Background

Protection of aquatic resources.

Cross Divisional Implications

Health certification procedures necessitate involvement of staff and resources of Berrimah Veterinary Laboratories.

Procedures

a. Permit to Import

Individuals or organisations (the Permit Holder) wishing to import yellowtail kingfish eggs from South Australia must hold a permit authorised by the Director of Fisheries.

b. Biosecurity

Establishment of Designated Biosecurity Area

An area will be set aside for holding of the eggs and tanks and equipment associated with the eggs. This area will be designated the designated biosecurity area. The biosecure area will remain a restricted site for the duration of the period the eggs and larvae or juvenile fish derived there-from are present in the holding tank or tanks.

Containment of eggs and larvae or fish derived there-from

Eggs and larvae or juvenile fish derived there-from will be placed in an isolated tank or tanks within the biosecure area. No eggs, fish or water will be removed or discharged from the biosecure area unless under the direct supervision of the permit holder or the Manager, Aquatic Animal Health.

Discharges of water and sterilisation of effluents

No discharge of effluent water will take place from the tank holding the eggs and larvae or juvenile fish derived there-from unless the water is subject to a process designed to inactive microbial or parasitic pathogens present.

For the purposes of sterilisation of effluents, the following are seemed satisfactory:

- Continuous low-level flow through a bed of chlorine tablets of sufficient number and density to give a resultant chlorine level in the effluent water of no less that 250 mg/l available chlorine
- Retention of effluent in a holding tank or vessel and subsequent chlorination using a minimum concentration of 200mg/litre of active chlorine for a minimum of 3 hours.
- Heating as per Attachment 1.

Physical security

The holding facility will be screened or delineated such that no unauthorised person shall enter the immediate region of the holding facility.

The biosecurity area holding the eggs and larvae or juvenile fish derived there-from will be clearly designated a restricted area with clearly visible signs stating that unauthorised entry is not permitted. Signs to this effect will be posted in clearly visible locations around the biosecurity area.

Authorised Persons

Only persons authorised by the Director of Fisheries or by the Permit Holder shall enter the biosecurity area.

Disinfection of eggs

On arrival, eggs will be maintained under biosecure conditions and subject to disinfection by ozonisation. Every effort will be made to ensure that there is no transfer of un-disinfected water from the eggs into the biosecurity tanks used for holding and hatching the eggs.

Physical containment of equipment

No item of equipment, including feedstuffs, nets, tanks, drums shall leave the immediate area of the holding facility, unless the items are subject to a satisfactory disinfection procedure as described under Disinfection of equipment.

Disinfection of packing materials and transport waters

All packing materials including boxes, bags used to trans-ship the eggs will be decontaminated in an approved manner (Attachment 1).

Disinfection of equipment

All equipment used in the handling or collection of eggs or larvae or juvenile fish derived there-from, and all items of equipment which come into contact with water from the tank holding the eggs or larvae or juvenile fish derived there-from, shall be decontaminated by an approved process (Attachment 1).

Disinfection of Personnel

Footbath

A footbath containing an approved disinfectant at an approved concentration (Attachment 1) will be placed at the entrance to the containment area. The disinfectant in the footbath will be maintained at all times and replenished as may be necessary.

All footwear will be disinfected on entering and leaving the containment area. Persons entering or leaving the area in bare feet will be required to disinfect their feet then wash with water.

Handwash

Hands and body surfaces contaminated with water from the holding tank/s will be washed thoroughly prior to leaving the biosecurity area and entering other regions of the hatchery.

Shower

In the event of gross contamination, facilities will be available for a full body shower with collection of contaminated clothing for laundering in hot water.

Restrictions on personnel

Where possible, personnel involved in the feeding and maintenance of the eggs or larvae or juvenile fish derived there-from will not come into contact with other fish or water in the general hatchery area after working with the imported eggs or larvae or juvenile fish derived there-from, on any one day.

Activities involving other handling and / working with fish and/or water supplies in other areas of the hatchery should be completed prior to entering and working in the containment facility.

Where this is not possible, disinfection of personnel as described above shall apply.

Duration of biosecurity

Biosecurity as described above will be maintained to the time the eggs or larvae or juvenile fish derived there-from, are ready for transfer to the nursery area of the hatchery, a period of approximately four weeks, unless otherwise authorised.

c. Disease monitoring of fish

Daily observations

Larvae and juvenile fish derived from the eggs will be observed on a daily basis for any evidence of disease. For the purposes of this protocol, disease is considered as any deviation from normality in terms of behavior, mobility, growth or in physical or morphological characteristics.

Clinical examination and records

Daily records of the health and clinical status of all imported fish will be maintained. Numbers of deaths will be monitored and recorded daily. At the larval stage, an estimate

of the deaths will be satisfactory. Clinical observations including motility, colouration, feeding behavior, condition of fins and integument will be recorded.

d. Sampling

Daily sampling of normal fish

On a regular basis and not more that 3 days apart, approximately 30 fish will be sampled and preserved in 10% seawater formalin as on on-going record of the development of the fish and to serve as a source of samples for retrospective histopathological analysis should a disease event occur.

Sampling for health monitoring

a. Normal fish: No evidence of disease

Periodic sampling of fish and water will be undertaken for examination for specific pathogens or parasites according to Attachment 2. For this purpose a representative sample of live larvae / fry / fingerlings will be collected, together with a representative sample placed into 10% seawater formalin.

b. Diseased fish

In the event of an outbreak of disease, typically affected, moribund fish will be collected and examined as described under Disease Testing. Typically affected fish will be collected live for parasitological examination, live on ice for bacteriological and / or virological examination and in 10% seawater formalin for histopathological examination

Collection of Dead Fish

Unless otherwise specified, where possible, all larvae, fry and fingerlings which die during routine management and rearing of the fish will be collected into 10% formalin solution. Fish will be collected on a daily basis and stored in formalin for subsequent sub-gross / histopathological examination as may be deemed necessary.

Mortalities exceeding “background" mortality associated with handling or other husbandry procedures will be subjected to gross, sub-gross and histopathological examination at the discretion of the fish pathologist.

e. Disease Testing

Gross pathological examination

Fry and fingerlings observed to show clinical abnormalities will be subject to a gross evaluation for evidence of lesions or abnormalities

Sub-gross examination

Sub-gross examinations will be conducted on a representative sample of larvae, fry or fingerlings to evaluate normal development and to examine for sub-gross lesions and to evaluate feeding or other morphological changes.

Microscopic examination

Microscopic examinations will be conducted on a representative sample of larvae, fry and fingerlings to examine protozoan or metazoan parasites on skin and/or gills

Histopathological examination

Histopathological examinations will be conducted on a representative sample of larvae, fry or fingerlings to examine for the presence of cellular changes consistent with or indicative of specific disease states.

Specifically, fish will be examined for evidence of grey matter vacuolation consistent with vacuolating encephalopathy and retinopathy nodavirus infection or for other changes consistent with infectious disease.

Microbiological examination

Larvae, fry and fingerlings will be cultured or otherwise examined for the presence of recognised bacterial and viral pathogens.

f. Destruction of fish

If at any time a disease event occurs, consultation between the Director of Fisheries, the Manager, Aquaculture, the Project Manager and the manager, Aquatic Animal Health will determine and evaluate the cause and nature of the disease and whether destruction of all fish is warranted.

The confirmation of microbial pathogens not known to occur in Northern Territory waters, including non-endemic strains of *Streptococcus iniae* or nodavirus or will result in the immediate destruction of all fish.

A decision to destroy the fish will result in the immediate treatment of the all tanks holding imported fish with an anaesthetic agent followed by introduction of chlorine at a minimum concentration of 200 mg/litre of active chlorine and held for a period of no less than six hours (overnight). Following chlorination, fish will be collected and disposed of by incineration or burial.

Attachment 1. Approved Disinfection Procedures

APPLICATION	AGENT	CONCENTRATION	PROCEDURE
Footbaths	Iodophor[1]	200-250 mg/L available iodine	Replenish footbath daily
	Hypochlorite	50-100 mg/L available chlorine	Brush boots prior to immersion
	Chloramine - T	50g/L	Leave to dry on boots
Nets	Hypochlorite	200 mg/L available chlorine	Dip for > 2 minutes then rinse
	Iodophor[1]	200-250 mg/L available iodine	Dip for > 10 minutes
Equipment,buckets & trays	Hypochlorite	100–200 mg/L available chlorine	Follow by rinse in fresh water
	Iodophor1	100-250 mg/L available iodine	Spray or rinse on previously cleaned and dried equipment
	Boiling water		Short dip
Hand-wash	Benzalkonium chloride	0.1-1 g/litre	Apply for 1 minute
	Chlorhexidine	4%w/v chlorhexidine	Apply and rinse for 1 minute
	Iodophor[1]	200 mg/L available iodine	Apply for a few seconds
	Antiseptic soap		Thoroughly wash and rinse
Hard surfaces & holding tanks (cleaned first with soap & hot water)	Benzalkonium chloride	2-5g/litre	Apply for >15 minutes
	Iodophore[1]	200 - 250 mg/L available iodine	Apply for 1-2 minutes
	Hypochlorite	100-250 mg/L available chlorine	Hold for 3 hours
	Steam cleaning	115-130 ^{o}C	
Transport vehicles	Hypochlorite	50-100 mg/L available chlorine	
	Steam cleaning	115-130 ^{o}C	
Protective clothing	Laundering	50-60 ^{o}C minimum with detergent	Commercial laundering
	Iodophor[1]	200-250 mg iodine/litre	
Boots & footwear	Iodophor[1]	200-250 mg/L available iodine	Scrub boots before treatment
	Hypochlorite	50-100 mg/L available chlorine	
Solid / semi-solid wastes	Incineration		
	Burial		Limit access by birds & vermin
	Heating	Min. 60 ^{o}C for 1 hour	
	Rendering as fertilizer	Approved process	Currently unavailable in NT
Water & washings	Hypochlorite	100 mg/L active chlorine	Hold >24 hours before discharge
	Ozone	Levels of 0.08-1.0 mg/litre	Caution: Significant OH&S issues
	Heat	60^{o}C for 10 minutes, 70 ^{o}C for 6 minutes, 75^{o}C for 5 minutes, or 80 ^{o}C for 4 minutes	

[1] Suitable products include Wescodyne®, Betadine® or Phoraid®

References:

Department of Agriculture, Fisheries and Forestry (2008). Operational Procedures Manual — Decontamination (Version 1.0). In: Australian Aquatic Veterinary Emergency Plan (AQUAVETPLAN), Australian Government Department of Agriculture, Fisheries and Forestry, Canberra, ACT.

[http://www.daff.gov.au/animal-plant-health/aquatic/aquavetplan/operational_procedures_manual_-_decontamination]

Methods for Disinfection of Aquaculture Establishments. OIE Manual of Diagnostic Tests for Aquatic Animals 2006 [http://www.oie.int/eng/normes/fmanual/A_00014.htm]

Attachment 2: Protocol for Routine Health Monitoring of Clinically Normal Fish

Introduction

This protocol describes the samples required for the routine health monitoring of each batch of yellowtail kingfish held in quarantine.

The protocol specifies the times of sampling after spawning and the nature and number of specimens to be collected. It also specifies details of preservation, storage and transport of samples for laboratory examination. The protocol provides for collection and storing of sequential samples for retrospective studies, as well as the strategic collection of samples for laboratory testing.

Specimens to be collected for laboratory examination

Sample	Age	No	Preservation	Procedure
Larvae	Approx 10 d.o.	>60	Freeze -20^{o}C	Hold for future examination
		>60	Freeze –20^{o}C or fresh in chilled (4^{o}C) water	Virus isolation in cell culture
		>60	Freeze –20^{o}C or fresh in chilled (4^{o}C) water	PCR Nodavirus
		>60	10% seawater formalin	Histopathology
		>60	Chilled (4^{o}C) water	Bacteriology
Fingerlings	5-6 weeks	>30	10% seawater formalin	Histopathology

Information Required

All samples must be labelled with the batch identification, the age, date of collection and the container or tank number of identifier.

Transport to the Laboratory

Frozen or chilled samples must be submitted to the laboratory on ice and must arrive frozen or chilled. Formalin fixed specimens may be submitted at ambient temperature. Formalin fixed and frozen / chilled samples should be stored separately.

Improvements in Yellowtail Kingfish Larval Survival and Juvenile Quality

Appendix E – IMAS component

Dr Stephen Battaglene and Dr Jennifer Cobcroft

Project No. 2009/749.20

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

This project was conducted by Institute for Marine and Antarctic Studies

The Australian Seafood CRC is established and supported under the Australian Government's Cooperative Research Centres Programme. Other investors in the CRC are the Fisheries Research and Development Corporation, Seafood CRC company members, and supporting participants.

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CRC AUSTRALIA

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Acknowledgements

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Morten Deichmann is thanked for his insightful and critical engagement with the IMAS (formerly TAFI) researchers and support of the project. The comprehensive sampling of Yellowtail Kingfish larvae and juveniles in commercial production was ably supported and conducted by Jamie Crawford, Alex Czypionka, Konrad Czypionka, Travis Dymmott, Allan Mooney, and Adrian McIntyre. Several technical staff members from IMAS were instrumental in the cataloguing, processing and assessment of samples; we thank Mel Evans, Tanaz Jungalwalla, Tom Litjens, and Anna Overweter. Mark Thomas and Erin Bubner at the Lincoln Marine Science Centre provided logistical support for liquid nitrogen and dry ice for transport of biochemical samples. Malcolm Brown, Mina Brock, Peter Mansour and Peter Nichols of CSIRO Marine, Hobart are thanked for processing the biochemical samples for lipid and vitamin content. We thank Graeme Knowles for his thorough processing of the histopathology samples (special thanks for the late-night slide reading).

Abbreviations

AB	Arno Bay hatchery
ARA	arachidonic acid
BHT	butylated hydroxy-toluene
BTBT	bromothymol blue teepol agar
CST	Clean Seas Tuna Ltd
CT	contact time (for ozonation; calculated as exposure in ppm x time in mins)
DAC	Darwin Aquaculture Centre
DHA	docosahexaenoic acid
DM	dry matter
DPH	days post-hatch
DPIPWE	Department of Primary Industries, Parks, Water and Environment
EPA	eicosapentaenoic acid
HPF	hours post-fertilization
PA	Port Augusta hatchery
ppm	parts per million
ppt	parts per thousand
SARDI	South Australian Research and Development Institute
SOP	standard operating procedure
TAFI/IMAS	Tasmanian Aquaculture and Fisheries Institute / Institute for Marine and Antarctic Studies
YTK	Yellowtail Kingfish
ZMA	Zobell's marine agar

Non-Technical Summary

PROJECT 2009/749.20 Improvements in Yellowtail Kingfish larval survival and juvenile quality (IMAS component)

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NON TECHNICAL SUMMARY:

Jaw malformation assessment was completed by IMAS (formerly TAFI) for all Yellowtail Kingfish (YTK) larval tanks for five Clean Seas Tuna (CST) hatchery runs (from the Arno Bay and Port Augusta hatcheries) in 2008, and for most larval rearing tanks in five hatchery runs at CST in 2009. The overall proportions of fish with commercially significant jaw malformations at the end of the larval stage in each of the hatchery runs were 27 to 29% in 2008, and 32 to 35% in 2009. These values correlated with IMAS assessments using the same jaw malformation classification system at the end of the nursery phase of 17 to 35% and 25 to 38% in 2008 and 2009, respectively. As in 2007, there was considerable variation in malformation rates between larval tanks (7 to 64%), even when they were stocked with larvae from the same batch of eggs.

In 2008 and 2009, fertilised YTK eggs were transported from CST to the Darwin Aquaculture Centre (DAC) and South Australian Research and Development Institute (SARDI) for rearing trials. Samples of larvae (from 1, 5, 10, 15, 20 DPH, and at transfer to the nursery and at handsorting) were sent to IMAS for assessment of jaw and other malformations. There was a high incidence of jaw (44%) and spinal (>30%) malformations in the YTK cultured at DAC in Jan-Feb 2009. Significant notochord (spinal) malformations were observed from Day 1, supporting other evidence such as low hatching rate, that the batch was compromised before larval rearing commenced. There was no effect of the *Artemia* feeding regimes tested at SARDI in 2008 on jaw malformation (25-41%) which was similar to the incidence in commercial hatcheries.

Bacteriology methods were demonstrated to CST staff for diluted plate counts of water and homogenised rotifers, and serial dilution was recommended for ongoing testing.

Biochemical analyses were completed for 20 fertilised egg samples and 36 samples of live feeds, with lipid composition, lipid class and vitamin content analysed. There were indications of variability between different batches of fertilized eggs and enriched live feed profiles, although it is not known how much these may have been due to differences in the way and duration for which the samples were stored prior to analysis. The implications (if any) of differences in these parameters to YTK culture are unknown without further experimentation.

Histopathology of over 2,000 larvae and juveniles from commercial production did not indicate any significant pathologies, with the most notable finding being non-inflated swimbladders.

KEYWORDS: Yellowtail Kingfish, aquaculture, fingerling production, juvenile quality, jaw malformation, live feeds, biochemical composition, swimbladder

1. Background

This report describes the work done by IMAS (formerly TAFI) in association with the CST hatcheries at Arno Bay and Port Augusta and project collaborators. The work described in this report was carried out in the 2008 and 2009 YTK spawning seasons, therefore the procedures described reflect the YTK larval rearing practices at that time. As a result of this project, and the others carried out by CRC collaborators, many of these practices and procedures will since have been modified as necessary. In this way, the hatchery manuals and SOPs developed by CST are very much 'living documents'.

Survival rates of YTK from hatched larvae to weaned juveniles is very low (~5-10%) with deaths in the tank, and culls due to deformities, being major losses. Of particular note, several skeletal malformations particularly of the spine, jaws and opercula, have been reported in Australian and New Zealand marine hatcheries (Cobcroft *et al*., 2004), with a classification system completed for YTK jaw malformations in a recent CRC project (Battaglene and Cobcroft, 2008).

A number of biotic and abiotic factors have been shown to be associated with higher levels of deformities at the hatchery stage in other species, and it seems likely that some of these factors are also likely to be important for YTK. Whilst trials in specialist R&D facilities are extremely useful in trying to understand the effect of these various factors, either alone or in combination, there may also be value in looking at the production performance data collected by commercial hatcheries to see if there are some clues to indicate which factors might be more, or less, important. The variety of biotic and abiotic factors examined in this project include:

- Egg quality factors
- Light conditions: Artificial versus Natural
- Rearing temperature and salinity
- Tank setup and hydrodynamics
- Tank colour and pattern
- Microbial characterisation and microbial management strategies in rearing and live food systems
- Nutrition: e.g. INVE Culture Selco®, yeast and live algae versus algal pastes, enrichment strategies
- Larval stocking rates and live food (prey) density interactions

Some of these factors were assessed via additional sampling of normal commercial production runs of YTK eggs, larvae and juveniles by hatchery R&D staff, and using the inherent variability in culture conditions to correlate with larval survival and deformity rates. The effect of other factors were assessed via replicated (where possible) tank experiments, where one or more factors had been deliberately changed or manipulated by the R&D agency staff.

IMAS researchers have extensive experience in the identification, description and quantification of finfish egg quality and larval malformations (e.g. Trotter et al., 2001; Morehead et al., 2001; Cobcroft et al., 2004; Battaglene and Brown, 2006, Battaglene and Cobcroft, 2008; Cobcroft and Battaglene, 2009), so the aim of the current sub-project was to supply information for the above analysis through:

- Assessment of jaw malformation rates in CST production runs and from YTK larvae cultured by partner organisations
- Biochemical analyses of fertilised YTK eggs and live feeds
- Histopathology assessment of YTK larvae samples collected by CST R&D staff

1.1 Need

The costs of producing juvenile YTK which are fit (i.e. having no or low level of deformity) to be stocked at sea for growout need to be reduced. Currently, the low survival rates and high deformity rates of YTK larvae and juveniles is constraining the growth and profitability of this industry in price-sensitive markets. This project aims to determine the factors that contribute most to the low survival and high deformity rates, and to seek industry-relevant ways in which to address them.

1.2 Objectives

1. To identify improvements to be made to commercial scale YTK larval and juvenile rearing systems and procedures resulting in higher survival (> 25% by end 2010), better growth, reduced levels (<5% by end 2010) and severity of malformations and more cost efficient juvenile production.
2. To assess the suitability of some novel larval and juvenile rearing techniques for YTK: recirculating intensive larval rearing system with semi-automatic feeding, artificial light, algal paste, small rotifer, high prey density and early weaning.
3. To test a range of key biotic and abiotic factors and rearing strategies on YTK larvae and juveniles in replicated tanks and identify optimal regimes for adoption in commercial scale hatcheries.

2. Methods

IMAS staff conducted 4 field trips for project planning, data collection, analysis and project progress reporting.

- Trip 1, 4-7 August 2009, Assoc Prof Stephen Battaglene and Dr Jenny Cobcroft (Larval Research Steering Committee meetings, Arno Bay)
- Trip 2, 12-19 October 2009, Assoc Prof Stephen Battaglene and Dr Jenny Cobcroft (Field work, Arno Bay and Port Augusta)
- Trip 3, 28 October-2 November 2009, Dr Jenny Cobcroft and Ms Polly Hilder (Sample collection and PhD project planning, Arno Bay)
- Trip 4, 24-30 November, Dr Jenny Cobcroft and Ms Melanie Evans (Sample collection, analysis and reporting, Arno Bay and Port Lincoln).

In addition, there were several other trips associated with this project:

- 1-2 December 2009, Assoc Prof Stephen Battaglene, Dr Jenny Cobcroft and Ms Polly Hilder attended the Tuna Symposium in Adelaide.
- 2-3 December 2009, Dr Jenny Cobcroft attended the Seafood CRC Aquaculture Innovation Hub meeting for reporting of travel grant outcomes and research planning.

2.1 Malformation Assessment

Larval and juvenile Yellowtail Kingfish (YTK) were sampled by CST staff from most hatchery runs (3 runs at Arno Bay and 2 runs at Port Augusta) in 2008, and 3 runs at Arno Bay and 2 runs at Port Augusta in 2009. Larvae were sampled on days 5, 10, 15, 20 and at the end of the larval tank phase (Day 20-28), and again from nursery tanks prior to hand sorting and transfer to sea cages. Samples were fixed in 10% neutral buffered formalin and transported to IMAS, Hobart, for malformation assessment using the scoring system described in the previous project (Battaglene and Cobcroft, 2008), and briefly described below.

Jaw malformation scoring system

Jaw appearance was scored from whole fish fixed in formalin, with a scoring system modified from Cobcroft et al. (2004) from 0 to 3, where 0 is normal and 3 is a severe malformation (Table 1).

Table 1. Jaw malformation scoring system used to classify Yellowtail Kingfish, including descriptions of the common malformations for a particular score and the colour code used for graphs throughout this report.

Score	Description
0	normal, perfect jaw
0.5	very minor malformation (slight variation from normal), would not be culled as a commercial malformation; most short lower jaw (lower >95% length upper)
1	minor malformation, would not be culled; most short lower (lower 85-95% upper), or long lower, or lower jaw bent down or twisted sideways on slight angle
2	moderate malformation, most culled; very short lower (lower 60-85% upper), or very long lower jaw often with upward 'hook' at tip of lower jaw, fused maxilla (mandible) on one side
3	severe malformation; most with fused maxilla both sides, or open with broken/bent lower jaw

All score 3 and most score 2 should be culled according to commercial hatchery protocols. Examples of the malformations and related scores are shown in Figures 1, 2 and 3.

Regarding short lower jaws, the severity score is related to the relative length of the lower jaw to the upper jaw. It is not appropriate to use a fixed distance difference from the tip of the lower jaw to the tip of the upper jaw when examining larvae of different ages and sizes, but is used as a commercial guide when hand-sorting juvenile fish of approximately the same size (eg if the juvenile fish are > 5 cm in length, cull if > 0.5-1.0 mm gap in jaw length). The equivalent length difference (gap) is indicated below for different scores for a 5 cm juvenile (examples of each score are shown in Figure 3). Upper jaw length is measured from the tip of the upper jaw to the end of the maxilla (upper jaw bone). Lower jaw length is measured from the tip of the lower jaw to the end of the maxilla for this relative index.

Score 0 – normal; tip of upper and lower jaw meet and are same length, lower jaw is 100% length of upper jaw (5.0 mm, no gap)
Score 0.5 – lower jaw length >95% upper jaw length (>4.8 mm, <0.2 mm gap)
Score 1 – lower jaw length 85-95% upper jaw length (4.3-4.8 mm, 0.2-0.7 mm gap)
Score 2 - lower jaw length 60-85% upper jaw length (3.0-4.3 mm, 0.7-2.0 mm gap)
Score 3 - lower jaw length <60% upper jaw length (<3.0 mm, >2.0 mm gap)

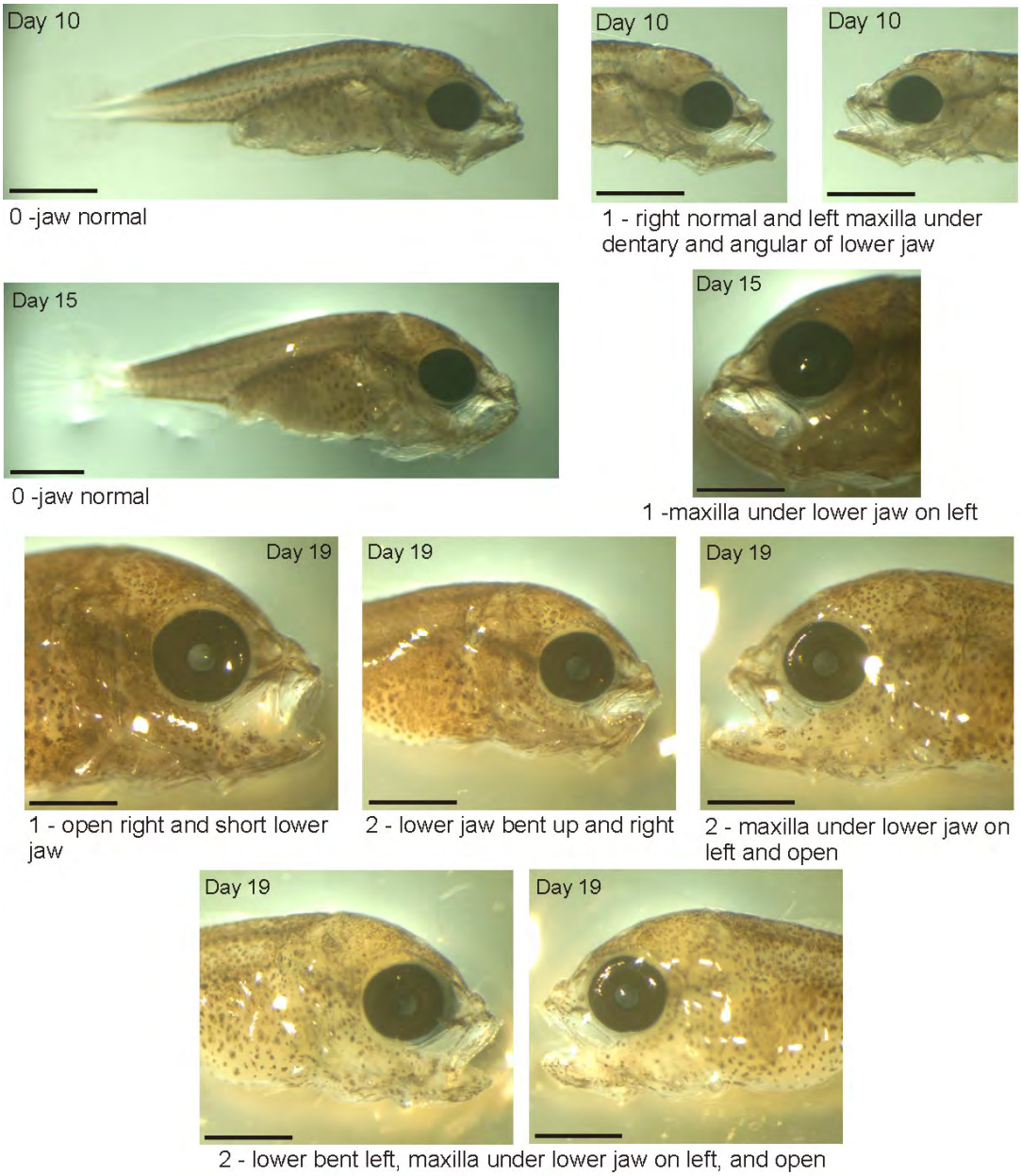


Figure 1. Scoring system for jaw malformations in Yellowtail Kingfish larvae. Examples of jaw malformations in larvae on Days 10, 15 and 19. Scale bars are 1 mm.

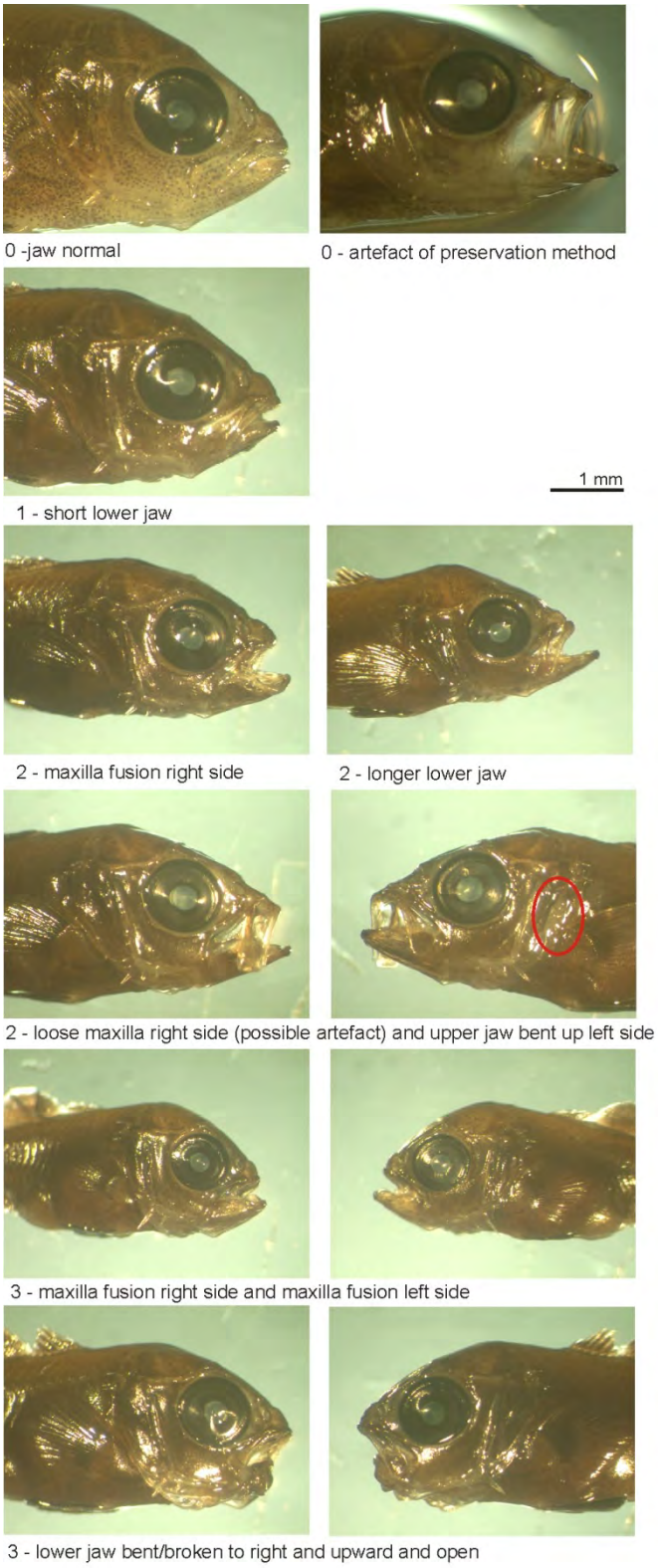


Figure 2. Scoring system for jaw malformations in Yellowtail Kingfish juveniles. Examples of jaw malformations in Day 30 juveniles. Red outline indicates shortened operculum exposing the gills. All images same scale.

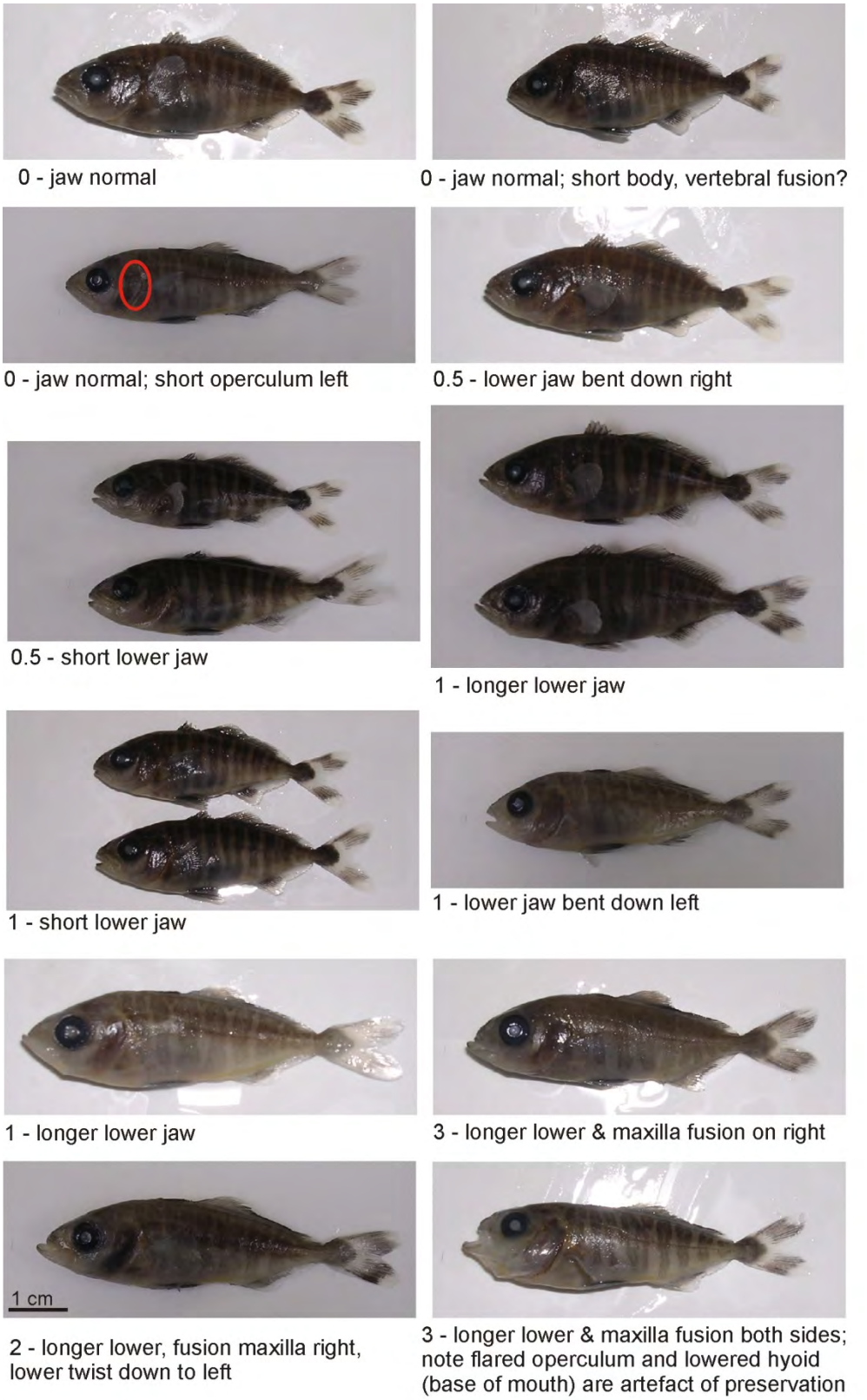


Figure 3. Scoring system for jaw malformations in Yellowtail Kingfish juveniles. Examples of jaw malformations in Day 41 and Day 51 juveniles. Red outline indicates shortened operculum exposing the gills. All images same scale.

2.2 Bacteriology

Samples were taken from two different rotifer culture systems to demonstrate a method to assess culturable bacteria per rotifer, as well as comparing plating methods of CST and IMAS for the rotifer culture water. For additional details of this activity see the IMAS Trip 4 report provided to CST (15/12/09) (Appendix 3).

2.3 Biochemistry

Samples from different spawning groups and times were assessed for lipid content and fatty acids (including mg g^{-1} DHA, EPA and ARA), and vitamins C, E and A (ascorbic acid, alpha tocopherol and retinol, respectively). Throughout the project it was intended to obtain samples from the five broodstock groups, at a minimum of two sampling times each, using duplicate samples (i.e., minimum of 20 samples) and this was achieved through sampling in Arno Bay and Port Augusta in 2008, and from Arno Bay in 2009 after consolidation of broodstock to one site. Analysis of live feeds was also undertaken for fatty acids and vitamins. The minimum samples to be processed were for rotifers and *Artemia*: four replicates for a minimum of four batches (i.e., minimum of 32 samples), and 36 samples were actually analysed. All analyses were conducted at CSIRO Marine Research in Hobart, with Dr Malcolm Brown as the primary contact.

Samples were collected by CST hatchery research staff at Arno Bay and Port Augusta. Samples were washed in ammonium formate to remove salt crystals and stored in domestic freezers for up to three months before transfer to a low (-80^{o}C) temperature freezer in the Lincoln Marine Science Centre. Samples were then shipped to Hobart on dry ice.

Ascorbic acid (vitamin C) analysis

In brief, samples (10 mg dry weight; DM) were extracted using metaphosphoric acid (3%) + acetic acid (8%) and derivatized (Brown et al., 2005). Samples (150 µl injection) were analysed using a Waters Model 600E HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Waters Model 475 scanning fluorescence detector set for an excitation maximum at 355 nm and emission maximum at 425 nm. The column used was an Alltima C18 (250 x 4.6 mm; Alltech Associates) which was eluted isocratically with 80:20 (v/v) 0.08 M potassium dihydrogen phosphate (pH 7.8): methanol, at a flow rate of 1.0 ml min^{-1}.

Tocopherol (vitamin E) analysis

Samples were analysed as described in Brown et al. (2005). In brief, samples (10 mg DM) were added to 10 ml polypropylene test-tubes together with 2.0 ml methanol containing 2 mg butylated hydroxy-toluene (BHT) as antioxidant. Samples were sonicated (Labsonic 1510 sonic probe; 30 s at 100 W), vortexed (10 s) then left to extract at room temperature for 1 h. The tubes were vortexed again, centrifuged (1000 x g, 10 min) and the supernatant was filtered through a 0.45 µm syringe filter (Alltech). Samples (150 µl injection) were analysed using the HPLC system previously described, equipped with the fluorescence detector set for an excitation maximum at 292 nm and emission maximum at 330 nm. The column used was an Alltima C18 (250 x 4.6 mm; Alltech Associates) which was eluted isocratically with 100% methanol at a flow rate of 1.0 ml min^{-1}.

Retinoid (vitamin A) analysis

Samples were analysed essentially as described in Fernandez et al. (2008). All operations were carried out under subdued light. Samples (150 mg DM) were placed into 20 ml glass extraction tubes, together with 0.85 ml of Milli-Q™ purified water (Millipore). Tubes were placed on ice for 10 mins, then 7.5 ml of chloroform: methanol (C:M; 2:1 v/v) was added. Suspensions were homogenised with a high speed blender (5-10 s), then the blender was rinsed with a further 2.5 ml of C:M, which was drained back into the same sample tube. Potassium chloride (2.5 ml of a 0.88% solution) was added, tubes were re-vortexed, and left on ice for a further for 1 h. The lower chloroform phase was transferred to a 10 ml glass test tube, the volume made up to 10 ml, then a subsample of 4.0 ml was transferred to a 5.0 ml Mini-Vial and evaporated under a stream of N_2 gas. When samples were dry, an additional 0.5 ml of fresh chloroform (not the extract) was added and the drying process repeated. Samples were stored at -20°C, under N_2 gas.

Prior to analysis, the samples were redissolved into 2.0 ml of C:M (2:1) + BHT (0.01%), then filtered through 0.45 µm nylon syringe filters. For HPLC, 100 µl was injected. The column used was an Alltima C18 (250 x 4.6 mm). Solvent A was 98% methanol containing 0.5% ammonium acetate; solvent B was the same solvent except 20% of the volume was replaced by chloroform. The separation was carried out at a flow rate of 1.5 ml min^{-1} at 30°C with a gradient of 100% A to 100% B over 20 mins (using Waters gradient program 5), and from 20 to 26 mins isocratically at 100% B. Retinoids were detected using a Waters photodiode array detector, set for routine detection at 325 nm. The identity and quantification of retinoids were affected

by comparison of both retention times and spectral properties of the peaks in test samples to those within Sigma standards (i.e. retinoic acid, retinol, retinal and trans-retinol palmitate). The analysis of retinoids is currently being optimised and validated. Hence, there is no detection and quantification of retinoic acid, retinol and retinal in samples. Instead retinyl palmitate is reported in a number of samples but these data should be regarded as preliminary.

Lipids and fatty acids

At CSIRO freeze-dried samples (100 mg) were rehydrated with Milli-Q^{TM} purified water and fish egg samples were disrupted using a blender prior to adding methanol and chloroform to give a chloroform-methanol-water combination of (1:2:0.8, v/v/v). The samples were allowed to extract overnight in separating funnels in by a modified version of the method of Bligh and Dyer (1959). To initiate phase separation, additional chloroform and Milli-Q^{TM} purified water were added to give a final chloroform-methanol-water ratio of 1:1:0.9 (v/v/v). The chloroform layer was rotary-evaporated and the yield of lipid determined gravimetrically. The extracts were then reconstituted in chloroform and stored under nitrogen at -20°C until required for analysis. FAMEs were formed by transmethylation of an aliquot of lipid extract (1 mg) and heating under nitrogen with methanol : hydrochloric acid : chloroform (10:1:1, v/v/v) at 80°C for 2 h. FAME were extracted three times with hexane : chloroform (4:1, v/v), dried with a stream of nitrogen gas and an internal injection standard (methyl tricosanoate, C23:0) added. FAME were analysed by gas chromatography (GC) using an Agilent Technologies 6890N GC fitted with an 7683 autosampler operated in split/splitless injector mode, a flame ionization detector and an Equity^{TM} -1 (Supelco) cross-linked methyl silicone fused silica capillary column (15 m $\times$ 0.1 mm ID and 0.1µm film thickness). Helium was the carrier gas. The oven temperature was raised from 120°C to 270°C by 10°C min^{-1}, then to 310°C by 5°C min^{-1} were it was held for 5 min. Fatty acids were quantified using GC-Chemstation software. Individual components were identified by comparing retention times and mass spectral data (Finnigan Thermoquest GCQ GC Mass Spectrometer fitted with an on-column injector, Texas, U.S.A) with data obtained from authentic and laboratory standards.

2.4 Histopathology

Yellowtail Kingfish larvae were routinely sampled every five days from all commercial production tanks at CST in both Arno Bay and Port Augusta hatcheries. Selected juveniles were also sampled from the nursery systems. Sampled fish were anaesthetised and fixed in 10% neutral buffered formalin. They were transferred to tissue cassettes and submitted to the Department of Primary Industries, Parks, Water and Environment (DPIPWE), Fish Health Unit in Launceston, Tasmania for histopathology assessment. Specimens were sent in two batches for samples collected in 2008 (Table 2) and in 2009 (Table 3).

Twelve sagittal section levels were cut for each larva, except for the largest juvenile samples which only needed two to three levels. This was the most thorough method to examine all organs, as best as possible. The scoring system for tissue and organ assessment was the following:

Grading:
0 = normal
1 = mild changes
2 = moderate changes
3 = marked / significant changes

Swimbladder was scored as 0 = inflated (normal), and 1 = non-inflated.

Table 2. Yellowtail Kingfish larvae and juvenile samples collected in Arno Bay, Port Augusta and SARDI hatcheries in 2008, processed for histopathology by DPIPWE, Fish Health Unit. n = 5 fish per sample occasion, and overall n = 758 fish examined.

Factors	Fish Age	Hatchery	Run/Expt	Number cassettes	Factor comment	Comment
Salinity & Light	Juveniles	PA	Run 1	5	hatchery 35 to nursery 42-47	Subsample of 5 fish from malformation series
Salinity & Light	Juveniles	AB	Run 1	5	hatchery and nursery 36-37	Subsample of 5 fish from malformation series
SARDI Expts	Larvae	SARDI	Rot	9		
	5,10,15,20	PA	Run 1	33		No cass = 8 tanks x 4 ages = 32 + 1 extra L7 = 34
	5,10,15,20	PA	Run 2	32		No cass = 8 tanks x 4 ages = 32
	5,10,15,20	AB	Run 1	19		No cass = 5 tanks x 4 ages = 20 + 2 extra L2 = 22 (some missing)
	5,10,15,20	AB	Run 2	28		No cass = 7 tanks x 4 ages = 28 + 2 extra L7 = 30 (some missing)
	5,10,15,20	AB	Run 3	10		No cass = 2 tanks x 4 ages x 1 or 2 cassettes/age = 10
	5,10	AB	pre Run 1	19		No cass = 4 tanks x 2 ages + 2 tanks x 1 age = 10 + 9 duplicate samples
			TOTAL	**160**		

Table 3. Yellowtail Kingfish larvae and juvenile samples collected in Arno Bay and Port Augusta hatcheries in 2009, processed for histopathology by DPIPWE, Fish Health Unit. n = 10 fish per sample occasion, and overall n = 1307 fish examined.

Fish Age	Hatchery	Run/Expt	Number cassettes	Factor comment	Comment
1, 5,10,15,20	AB	Run 1	65	natural vs artificial; system	13 tanks x 5 ages = 65 cassettes
1, 5,10,15,20	AB	Run 2	35		7 tanks x 5 ages = 35
1, 5,10,15,20,21	AB	Run 3	38		7 tanks x 4-6 ages = 38
27	PA	Run 1	22		3 tanks with 4 cassettes each and 2 tanks with 5 cassettes
		TOTAL	**160**		

3. Results and Discussion

3.1 Malformation Assessment

3.1.1 Overview of both CST hatcheries 2008 and 2009

Jaw malformation assessment was completed by IMAS for five Clean Seas Tuna (CST) larval runs in 2008 and for most larval rearing tanks in five hatchery runs at CST in 2009. The overall proportions of fish with commercially significant jaw malformations at the end of the larval stage in each of the hatchery runs were 27 to 29% in 2008, and 32 to 35% in 2009. These values correlated with IMAS assessments using the same jaw malformation classification system at the end of the nursery phase of 17 to 35%, and 25 to 38%, in 2008 and 2009, respectively.

Jaw malformation rates at each of the larval/juvenile stages were generally similar between equivalent runs in each year at the Port Augusta hatchery, with Run 1 tending to have higher levels (25-49%) than Run 2 (17-30%)(Table 4). Malformation rates at the Arno Bay hatchery were relatively low in 2007 (5-28%), again with higher values being evident in Run 1 (20-28%) compared to Runs 2 and 3 (5-19%). However, in 2008 and 2009 all Arno Bay hatchery runs had high levels of deformities in the larval, nursery and/or juvenile stages (26-50%), and there was no indication that Runs 2 and 3 (at least 30-32% malformation rate) were any better than Run 1 (26 to 50%)(Table 4). This suggests that no improvement in larval or juvenile YTK malformation rate was made during the 2008 or 2009 hatchery seasons.

In most cases the IMAS estimate of the proportions of fish with ‘commercially significant’ malformations (i.e. scores of 2 or 3) was higher in the nursery than the larval stage. An increase in malformation rate would occur in those cases where the severity of the malformation increases with fish age, such that a previously minor malformation (i.e. a score of 0.5 or 1) would become significant with developmental age, which is certainly the case with fusion of the jaw. The reason for the decrease in proportions of malformation in some runs is unclear and may reflect issues with non-random sample collection or higher mortality of malformed fish in the nursery.

In general, the IMAS estimates of the proportion of fish to be culled in the nursery are close to the CST production figures of actual culls (Table 4), and variation may be explained by 1) IMAS assessments averaged across all tanks without any allowance

for the number of fish that were in each tank, 2) difficulty in obtaining a truly random sub-sample from nursery tanks, and 3) wet weight calculation of culled fish may cause some variability in production figures. Nevertheless, both values indicate similar patterns and are vital for the comparison of juvenile quality between runs.

3.1.2 Arno Bay 2008

There was a high variation in the proportion of malformed fish between larval tanks, ranging from 7 to 44%, 10 to 44% and 8 to 45% in Runs 1, 2 and 3, respectively (Table 5, Figure 4). In Run 1, L2 was a blue walled tank and all others were marble. There was a similar proportion of malformed fish from the blue (38%) and marble (44%) tanks stocked with larvae from the same egg batch, which contrasted with 2007 results which indicated a potential benefit of marble over blue walled tanks. The effect of tank colour remains to be tested in a controlled and replicated environment. Following low survival in the blue tank in Run 1, all tanks were marble for the next two runs at Arno Bay.

There was no apparent effect of egg batch on jaw malformation rates. In some cases malformation rates were similar for larvae from the same batch stocked into different tanks (e.g. AB 2(14) 44% in L6 and 42% in L7), and in others there was a large difference (e.g. AB 3(4) 28% in L1 and 8% in L2).

The severity of jaw malformation reduced between larval (Figure 4) and nursery (Figure 5) tank assessments, likely due to differential higher mortality of more severely malformed fish through the weaning phase in the nursery. There was substantial variability in malformation rates between tanks in the nursery made apparent by grading, which separates larger, fast growing fish with low malformation rates from the smaller fish with a higher incidence of malformation (Table 5, Figure 5).

Table 4. Summary of jaw malformation assessment in YTK in larval and nursery tanks from CST production runs in 2007, 2008 and 2009. Values are the weighted proportion (%) of fish that had malformation scores of 2 or 3, or were culled as malformed juveniles by CST.

		2007			2008			2009		
		Larval (2+3)	**Nursery**	**CST data malformed culled (*)**	**Larval**	**Nursery**	**CST data malformed culled**	**Larval**	**Nursery**	**CST data malformed culled**
Arno Bay	Run 1	20	28	26 (21)	25	15	20	26	35	50
	Run 2	8	19	13 (11)	32	12	15	44	46	58
	Run 3	5	12	14 (12)	30	25	24	nd	34	41
	Overall	**11**	**20**	**16**	**29**	**18**	**19**	**35**	**39**	**53**
Port Augusta	Run 1	30	49	27 (23)	35	42	42	32	25	39
	Run 2	22	29	17 (13)	19	28	24	33**	nd	36
	Overall	**26**	**39**	**23**	**27**	**35**	**31**	**32****	**25****	**38**

* indicates 2007 data used within CST and based on incorrect calculation.
** indicates data are incomplete (preserved larvae from 3 of the 8 tanks were not assessed at the time of writing)

Table 5. Summary of jaw malformation assessment in YTK in all larval tanks from CST production runs at Arno Bay in 2008 at the end of the larval phase. The numbers of fish (total and malformed) transferred to the nursery and weights of fish from each nursery tank allow a weighted proportion to be estimated for the whole hatchery run.

Run	Tank	Egg Batch	Age (DPH)	Malformation Rate (%)	Number of Fish Transferred to Nursery	Number of Malformed Fish Transferred	Weighted Malformation Rate (%)
1	L1	1(6)	22	44	67,077	29,514	
	L2	1(6)	22	38	23,523	8,939	
	L3	1(9)	23	33	70,457	23,251	
	L5	1(7)	22	7	69346	4,854	
	L4	1(7)	22	16	72,219	11,555	
			Overall	**28**	**302,622**	**78,113**	**25**
2	L3	2(6)	24	31	57,783	17,989	
	L4	2(7)	23	10	45,283	4,655	
	L5	2(7)	23	11	44,634	4,782	
	L2	2(14) 2(15)	23	19	58,298	11,336	
	L6	2(14)	23	44	147,914	64,901	
	L7	2(14)	23	42	162,126	67,419	
	L1	2(6)	24	21	69,710	14,639	
			Overall	**25**	**585,748**	**185,721**	**32**
3	L3	3(5)	22	33	96,281	31,773	
	L7	3(1)	25	29	67,639	19,615	
	L4	3(7)	24	41	78,474	32,174	
	L1	3(4)	24	28	87,037	24,370	
	L6	3(1)	25	15	62,762	9,414	
	L2	3(4)	22	8	30,000	2,400	
	L5	3(7)	24	45	52,371	23,567	
			Overall	**28**	**474,564**	**143,314**	**30**
Arno Bay 2008 average				**27**			**29**

Larval rearing

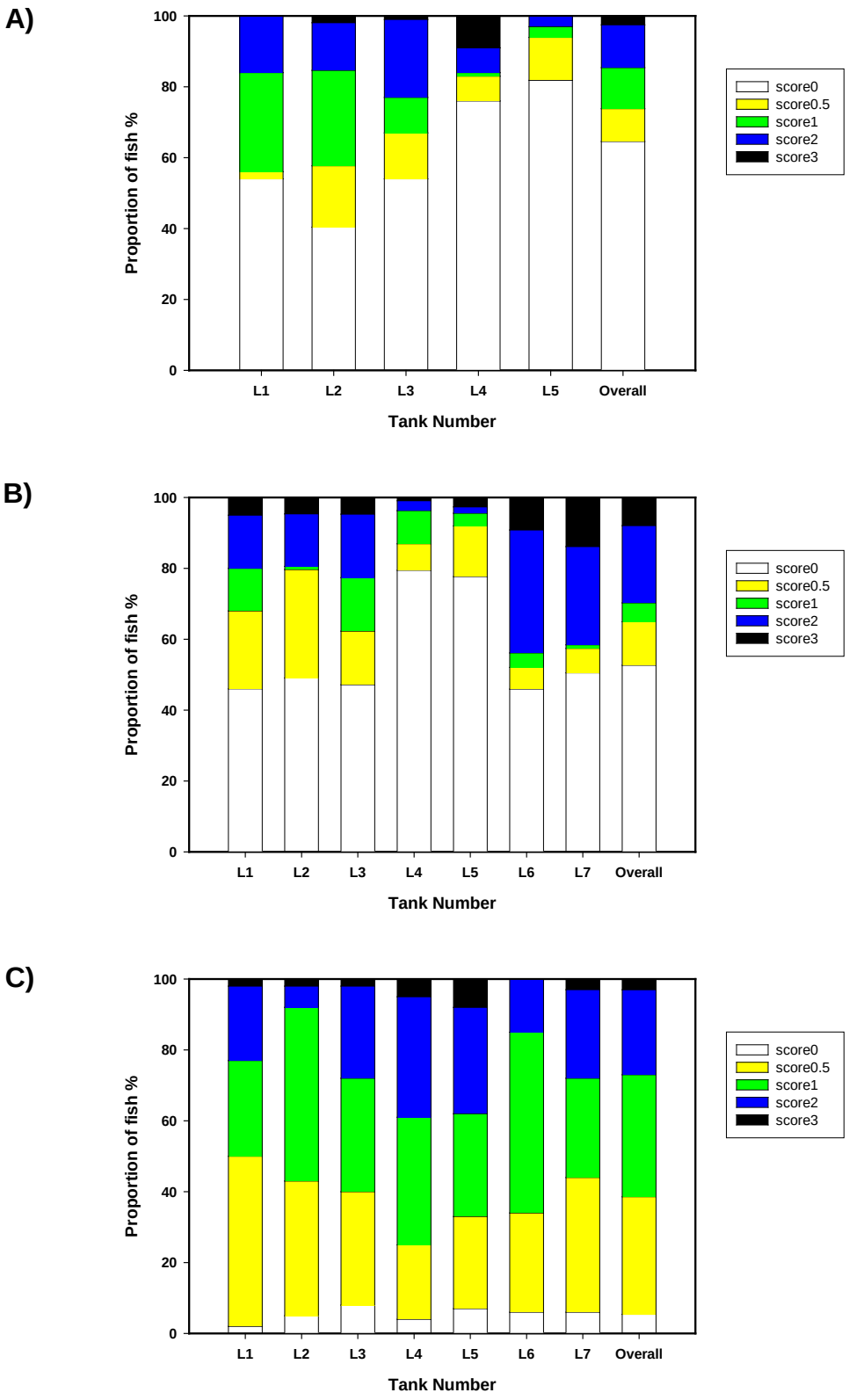


Figure 4. Incidence and severity of jaw malformation in YTK at the end of the larval tank phase from Arno Bay in (A) Run 1 (n = 401), (B) Run 2 (n = 732), and (C) Run 3 (n = 700) in 2008.

Nursery

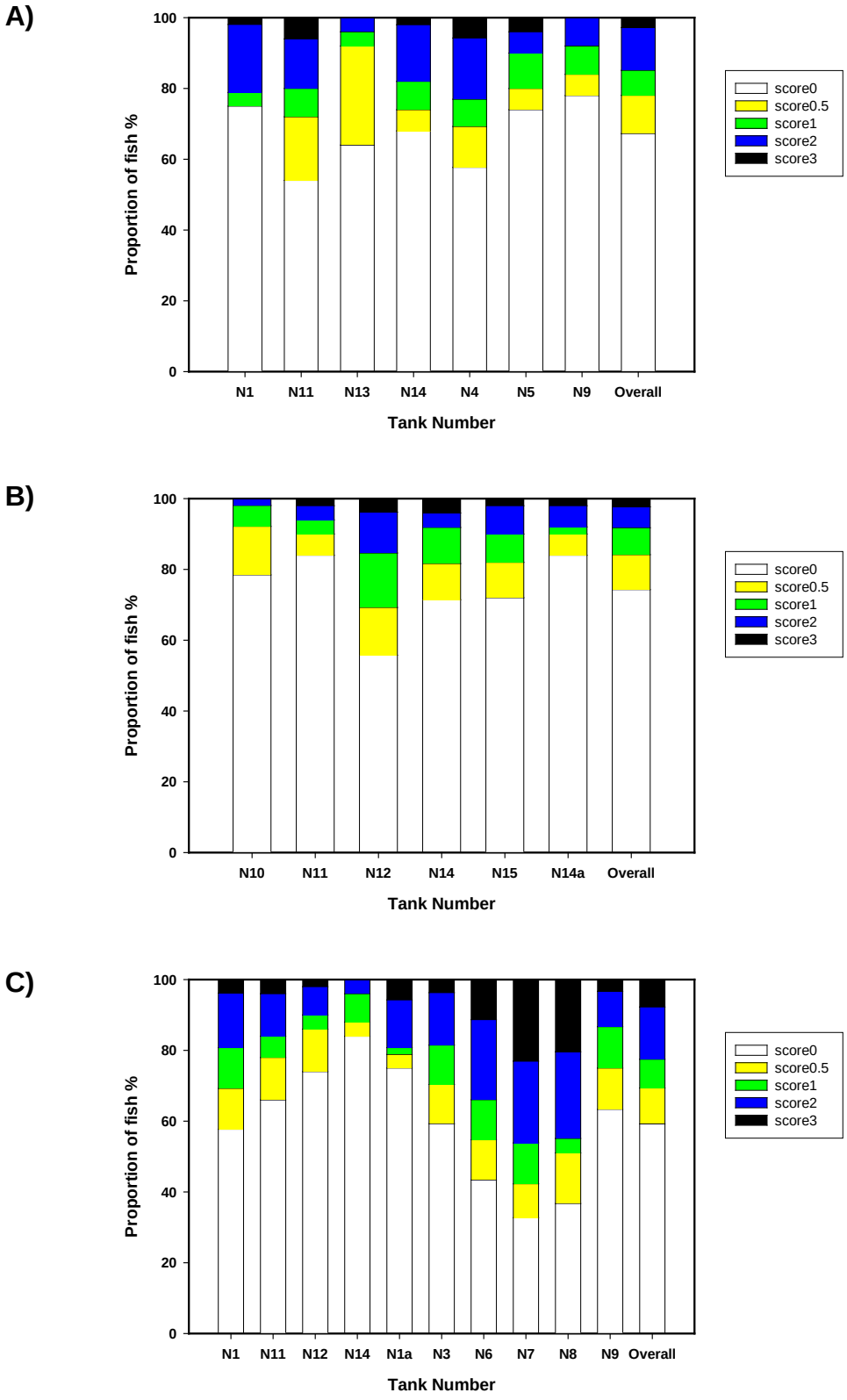


Figure 5. Incidence and severity of jaw malformation in YTK at the end of the nursery tank phase from Arno Bay in (A) Run 1 (n = 354), (B) Run 2 (n = 302), and (C) Run 3 (n = 522) in 2008.

3.1.3 Port Augusta 2008

There was an overall higher proportion of malformed fish and less variation between larval tanks at Port Augusta compared with Arno Bay T(table 6)(Figure 6). Tank L7, the larger 9,600 L outside tank, had a lower proportion of malformed fish in both runs compared to all the 5,000 L tanks. This trend was also seen in Run 2 in 2007 when the large tank was marble, but not in Run 1 when it was white.

Table 6. Summary of jaw malformation assessment in YTK in all larval tanks from CST production runs at Port Augusta in 2008 at the end of the larval phase. The numbers of fish (total and malformed) transferred to the nursery and weights of fish from each nursery tank allow a weighted proportion to be estimated for the whole hatchery run.

Run	Tank	Egg Batch	Age (DPH)	Malformation Rate (%)	Number of Fish Transferred to Nursery	Number of Malformed Fish Transferred	Weighted Malformation Rate (%)
1	L7	1(16)	27	19	262,126	49,311	
	L2	1(10)	27	53	65,031	34,769	
	L5	1(13)	27	44	48,237	21,439	
	L1	1(10)	27	38	45,418	17,259	
	L6	1(13)	27	46	60,212	27,423	
	L8	1(16)	27	50	44,108	22,054	
	L3	1(10)	27	48	49,436	23,518	
	L4	1(13)	27	43	47,366	20,367	
			Overall	**43**	**621,934**	**216,140**	**35**
2	L4	2(39)	27	27	51,565	14,018	
	L3	2(37)	27	21	63,900	13,175	
	L5	2(39)	27	22	56,687	12,417	
	L6	2(39)	27	21	43,613	9,068	
	L2	2(37)	27	30	53,580	16,236	
	L7	2(41)	28	11	124,405	13,549	
	L8	2(42)	27	14	43,543	6,096	
	L1	2(37)	27	15	55,486	8,160	
			Overall	**20**	**492,779**	**92,719**	**19**
Port Augusta 2008 average				**31**			**27**

Larval rearing

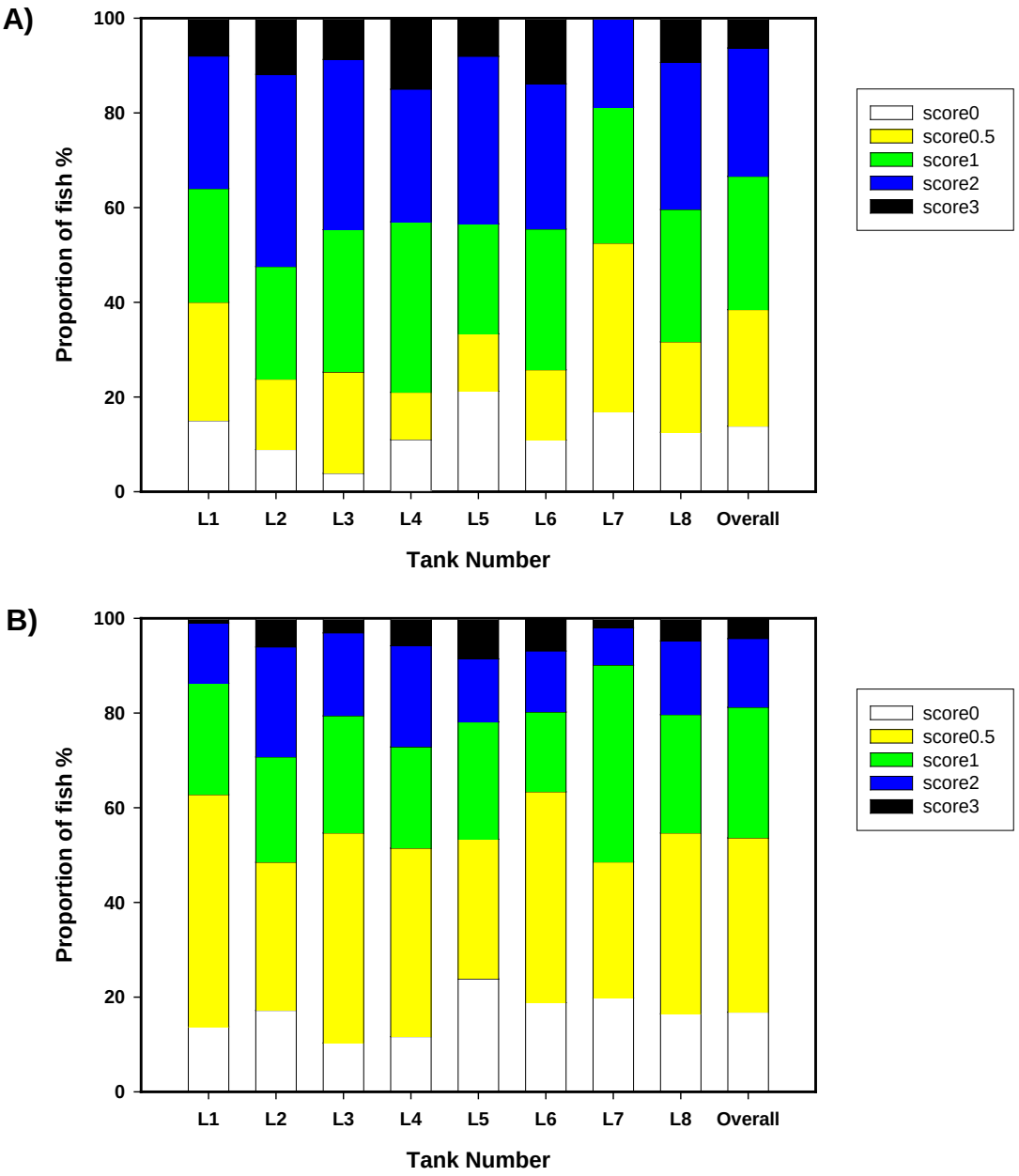


Figure 6. Incidence and severity of jaw malformation in YTK at the end of the larval rearing tank phase from Port Augusta in A) Run 1 (n = 805), and B) Run 2 (n = 811) in 2008.

Across all larval rearing tanks Run 2 in 2008 had half the malformation rate of Run 1, which was again similar to the pattern observed in 2007.

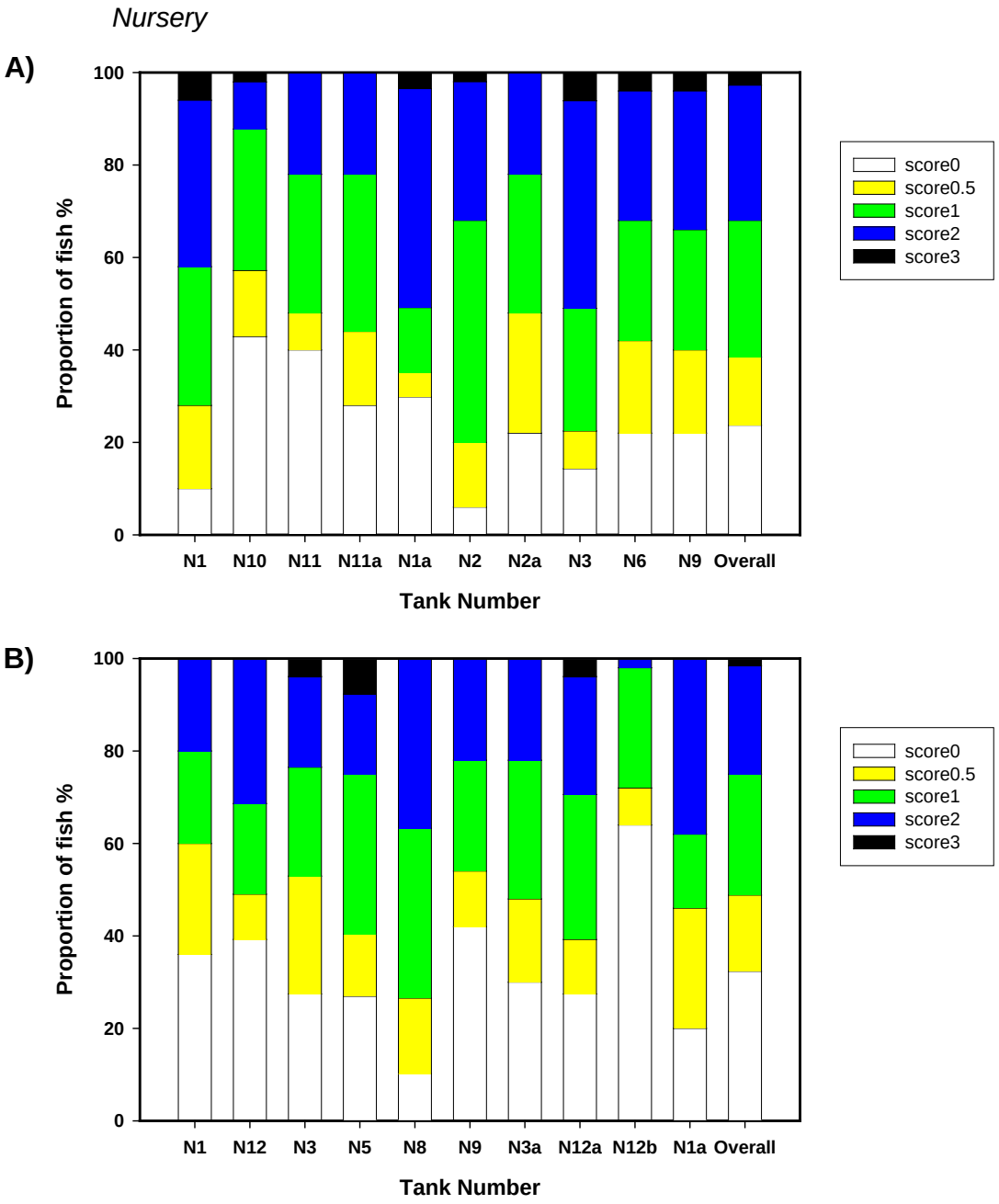


Figure 7. Incidence and severity of jaw malformation in YTK at the end of the larval rearing (nursery tank phase) from Port Augusta in (A) Run 1 (n = 505), and (B) Run 2 (n = 504) in 2008.

3.1.4 Arno Bay and Port Augusta 2009

There was a similar proportion of malformed fish in the larval rearing tanks in Run 1 at Arno Bay over the 3 years 2007-2009, however the proportion culled from the nursery for Run 1 in 2009 was approximately twice as high as in previous years (Table 4). Nursery data suggested that there was differential mortality of good (i.e. not malformed) fish, either through weaning or at swim bladder floating stages. This hypothesis, however, is not supported by visual assessment of the fish culled at the swim bladder float stage in Run 1, which revealed that many of the culls had both jaw malformations as well as a non-functional swim bladder (Alex Czypionka, pers. comm.). An alternative suggestion for the variation between the end of larval tank assessment and culling proportion is that the criteria for culling during hand-sorting was more rigorous in AB Run 1 2009 than previous years. For Port Augusta, both the assessment of malformation at the end of the larval phase and the proportion culled from the nursery in 2009 were similar to previous years.

There were differences in malformation rates between larval tanks in 2009 (Table 7, Figs. 7-9). In Arno Bay Run 1, tanks L1 to L5 were stocked from three different egg batches and cultured with air or air pattern hydrodynamic configurations, had the highest level of jaw malformation (32-37% commercially significant) (Fig. 7). Whereas, both tanks L6 and L7 which were stocked from two different egg batches and cultured using the water-driven hydrodynamic configuration, had lower incidences of malformation than the other outside tanks, and malformation rates similar to most inside (nursery) tanks.

There was no consistent pattern of jaw malformation incidence with egg batch or system type in inside tanks; the highest (N1, 39%) and lowest (N5, 12%) incidences of jaw malformation were in ‘air’ tanks stocked from the same egg batch, once again highlighting the impact of individual tank effects (e.g. differential mortality, density dependent access to food, yolk sac larvae handling at stocking, bacterial flora, etc). The overall incidence of malformation was higher in outside tanks (30%) than inside tanks (23%), and there was also higher survival inside than outside, but lower swim bladder inflation inside.

In Arno Bay Run 2, most tanks had similar levels of severity of jaw malformation (Table 7, Fig. 6), with 37 to 47% the range of commercially significant malformations, while L4 had a higher level of 64%.

In Port Augusta Run 1, the jaw malformation rate was highly variable between tanks, ranging from 17% in L1 to 52% in L8 (Fig. 7). Of the 5 tanks examined, there was less variability in Port Augusta Run 2, with the jaw malformation rate ranging from 22% in L1 to 39% in L4 (Fig. 8).

Table 7. Summary of jaw malformation assessment in YTK in larval rearing tanks from CST production runs at Arno Bay (AB) and Port Augusta (PA) in 2009.

Hatchery	Run	Tank	Broodstock and Egg Batch	Age	Commercially Significant Malformation (%)	Number of Fish Transferred to Nursery	Number of Malformed Fish Transferred	Weighted Malformation Rate (%)
AB	1	L1	BS2 (5&6)	27	36	28,822	10,376	
		L2	BS2 (5&6)	27	32	18,276	5,848	
		L3	BS2 (10)	25	37	59,269	21,930	
		L4	BS2 (10)	25	33	30582	10,092	
		L5	BS2 (9&10)	25	33	45464	15,003	
		L6	BS3 (6)	20	17	10000	1,700	
		L7	BS2 (12&13)	26	21	41746	8,767	
		N1	BS2 (2&3)	21	39	34572	13,483	
		N2	BS2 (2&3)	25	24	57500	13,800	
		N3	BS2 (2&3)	22	23	29551	6,797	
		N4	BS2 (1)	22	18	51623	9,292	
		N5	BS2 (2&3)	21	12	33388	4,007	
		N6	BS2 (1)	22	20	66291	13,258	
				Overall	**27**	**507,084**	**134,352**	**26%**
	2	L1	BS2 (23)	24	37	71,502	26,456	
		L2	BS2 (23)	24	41	100,129	41,053	
		L3	BS2 (23)	24	47	57,300	26,931	
		L4	BS3 (16)	23	64	81,930	52,435	
		L5	BS2 (25) & BS3 (19)	24	39	173,581	67,697	
		L6	BS2 (25) & BS3 (19)	24	44	100,617	44,271	
		L7	BS2 (25) & BS3 (19)	24	41	95,160	39,016	
				Overall	**45**	**680,219**	**297,859**	**44%**
		2009 overall for Arno Bay			**36**			**35%**
PA	1	L1		27	17	33,407	5,679	
		L2		27	39	40,100	15,639	
		L3		27	46	27,536	12,667	
		L4		27	38	26,579	10,100	
		L5		27	19	53,982	10,257	
		L6		27	26	69,187	17,989	
		L8		27	52	38,647	20,096	
				Overall	**34**	**289,438**	**92,426**	**32%**
	2	L1			22	17,950	3,949	
		L2			29	40,384	11,711	
		L3			26	40,430	10,512	
		L4			39	61,706	24,065	
		L5			36	55,173	19,862	
				Overall	**30**	**215,643**	**70,100**	**33%**
		2009 overall for Port Augusta			**32**			**32%**

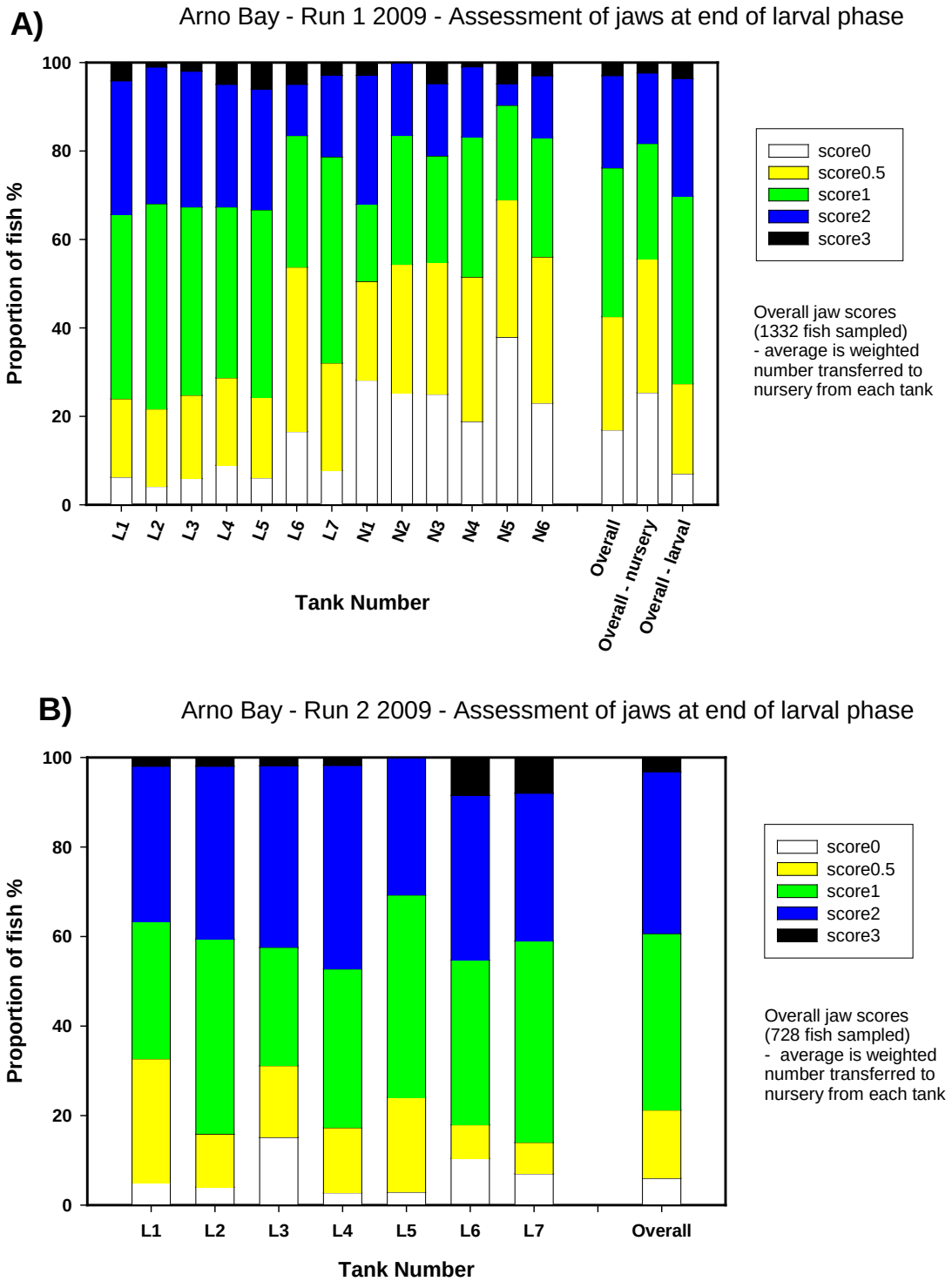


Figure 7. Severity of jaw malformations in Yellowtail Kingfish at the end of the larval rearing tank phase in (A) Run 1, and (B) Run 2, at Arno Bay in 2009.

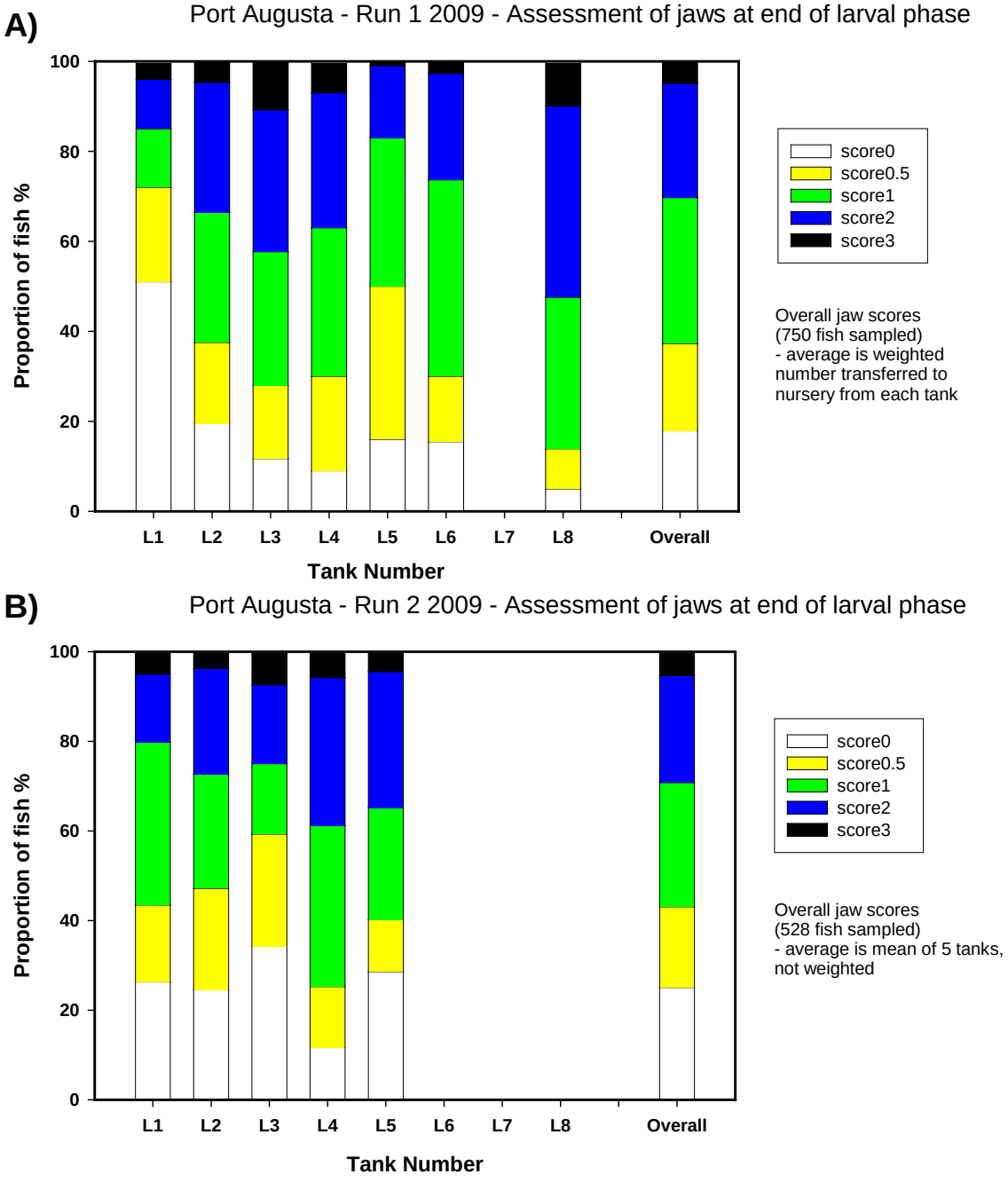


Figure 8. Severity of jaw malformations in Yellowtail Kingfish at the end of the larval rearing tank phase in (A) Run 1, and (B) Run 2, at Port Augusta in 2009.

The most common types of severe jaw malformations (score 2 or 3) in Arno Bay Run 1 were fusion (including the precursor stage with maxilla inside the lower jaw; 46-68% of the deformed fish), longer lower (13-14%), and maxilla (upper jaw) bent forward (8-24%) (Table 8). Of the fish with minor malformations (score 0.5 or 1, not normally culled at hand-sorting), maxilla bent forward (56-60%), longer lower jaw (18-26%) and short lower jaw (9-24%) were common. In Arno Bay Run 2, of the severe jaw malformations there was a lower incidence of fusion (26-29%) than in Run 1, but higher incidences of maxilla forward (18-35%) and longer lower (20-23%) often associated with an open mouth condition (4-26%). In Run 2 the most common minor malformations were maxilla forward (40-65%), short lower (12-50%) and longer lower (10-19%).

The most common types of severe jaw malformations (score 2 or 3) in Port Augusta Run 1 were similar to Arno Bay Run 1; fusion (40-45% of the deformed fish), maxilla bent forward (16-29%), and longer lower (12-22%) (Table 8). Of the fish with minor malformations, maxilla bent forward (41-57%), short lower jaw (22-44%) and longer lower jaw (13-14%) were common. In Port Augusta Run 2 a higher proportion of severely malformed fish had fusion (41-74%) than in Run 1, and the other most common severe malformations were longer lower (18-21%), and maxilla forward (3-20%). Fish with minor malformations were similar to Port Augusta Run1, maxilla bent forward (59-62%), short lower jaw (9-34%) and longer lower jaw (4-28%).

Table 8. Proportion (%) of YTK larvae with different jaw malformation types within severity of jaw score, and with opercular deformities, from CST production runs in 2009 at the end of the larval phase. Note fish could have more than one type of jaw malformation, but only one jaw score.

Proportion (%) of fish with different malformation types					
	Severity of jaw malformation (score)				
	0.5	**1**	**2**	**3**	**Overall**
Arno Bay Run 1					
Malformation type					
Operculum					14
Longer lower	18	26	14	13	20
Short lower	24	9	3	0	11
Fusion	0	0	46	68	16
Open	1	2	3	8	2
Lower twist	2	4	10	5	5
Other upper	56	60	24	8	47
Overall	100	100	100	100	100*
Arno Bay Run 2					
Malformation type					
Operculum					nd
Longer lower	10	19	23	20	20
Short lower	50	12	4	2	12
Fusion	0	1	29	26	16
Open	0	2	4	26	4
Lower twist	0	1	4	8	3
Other upper	40	65	35	18	46
Overall	100	100	100	100	100*
Port Augusta Run 1					
Malformation type					
Operculum					7
Longer lower	14	13	12	22	13
Short lower	44	22	5	0	18
Fusion	0	1	40	45	18
Open	0	5	11	10	7
Lower twist	1	2	3	7	3
Other upper	41	57	29	16	41
Overall	100	100	100	100	100*
Port Augusta Run 2 - 5 tanks only					
Malformation type					
Operculum					9
Longer lower	4	28	21	18	20
Short lower	34	9	1	0	10
Fusion	0	0	41	74	20
Open	0	1	4	3	2
Lower twist	0	3	13	3	6
Other upper	62	59	20	3	41
Overall	100	100	100	100	100*

* excludes opercular deformity which can occur in fish with any jaw malformation category

3.1.5 Darwin Aquaculture Centre 2009

IMAS received samples from the batch of YTK cultured at the Darwin Aquaculture Centre (DAC) early in 2009. The fixed larvae (1-20 DPH) were assessed 'grossly' before clearing and staining the cartilage and bone (technique modified from Taylor and Van Dyke, 1985) to determine the types of malformations present.

The IMAS malformation assessment revealed a high proportion of fish with jaw and spinal deformities. These results are in agreement with DAC's observations and histopathology assessment. The batch had a very low hatching rate (10-15%), compromised swimbladder inflation and abnormal behaviour (see DAC component).

The overall rate of commercially significant jaw malformations at the end of the YTK run at DAC was 44% at 20 DPH (see Figure 9 and Table 9). The most common severe malformations were an open mouth, often combined with the maxilla (upper jaw) aligned under (inside) the lower jaw which is an early stage of fusion. In addition, there was a high proportion of fish with spinal malformations, 30% with severe malformations at Day 20. While the jaw malformations appeared to be similar to those observed at CST hatcheries, the severe spinal malformations (30%) have not been seen previously in YTK from CST.

The early signs of the spinal malformations were visible from Day 1 (Fig. 10A), and clearly apparent from Day 5 with hypertrophy (enlargement) of the notochord (Figure 10B). The spinal malformations developed into abnormal vertebrae, fusion, scoliosis, kyphosis (upward/humpback curvature of spine) and irregularly twisted neural and haemal spines (Figures 10C-E). Abnormal head shape (cranial development) was also apparent.

The most likely reasons for the spinal deformities and high jaw malformation rate were:

- Poor egg quality – either due to the egg batch or issues in transport and handling (mechanical damage, low pH, high ammonia, temperature, aeration in incubation). These are the most likely contributing factor(s), as malformations were evident in Day 1 larvae before feeding.
- Poor nutritional quality of rotifer diet.

Many larvae, especially at 5-15 DPH appeared thin, indicative of poor feed intake or nutritional quality of the diet. However, this may be related to the compromised feeding ability of larvae with abnormal skeletal development. On 5 DPH, 22% of fish had an unusually large lipid drop remaining. This was recently linked to excessive aeration during incubation of striped trumpeter yolksac larvae, where the oil drop was apparently dislodged due to mechanical damage.

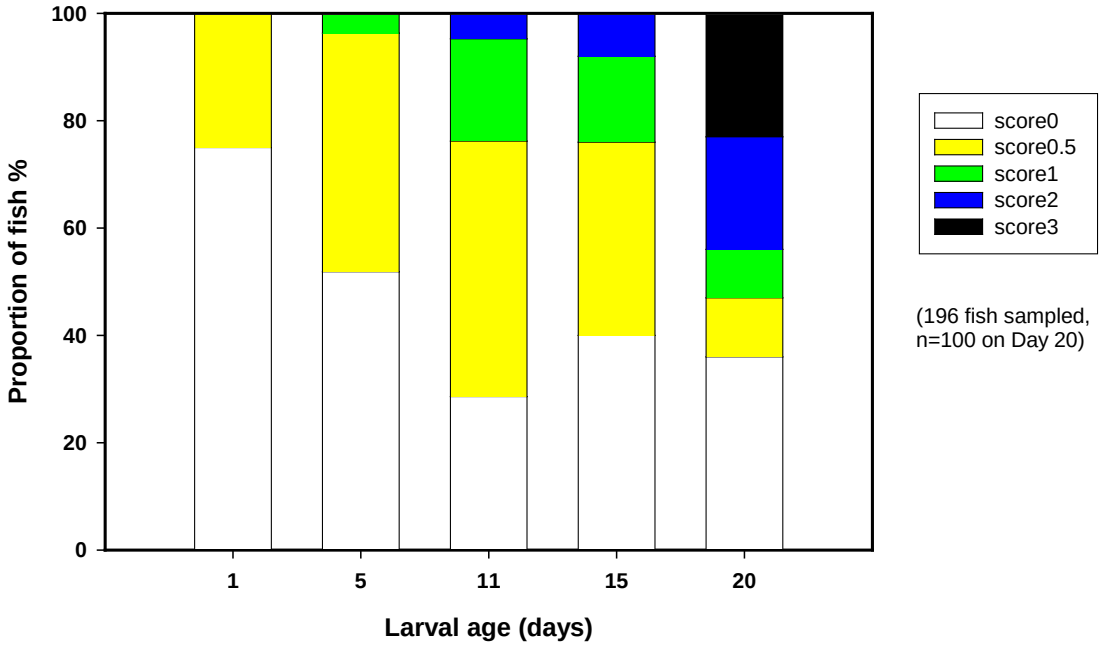


Figure 9. Incidence and severity of jaw malformation in larvae at different ages from DAC Run 1 in 2009. Jaw scores indicate: 0 = normal, 0.5 = very minor, 1 = minor, 2 = moderate, and 3 = severe malformation. Scores 2 and 3 are usually commercially significant. (n = 20-27 fish each day, except n = 100 on Day 20.)

Table 9. Jaw and spinal malformation assessment of Yellowtail Kingfish larvae from DAC Run 1 in 2009.

Fish Age (DPH)	Sample date	Number sampled	Total length (mm)		Commercially significant jaw malformations	Proportion jaw score (%)					Proportion (%) spinal malformations by severity*		
			Mean	± SD	(%)	0	0.5	1	2	3	none 0	minor 1	severe 2
1	29/1/2009	20	4.47	0.16	0	75	25	0	0	0	80	20	0
5	2/2/2009	27	4.73	0.21	0	52	44	4	0	0	80	15	5
11	8/2/2009	21	5.53	0.30	5	29	48	19	5	0	90	5	5
15	12/2/2009	25	6.94	0.62	8	40	36	16	8	0	45	40	15
20	17/2/2009	100	9.53	1.64	44	36	11	9	21	23	10	60	30
Overall		**196**											

*n=20 each age for spinal assessment

The jaw malformations present were:
Day 1 – short lower jaw
Day 5 – short lower jaw or short upper jaw
Day 11 – short lower jaw and maxilla under (early stage of fusion)
Day 15 – short lower jaw and maxilla under and longer lower; 1 in 25 fish with operculum deformity
Day 20 – open mouth, maxilla under (one or both sides), lower jaw twisted to one side, short lower jaw.

Spinal malformations were present in fish with and without jaw deformities, so that the total proportion of commercially significant malformations was >50%.

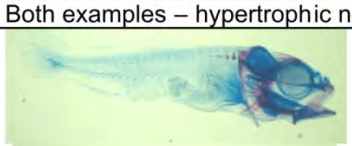
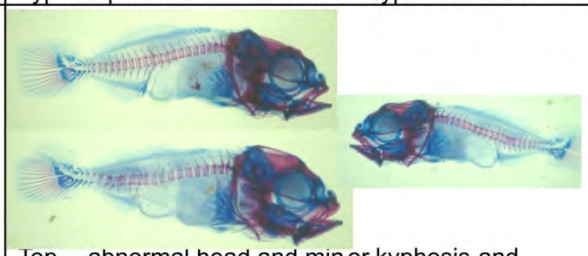
Age	Not cleared and stained	Cleared and stained
A) Day 1 Good Malformed		 Short lower jaw, curled down. Ethmoid cartilage (nose) bent down. Irregular notochord posterior to head.
B) Day 5 Good Malformed		  Both examples – hypertrophic notocord
C) Day 11	 	  Both examples – abnormally shaped head and hypertrophic notochord. Severe kyphosis in lower.
D) Day 15	 Apparently normal head and short body.	 Top – abnormal head and minor kyphosis and hypertrophy. Right and lower, abnormal developing vertebrae, vertebral compression and fusion, and hypertrophy towards tail.
E) Day 20	 	    Top left – normal. Right – severe kyphosis and irregular vertebrae. Lower left – compression, fusion, scoliosis.

Figure 10. Spinal malformations in Yellowtail Kingfish at different ages from DAC YTK Run 1 in 2009.

3.1.6 Assessment of jaw malformation in SARDI Artemia experiment

[See SARDI sub-project report (Appendix C) for a detailed description of this experiment and the different feeding approaches (Artemia density, and duration of rotifer co-feeding).]

The proportion of larvae with severe jaw malformation (scores 2 and 3, almost all of which would be culled in a commercial situation), was significantly affected by rotifer and *Artemia* feeding regime (ANOVA, $F_{8,27} = 3.123$, $P = 0.012$) (Figure 11). The proportion was lowest in larvae fed rotifers only (15%). However, it should be noted that these larvae had the lowest survival (7%) and were smaller (7.8 mm TL) than larvae from other treatments (averaging around 40% survival and 9.2 mm TL) (Figure 12). It is possible that the larvae fed only rotifers had not achieved a sufficient size to manifest malformations, which increase in incidence with larval age (Battaglene and Cobcroft, 2008). Consequently, feeding exclusively rotifers to 21 DPH is not recommended as a method to reduce jaw malformations. Excluding the rotifer only treatment, there was no significant effect of either *Artemia* density ($P = 0.284$), or co-feeding with rotifers to day 21 ($P = 0.076$), nor was there an interaction of the two factors ($P = 0.362$) on the incidence of jaw malformation. The proportion of fish affected by jaw malformations in this experiment (25-41%) was similar to that observed in the commercial hatcheries.

A variety of malformation types were present in larvae from the experiment (Figure 13). Opercular malformations tended to be minor and there was no effect of treatment on their prevalence (9 ± 6%) (ANOVA, $F_{8,27} = 1.755$, $P = 0.131$). Spinal malformations were rare (1 ± 2%; indeed, 26 out of 36 tanks had none), found across most treatments with no treatment effect, and the maximum incidence in one tank was 14% (T10, A25). Signs of external bacterial infection were also rare, with a few fish exhibiting apparent bacterial growth around the eye (Figure 13).

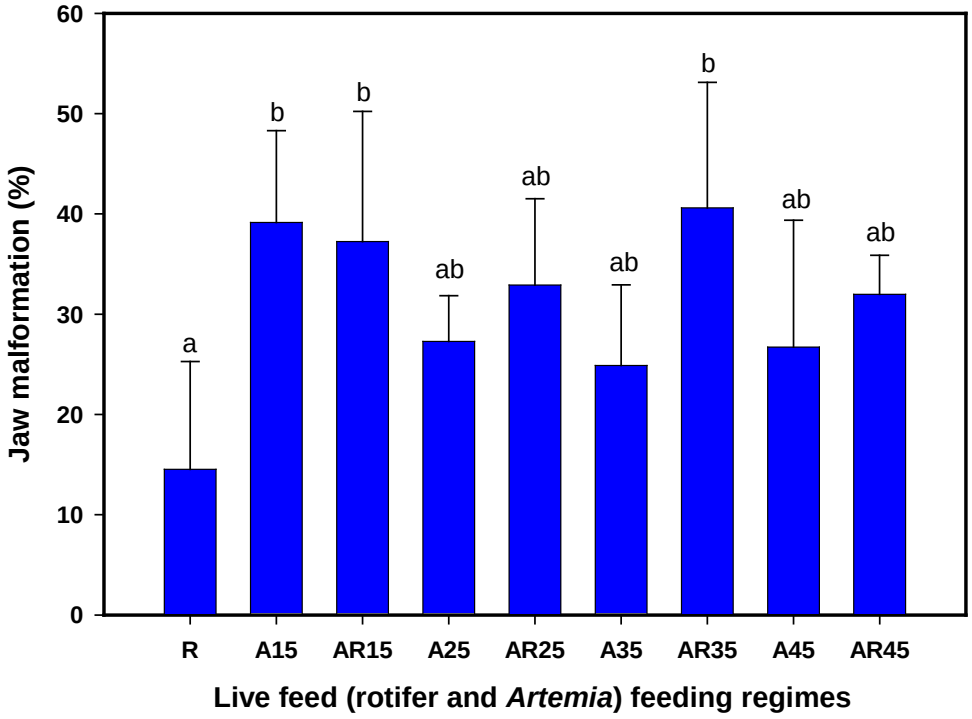


Figure 11. Severity of jaw malformations in YTK larvae at 21 DPH at the end of the rotifer and *Artemia* feeding regime experiment at SARDI. Values are means (+ SD) based on *n* = 50 larvae per replicate tank, *n* = 4 replicates per treatment. Bars with different letters are significantly different (P < 0.05).

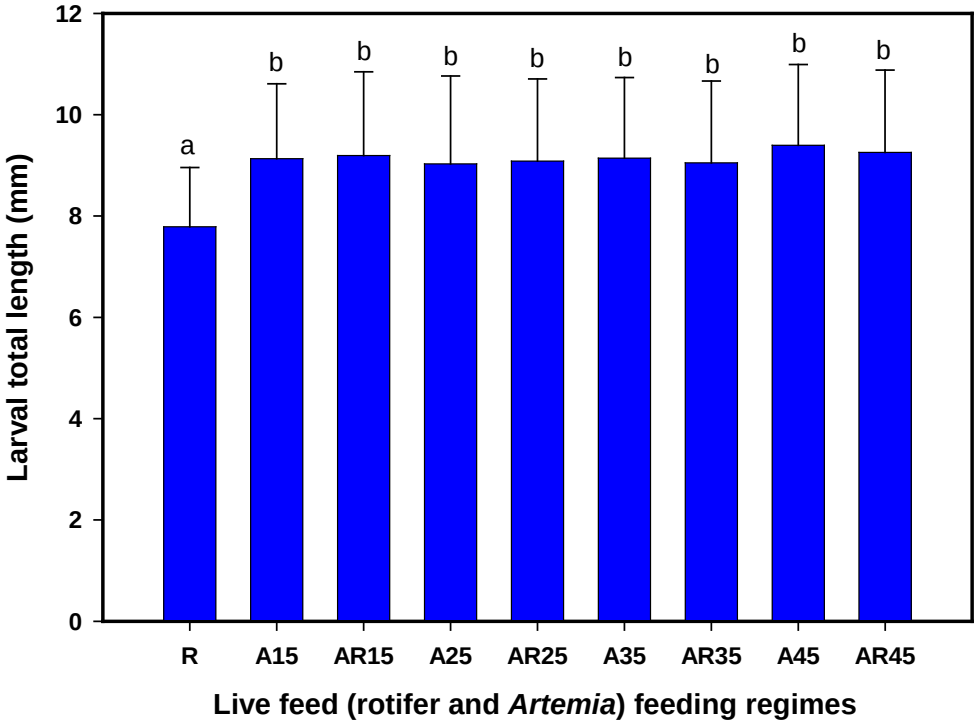


Figure 12. Total length (mean + SD) of YTK larvae fed different rotifer and *Artemia* regimes at 21 DPH. *n* = 50 larvae per replicate tank, *n* = 4 replicates per treatment. Bars with different letters are significantly different (P < 0.05).

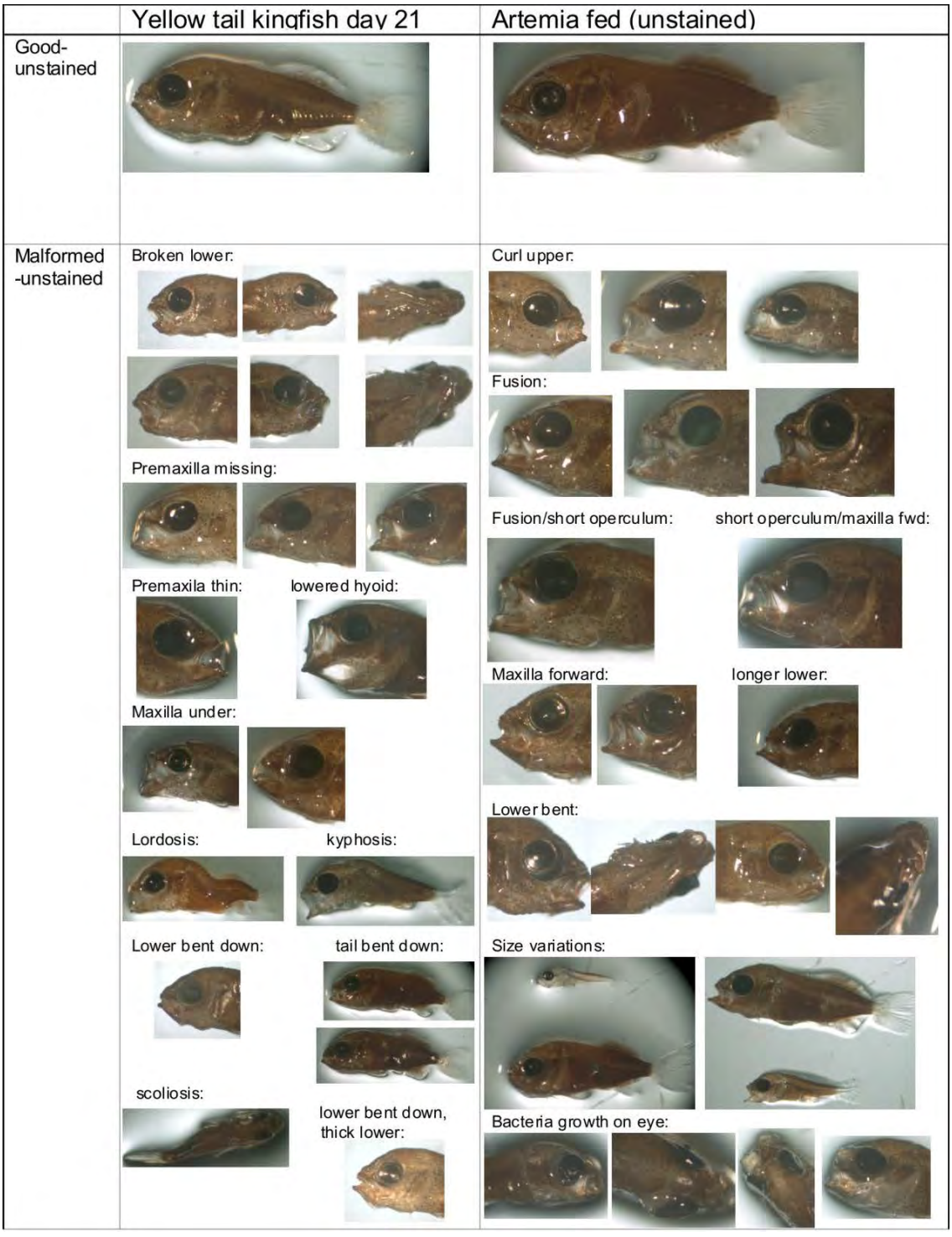


Figure 13. Jaw and spinal malformations in YTK larvae at 21 DPH from SARDI *Artemia* experiment.

3.1.7 Observation of CST grow-out fish (Port Lincoln)

IMAS staff observed weight checks of Mulloway and Yellowtail Kingfish in CST cages at Port Lincoln conducted by Chris Brooks (Marine Operations Manager, Port Lincoln) and crew on Friday 27 November 2009, and whole packing of 09YC (2009 year class) Yellowtail Kingfish at the processing factory in Port Lincoln on Monday 30 November 2009. Several jaw malformations were observed that would have been visible in those juveniles in the nursery and should have led to those fish being culled at hand-sorting. In contrast, however, the spinal deviations observed in the growout and harvested fish would not have been visible at hand sorting and would only have become more obvious as the fish got older. It was noted that the size and condition of the fish with jaw malformations was equivalent to other fish in the harvested batch at the processors. For additional details of this activity see the IMAS Trip 4 report provided to CST (15/12/09) (Appendix 3).

3.1.8 Discussions with INVE/Primo

Dr Cobcroft had an impromptu meeting with Tania De Wolf (INVE, Italy) and Liz Evans (Primo) and CST staff on 29th November 2009. The rotifer and *Artemia* enrichment profiles that were reported in CRC project 2007/718 were discussed. Tania indicated that the rotifer enrichment achieved was not optimal for the products (details discussed with Adrian and Soazig). It was noted that INVE no longer manufacture the Roti Selco Alg product and CST have purchased the last of the Australian supply for Arno Bay Run 3. The recommended replacement INVE product is S-presso, which is designed for species with high HUFA and DHA:EPA requirements. S-presso should provide HUFA at 40mg/g DW, DHA:EPA of 4.5-5.0:1 and vitamin C of 1,500 mg/g. This lipid profile would more closely match the egg profile of YTK. This is a potentially important area of research, as it follows recent research with grouper where jaw malformation (involving similar bones to those in some of the YTK deformities) was linked to inadequate DHA:EPA ratio and DHA content.

Testing of various commercial live feed enrichment products is recommended as a priority in replicated experimental trials. Investigating changes in survival and skeletal deformity with different enrichment products is important for CST to assess the cost effectiveness of alternative products or doses of products currently in use.

3.2 Bacteriology

All plates of rotifer culture and sump water from the CST method had too many colonies to count so that comparison of the two plating methods was not possible. The IMAS bacteriological approach produced numbers of colonies that could be enumerated (Table 9). There were no colonies on the CST control plates.

Table 9. Bacterial counts (IMAS methodology) from samples of rotifers and culture water from two different rotifer recirculation systems at Arno Bay. P2 used INVE Culture Selco and yeast, and Eco used algae paste (*Nannochloropsis* sp.)

		No. Colony Forming Units (CFU) per mL		No. Colony Forming Units (CFU) per rotifer	
Sample		**ZMA**	**TCBS**	**ZMA**	**TCBS**
rotifers	P2	6,400,000	1,700,000	427	113
	Eco	6,500,000	1,790,000	433	119
water	P2	360,000	262,000	na	na
	Eco	300,000	160,000	na	na

The IMAS method, particularly of homogenising rotifers was demonstrated as a potential tool for use at CST, but not recommended for routine assessment as it is time consuming. The serial dilution method for water samples is critical to obtain accurate results and we recommend should be routinely used when plating at CST. The CST method was an inefficient use of technical effort and time if the colonies could not be counted and the samples needed to be plated again the following day. Monitoring bacterial counts in rotifer and larval culture routinely (e.g. 2-3 times a week) could be a useful addition to the data records collected by CST during the hatchery season. However, if no management actions are in place to respond to changes in values then the need for this monitoring is questioned given the time involved to collect the data. Regular monitoring of existing water treatment systems would be worthwhile.

In comparison with previous trials, the colony counts for the rotifers themselves were more than twice as high as results using a similar technique at IMAS on enriched and rinsed rotifers (192 CFU/rotifer on ZMA and 53 CFU/rotifer on a *Vibrio* selective agar, BTBT). However, this difference could be due to the prior rinsing of rotifers in the IMAS study.

Further bacteriological work at CST is probably desirable, in order to better understand the nature of microbial loadings during the hatchery cycle. Simple studies, such as the benefit of rinsing harvested rotifers prior to them being fed to larval culture tanks, would add to the understanding of the system.

There was an indication of a higher *Vibrio* load in the water in the traditional INVE Culture Selco and yeast rotifer production system than in the algae paste rotifer production system. Further plating would be required if this is considered important to verify.

Consider adopting a routine microbial load monitoring approach to water treatment into egg incubation and larval systems (i.e. before and after 5µm and UV disinfection).

We recommend serial dilutions are used at CST whenever microbiological plating is required. If the bacterial load is unknown, every second dilution can be plated (in the range of 'neat' to 10^{-5}) as a range finder, then only one or two dilution levels plated on subsequent occasions.

3.3 Biochemistry

Three sets of samples were submitted to CSIRO Marine, Hobart for analysis of biochemical composition (Table 10). While more samples were collected by CST staff, some were poorly stored and not viable for analysis. Samples that were considered to be of most value to understanding larval rearing performance were selected for analysis, including duplicates of some samples to determine variability in analyses.

Table 10. Details of sampled batches of fertilised eggs, rotifers and *Artemia* sent to CSIRO Marine, Hobart for biochemical analyses.

	Arno Bay			Port Augusta		
Date	**Eggs**	**Rotifers**	***Artemia***	**Eggs**	**Rotifers**	***Artemia***
Jun-09	4	-	-	4	-	-
Oct-09	5	6	4	-	-	-
Nov-10	7	14	12	-	-	-
Total	16	20	16	4	0	0

3.3.1 Lipid profile of eggs

Overall, 16 samples of fertilised eggs were analysed from Arno Bay (2008 and 2009) and 4 samples from Port Augusta (2008 only). There were no significant differences in fatty acid profile between the Arno Bay and Port Augusta egg samples in 2008 (Table 11). Within the samples from Arno Bay, the proportion of EPA in the total fatty acids (TFA) was significantly higher in 2008 than 2009, also reflected in higher total n-3 polyunsaturates and higher ratio of n-3 to n-6 HUFAs. The proportion of total saturates was lower in 2008 than 2009. Within the samples from Port Augusta in 2008, 2 were nominated ‘poor’ and two ‘good’ according to subsequent larval performance. However, there were no apparent differences in lipid profile of eggs that went in the ‘good’ and ‘poor’ tanks.

The overall range in TFA in eggs analysed from 2008 and 2009 was 64.1 to 119.7 mg g^{-1} DW and mean of ~96 mg g^{-1} DW. This is 64% of the TFA content measured in fertilised eggs from 2007 in Arno Bay and Port Augusta (~150 mg g^{-1} DW; Battaglene and Cobcroft, 2008). Thus, the absolute amount of TFA available per larva, including essential FA, would have been lower in 2008 and 2009 than in 2007; however, the implication of the lower level is unknown.

The DHA:EPA ratio in 2008 and 2009 was 3.8:1 and 3.9:1, slightly lower than values in 2007 (being 4.2:1 in Arno Bay, and 4.7:1 in Port Augusta) (Battaglene and Cobcroft, 2008). This is similar to other cultured marine fish (Sargent, 1995; Morehead et al., 2001).

In 2009, two YTK egg samples were analysed for lipid class. In the limited sample size, the phospholipid content of 20 to 25% was less than half of the value reported for YTK in individual egg samples (58%) analysed in New Zealand (Hilton et al., 2008) (Table 12). This may be attributed to sample storage, analysis methodology, different broodstock diets, or time of spawning within the season. Phospholipids are known to be a vital component of larval fish diets as they improve lipid transport from the gut to larval tissues resulting in increased growth and survival (Tocher et al., 2008). Sargent (1995) argues that the ideal diet for larval fish is to match the biochemical composition of the egg, while Conceição et al. (2010) promote matching the biochemical profile of wild zooplankton (usually copepods). Consequently, a higher egg phospholipid content may be beneficial to subsequent larval performance. Further analyses would be required on YTK from CST if lipid class was considered a determinative factor in YTK larval performance.

Table 11. Summary of fatty acid composition of fertilised eggs of Yellowtail Kingfish sampled in 2008 and 2009. ANOVA on data from 2008 showed no significant differences between eggs from Arno Bay and Port Augusta. ANOVA on Arno Bay 2008 and 2009 data showed some significant differences (ns, not significant P>0.05; * P<0.05; ** P<0.01).

	2008		2008		2009		ANOVA (*P*)
	Arno Bay		Port Augusta		Arno Bay		Arno Bay
	Mean	SD	Mean	SD	Mean	SD	2008 vs 2009
Fatty acids (% TFA)							
20:4n-6 (ARA)	1.4	0.2	1.4	0.1	1.4	0.3	ns 0.938
20:5n-3 (EPA)	6.5	0.4	6.3	0.5	5.8	0.5	*** 0.032**
22:6n-3 (DHA)	24.4	2.0	23.7	0.6	22.7	2.0	ns 0.151
Total saturates	25.3	1.1	26.6	1.5	27.5	1.5	*** 0.018**
Total monos	31.9	2.6	31.3	1.4	32.3	2.1	ns 0.776
Total polys	42.8	2.2	42.0	1.4	40.2	2.8	ns 0.122
Total n-3 polys	37.1	1.9	36.0	1.6	33.3	3.0	*** 0.032**
Total n-6 polys	5.6	0.4	6.1	0.4	5.9	0.5	ns 0.245
Total fatty acids (mg/g)	105.8	8.4	90.2	18.7	92.7	15.2	ns 0.125
n-3/n-6 HUFA	6.6	0.4	5.9	0.6	5.6	0.5	**** 0.005**
DHA:EPA	3.8	0.4	3.8	0.3	3.9	0.1	ns 0.330
EPA:ARA	4.6	0.5	4.6	0.6	4.7	2.7	ns 0.978
Total lipid (mg/g)	166.5	10.5	138.0	29.9	162.2	14.7	ns 0.596

Table 12. Lipid class composition of two samples of fertilised eggs of Yellowtail Kingfish sampled in 2009 compared with published data from New Zealand.

YTK fertilised egg sample code	15/09/2009 2(12+13)		6/09/2009 2(5)		NZ YTK Hilton et al., 2008	
Lipid class (%)						
Triacylglycerides	28.4	± 0.1	30.8	± 0.3	14	
Diacylglycerides	0.3	± 0.1	0.4	± 0.0	-	
Monoacylglycerides	0.0	± 0.0	0.0	± 0.0	-	
Free fatty acids	1.3	± 0.0	2.9	± 0.0	0.5	
Sterols	3.9	± 0.1	3.8	± 0.0	4	
Hydrocarbons/ steryl esters	41.5	± 0.4	42.2	± 0.5	24	
Phospholipids	24.6	± 0.8	19.9	± 0.7	58	
Sum	100		100		-	
% lipid (dry wt.)	16.5		18.0		-	

3.3.2 Vitamin profile of eggs

The vitamin content (C, E and A) of fertilised eggs was measured from 4 samples each from Arno Bay and Port Augusta in 2008, and from twelve samples from Arno Bay in 2009 (Table 13). The α-tocopherol (vitamin E) concentrations were similar to those found in 2007 (80 – 111 µg g^{-1}; Battaglene and Cobcroft, 2008), although the minimum values in Arno Bay in both years (54 and 45 µg g^{-1}) were lower than in 2007. Overall, the averages were lower than for Striped Trumpeter eggs (127 ± 32 µg g^{-1}; range from 82 to 170 µg g^{-1} (Battaglene and Brown, 2006)). There was an association between α-tocopherol content of the eggs and 'good' and 'bad' larval performance in Port Augusta in 2008, with lower concentration (80 ± 2 µg g^{-1}) for 'good' fish and higher concentration (114 ± 2 µg g^{-1}) for 'poor' fish. This data should be interpreted with caution, given the small sample size and the instability of vitamins during storage. Based on the average content in Striped Trumpeter eggs, it is unlikely that the α-tocopherol content of the YTK eggs was driving differences observed in larval performance.

There was high variability in the measured ascorbic acid (vitamin C) content of the YTK eggs, especially in Port Augusta in 2008 and in Arno Bay in 2009, where there was a 14- and 28-fold difference in minimum and maximum values, respectively (Table 13). A similar level of variation was found in 2 YTK samples analysed in 2007 (30 and 443 µg g^{-1}). In comparison, Striped Trumpeter eggs contained 540 ± 200 µg g^{-1} (range 360 to 900 µg g^{-1}) (Battaglene and Brown, 2006). The variation in YTK egg content may be the result of the timing of feeding vitamins to broodstock (if not in every feeding event), the vitamin intake of individual broodstock, and the time of spawning relative to the last vitamin C feeding event. There was no association between ascorbic acid content of the eggs and larval performance in Port Augusta in 2008. It is possible that the low concentration of ascorbic acid measured in some batches in all years is an artefact caused by sub-optimal freezing and storing conditions (M. Brown; pers. comm..).

Retinol (vitamin A) was detected in YTK eggs in 2008 (Table 13), at lower levels than for retinol palmitate measured in 2007 (14-31 µg g^{-1}). The 2008 values are within the range (0.26 to 22 µg g^{-1}) reported in a review by Lubzens et al. (2003). Vitamin A, especially at high doses, has been linked with skeletal deformities, particularly jaw deformity, through the varied gene expression of retinoic acid receptors and retinoid X receptors in seabass (Villeneuve et al., 2006) and Japanese flounder (Haga et al., 2003) larvae. Vitamin A can be delivered by various routes, including: water bath (not

likely to be relevant in a commercial larval rearing situation), through live prey diet, and through the egg composition via manipulation of broodstock diet. Fontagné-Dicharry et al. (2010) found improved growth with low vitamin A incorporation in rainbow trout broodstock diet and eggs, whilst survival was lower in fry from eggs from broodstock fed higher vitamin A. The concentrations and the form of vitamin A in the egg (e.g. retinyl palmitate and retinol as storage forms vs retinoic acid the active and potentially teratogenic form), and species-specific responses to vitamin A, will likely impact the response of developing fish larvae to vitamin A. Taken together, the implications of YTK egg vitamin C, E and A content from the present study on larval quality and performance are unknown and would require additional research.

Table 13. Vitamin composition of fertilised eggs of Yellowtail Kingfish sampled in 2008 and 2009.

Sample information			Vitamin concentration (µg/g)				
Year	Hatchery		Ascorbic acid	Tocopherol		Total tocopherol	Retinol *
				α–	δ–		
2008	Arno Bay	Minimum	266	54	1	56	detected
	(n = 4)	Maximum	406	92	2	94	4
		Mean ± SD	**319 ± 62**	**71.9 ± 15.7**	**1.4 ± 0.7**	**73.3 ± 16.3**	
2008	Port Augusta	Minimum	58	79	1	80	detected
	(n = 4)	Maximum	809	115	1	116	≈ 1.6
		Mean ± SD	**381 ± 375**	**97.1 ± 19.4**	**1.1 ± 0.2**	**98.3 ± 19.5**	
2009	Arno Bay	Minimum	31	45	0	45	nd
	(n = 12)	Maximum	876	143	2	143	nd
		Mean ± SD	**277 ± 276**	**85 ± 33**	**0 ± 1**	**85 ± 33**	**nd**

nd = not determined

3.3.3 Lipid profile of rotifers

A total of twenty samples of rotifers were analysed for biochemical composition. Of those, seven were duplicate analyses of the same sample for replication. There was some variation in lipid content of S-presso enriched large-strain rotifers, which is the standard rotifer enrichment at CST. Variation was most notable in the proportions of DHA, total fatty acid and total lipids content (Table 14). Looking at the sampling dates, there was one day (26/10/2009) when the sampled enriched rotifers had a proportion of DHA (3.6%) and total lipid content (96.3 mg g^{-1}) that were lower than non-enriched rotifers (5.4% and 118.6 mg g^{-1}), and very low compared with other enriched samples where the content was 6.4 to 9.9% DHA and 108.3 to 144.6 mg g^{-1} (Table 15).

There were some differences in the profile of large- and small-strain rotifers enriched with S-presso, although results must be interpreted with caution as only one small-strain sample was analysed (Table 14). Small-strain rotifers had a higher proportion of DHA and lower proportion of EPA, which resulted in a higher DHA:EPA ratio than in enriched large-strain rotifers. The total fatty acid and total lipid content were similar.

The lipid composition of the single sample of algae-enriched rotifers (*Isochrysis* sp. and *Nannochloropsis* sp.) had a higher total lipid content (150mg/g), and DHA content (17.5% = 26.4mg/g) that was more than double that of other samples, resulting in an elevated DHA:EPA ratio of 3.7:1, which was the highest of all samples and closest to the composition of YTK egg samples. Further research would be required to determine if this is a repeatable enrichment result, and whether it may be an appropriate supplement or alternative to S-presso enriched rotifers.

Table 14. Summary of fatty acid composition of rotifers fed to marine finfish larvae at CST in 2009 and 2010. Uneven sample sizes and low numbers of replicates in some groups do not permit statistical analysis of these data.

	Overall									
Fatty Acids (mg/g)	**Large - non-enriched**		**Large - enriched**		**Large - Spirit enriched**		**Large - Iso & Nanno enriched**		**Small - enriched**	
sample dates	n = 3		n = 7		n = 1		n = 1		n = 1	
Fatty acids (% TFA)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
22:6n-3 (DHA)	5.4	3.0	7.5	2.4	1.9		17.5		8.8	
20:4n-6 (ARA)	1.2	0.4	1.2	0.2	1.8		1.6		1.3	
20:5n-3 (EPA)	5.4	2.2	4.3	0.5	7.7		4.7		3.5	
Total saturates	25.8	1.5	25.8	2.3	23.1		23.0		30.4	
Total monounsaturates	40.5	6.1	39.7	0.8	47.0		33.4		34.7	
Total polyunsaturates	33.7	4.5	34.4	2.8	29.9		43.7		35.0	
Total n-3 polyunsaturates	15.1	0.7	15.8	3.2	14.5		26.4		16.9	
Total n-6 polyunsaturates	18.2	4.1	17.7	0.9	14.6		16.7		18.0	
Total fatty acids (mg/g)	62.0	23.3	76.6	20.0	34.0		78.9		73.5	
n-3/n-6 HUFA	0.9	0.2	0.9	0.2	1.0		1.6		0.9	
DHA:EPA	1.2	0.9	1.7	0.4	0.2		3.7		2.5	
EPA:ARA	4.4	0.4	3.7	0.4	4.2		2.9		2.8	
Total lipid (mg/g)	118.6	24.6	128.9	19.2	84.2		150.7		136.1	

The single sample of large strain Spirit-enriched rotifers had a poor biochemical profile, similar to non-enriched rotifers, and additional trials would be needed to test whether this enrichment can be used in a commercial context to achieve appropriate lipid enrichment to support YTK survival, growth and development.

In relation to lipid class, just over 40% of the lipid content in S-presso enriched rotifers were free fatty acids, and 14.5% were phospholipids (Table 16). This is a very different composition to YTK eggs, which contained low free fatty acids (< 3%) and approx 20 to 25% phospholipids. The total lipid contents of rotifers and *Artemia* in this study were comparable to levels reviewed in Conceição et al. (2010), and were higher than reported values for copepod nauplii (8.6%) and adults (11%) (see Table 2 of Conceição et al., 2010).

Table 15. Fatty acid composition of rotifers sampled on thirteen different dates at CST in 2009 and 2010.

	24/03/2009		8/04/2009		2/09/2009		28/09/2009		12/09/2009		20/09/2009		21/10/2009		26/10/2009		18/12/2009		22/10/2009		3/02/2010		15/02/2010		15/02/2010	
Fatty Acids (mg/g)	**Large - enriched***		**Small - enriched***		**Large - enriched***		**Large - enriched***		**Large - enriched***		**Large - enriched***		**Large - non-enriched**		**Large - enriched***		**Large - enriched***		**Large - non-enriched**		**Large - Spirit enriched**		**Large - non-enriched**		**Large - Iso & Nanno enriched**	
Fatty acids (% TFA)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
22:6n-3 (DHA)	9.9		8.8		9.8	1.7	9.7	0.1	6.6	0.1	6.4	0.0	7.7	0.2	3.6	0.3	6.4	0.0	6.6		1.9		2.0		17.5	
20:4n-6 (ARA)	1.3		1.3		1.3	0.1	1.3	0.0	1.1	0.0	1.1	0.0	1.0	0.0	0.9	0.1	1.2	0.0	1.0		1.8		1.7		1.6	
20:5n-3 (EPA)	4.7		3.5		4.6	0.2	4.5	0.1	4.7	0.1	3.8	0.0	4.1	0.0	3.8	0.0	3.7	0.1	4.0		7.7		8.0		4.7	
Total saturates	22.9		30.4		24.0	1.8	24.6	0.1	25.4	0.1	29.1	0.1	27.1	0.1	26.5	1.1	28.3	0.0	26.2		23.1		24.1		23.0	
Total monounsaturates	39.0		34.7		39.9	1.2	38.6	0.2	39.5	0.1	40.4	0.4	35.5	0.1	40.7	2.1	40.0	0.1	38.7		47.0		47.2		33.4	
Total polyunsaturates	38.0		35.0		36.1	3.0	36.8	0.2	35.1	0.2	30.5	0.3	37.4	0.2	32.8	3.2	31.7	0.2	35.1		29.9		28.7		43.7	
Total n-3 polyunsaturates	19.1		16.9		18.8	2.4	19.0	0.3	15.4	0.1	13.6	0.1	15.9	0.3	11.0	0.8	13.6	0.2	14.7		14.5		14.8		26.4	
Total n-6 polyunsaturates	18.5		18.0		17.1	0.4	17.6	0.3	19.2	0.1	16.4	0.3	21.1	0.0	17.5	1.2	17.6	0.0	20.0		14.6		13.4		16.7	
Total fatty acids (mg/g)	106.4		73.5		92.2	21.7	87.7	8.3	73.6	0.2	69.2	4.4	83.3	7.9	49.7	11.1	57.5	3.4	65.8		34.0		37.1		78.9	
n-3/n-6 HUFA	1.0		0.9		1.1	0.1	1.1	0.0	0.8	0.0	0.8	0.0	0.8	0.0	0.6	0.0	0.8	0.0	0.7		1.0		1.1		1.6	
DHA:EPA	2.1		2.5		2.1	0.3	2.2	0.1	1.4	0.0	1.7	0.0	1.9	0.0	1.0	0.1	1.7	0.0	1.6		0.2		0.3		3.7	
EPA:ARA	3.6		2.8		3.6	0.2	3.5	0.1	4.3	0.1	3.6	0.0	4.2	0.0	4.1	0.3	3.1	0.0	4.1		4.2		4.8		2.9	
Total lipid (mg/g)	140.8		136.1		144.6	8.7	144.4	10.9	138.0	1.4	129.5	8.7	143.9	9.7	96.3	28.4	108.3	5.8	117.2		84.2		94.8		150.7	

* standard enrichment at Cleanseas Tuna is Spresso (INVE)

Table 16. Lipid class composition of two samples of live feeds collected in Arno Bay in 2009.

Live food (sample detail)	Rotifers (2/9/2009 - Spresso enriched large strain)			*Artemia* (15/9/2009 - enriched)	
Lipid class (%)					
Triacylglycerides	33.2	± 0.2		65.7	± 1.5
Diacylglycerides	1.0	± 0.1		3.2	± 0.1
Monoacylglycerides	2.1	± 1.0		1.3	± 0.3
Free fatty acids	40.9	± 1.6		13.5	± 0.3
Sterols	2.2	± 0.1		3.9	± 0.0
Hydrocarbons/ steryl esters	6.2	± 0.3		0.3	± 0.0
Phospholipids	14.5	± 0.8		12.3	± 1.5
Sum	100			100	
% lipid (dry wt.)	15.1			35.1	

3.3.4 Lipid profile of Artemia

Enrichment of *Artemia* with A1 DHA Selco (INVE; formerly named DC DHA Selco), increased total lipid and total fatty acid contents by around 55%, with substantial increases in DHA, ARA and EPA (Table 17). S-presso enrichment, measured in a single sample, increased the proportion of DHA to almost double that of the DHA Selco enrichment, although the overall total lipid and total fatty acid contents were only slightly elevated over non-enriched *Artemia*. The DHA:EPA ratio of A1 DHA Selco enriched *Artemia* was around 1:1 (similar to 2007 sample results; Battaglene and Cobcroft, 2008), 10 fold higher than non-enriched, and reached 2.4 in the S-presso enriched *Artemia*. There was variation between sample days in A1 DHA Selco enriched *Artemia*, particularly in total lipid content which ranged from 250 to 457 mg g-1 (Table 18).

The A1 DHA Selco *Artemia* contained less DHA (6.2% equivalent to 16 mg g^{-1}) than Algamac-enriched *Artemia* (18 mg g^{-1}; Bransden et al., 2005), but was higher than the 2007 sample data (12.3 mg g^{-1}; Battaglene and Cobcroft, 2008). This suggests improvement in *Artemia* enrichment techniques between 2007 and 2010, however testing of new commercial and experimental products is recommended in the context of improving formulations to match larval requirements. For additional context of the *Artemia* results see review by Conceição et al. (2010) and appendices in Battaglene and Cobcroft (2008).

Table 17. Summary of fatty acid composition of *Artemia* fed to marine finfish larvae at CST in 2009 and 2010.

			3/02/2010		2/11/2009	
Fatty Acids (mg/g)	**DC DHA / A1 DHA - enriched**		**Spresso - enriched**		**non-enriched**	
Fatty acids (% TFA)	Mean	SD	Mean	SD	Mean	SD
22:6n-3 (DHA)	6.2	1.4	11.2		0.2	0.0
20:4n-6 (ARA)	1.1	0.2	1.6		0.3	0.0
20:5n-3 (EPA)	6.0	1.0	4.6		1.6	0.0
Total saturates	17.1	3.3	19.2		19.0	0.1
Total monounsaturates	41.3	2.2	29.5		34.8	0.2
Total polyunsaturates	41.6	3.9	51.2		46.2	0.3
Total n-3 polyunsaturates	29.9	3.6	32.8		37.9	0.3
Total n-6 polyunsaturates	11.3	0.9	17.9		7.7	0.0
Total fatty acids (mg/g)	264.5	73.9	173.4		167.3	24.4
n-3/n-6 HUFA	2.7	0.3	1.8		4.9	0.0
DHA:EPA	1.0	0.1	2.4		0.1	0.0
EPA:ARA	5.4	0.5	2.9		5.4	0.1
Total lipid (mg/g)	349.6	71.5	256.0		222.7	25.8

Table 18. Fatty acid composition of *Artemia* sampled on different dates at CST in 2009 and 2010.

	8/04/2009		12/09/2009		15/09/2009		15/09/2009		18/09/2009		24/09/2009		28/10/2009		29/10/2009		3/02/2010		2/11/2009	
Fatty Acids (mg/g)	**DC DHA / A1 DHA - enriched**		**DC DHA / A1 DHA - enriched**		**DC DHA / A1 DHA - enriched**		**DC DHA / A1 DHA - enriched***		**DC DHA / A1 DHA - enriched***		**DC DHA / A1 DHA - enriched**		**DC DHA / A1 DHA - enriched**		**DC DHA / A1 DHA - enriched**		**Spresso - enriched**		**non-enriched**	
Fatty acids (% TFA)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
22:6n-3 (DHA)	5.2		3.7	0.3	7.5		5.9		6.8	0.2	6.8	0.8	5.4	0.0	7.9	0.7	11.2		0.2	0.0
20:4n-6 (ARA)	0.9		0.9	0.1	1.4		1.2		1.2	0.0	1.2	0.1	1.0	0.0	1.1	0.1	1.6		0.3	0.0
20:5n-3 (EPA)	5.3		4.3	0.1	7.5		6.2		6.3	0.1	6.3	0.4	5.8	0.0	6.9	0.6	4.6		1.6	0.0
Total saturates	17.5		21.3	1.5	15.8		17.3		17.1	0.2	17.9	0.9	17.6	0.1	12.0	6.3	19.2		19.0	0.1
Total monounsaturates	39.0		44.8	0.5	39.2		42.8		41.4	0.4	39.3	1.2	41.1	0.1	42.7	2.9	29.5		34.8	0.2
Total polyunsaturates	43.5		33.9	1.0	45.0		39.9		41.5	0.6	42.8	2.1	41.3	0.0	45.2	3.4	51.2		46.2	0.3
Total n-3 polyunsaturates	31.5		22.7	0.4	33.4		28.2		30.0	0.6	32.1	1.7	28.9	0.0	32.3	2.3	32.8		37.9	0.3
Total n-6 polyunsaturates	12.0		10.2	0.6	11.6		11.0		10.8	0.0	10.4	0.0	11.8	0.0	12.3	1.0	17.9		7.7	0.0
Total fatty acids (mg/g)	193.8		413.7	67.5	272.6		251.3		255.4	6.2	215.6	19.2	224.1	6.0	289.7	40.8	173.4		167.3	24.4
n-3/n-6 HUFA	2.6		2.2	0.1	2.9		2.6		2.8	0.1	3.1	0.2	2.4	0.0	2.6	0.0	1.8		4.9	0.0
DHA:EPA	1.0		0.9	0.1	1.0		1.0		1.1	0.0	1.1	0.1	0.9	0.0	1.1	0.0	2.4		0.1	0.0
EPA:ARA	5.6		5.0	0.3	5.3		5.0		5.2	0.1	5.2	0.0	6.0	0.2	6.2	0.2	2.9		5.4	0.1
Total lipid (mg/g)	250.3		417.0	17.9	350.6		341.8		371.7	1.6	284.3	17.1	324.3	4.9	456.5	94.4	256.0		222.7	25.8
* assumed to be enriched samples																				

3.3.5 Vitamin profile of rotifers

The four large-strain rotifer samples analysed contained similar concentrations of α-tocopherol (237 to 839 µg g^{-1}), with two samples higher than the range from 2007 samples (250 to 323 µg g^{-1}; Battaglene and Cobcroft, 2008) and with one sample higher than the range of concentrations we have reported in rotifers cultured and enriched with Algamac at IMAS's laboratories (60 to 400 µg g^{-1}; Battaglene and Brown, 2006). The small strain rotifers had a lower level of α-tocopherol (51 µg g^{-1}).

Concentrations of ascorbic acid in enriched rotifers were highly variable, both between the four large-strain enriched samples and within replicate samples (Table 19). Some variability is likely reflecting changes between enrichment dates, but other variation may be attributed to the rapid loss of vitamin C with suboptimal storage. Concentrations were within the same range as ascorbate measured in 2007 samples (710 to 1247 µg g^{-1}; Battaglene and Cobcroft, 2008), except for the sample on 2/9/2009 that was similar to unenriched rotifers (130 to 370 µg g-1; Brown and Lavens, 2000). Small-strain enriched rotifers ascorbate levels were below the 2007 sample values. Overall, the range of values was higher and lower than rotifers enriched with Algamac at IMAS (310 to 770 ug g-1; Battaglene and Brown, 2006).

3.3.6 Vitamin profile of Artemia

The concentration of ascorbic acid in *Artemia* was variable between days and between replicate samples, as seen in the rotifers. The range was 172 to 282 µg g^{-1}, with a mean of 228 µg g^{-1} (Table 20). This was approximately half of the concentrations found in 2007 samples (389 to 445 µg g^{-1}; Battaglene and Cobcroft, 2008) and suggestive of an enrichment product that does not contain additional vitamin C. Additional vitamin C supplementation in enrichment could be investigated for potential beneficial effects of YTK performance during *Artemia* feeding and weaning to formulated diets.

The concentration of α-tocopherol in *Artemia* was in the range of 126 to 206 µg g^{-1}, with a mean of 187 µg g^{-1} (Table 20), that was similar and a broader range than levels measured in 2007 (142 to 156 µg g^{-1}; Battaglene and Cobcroft, 2008). These levels were similar to those reported for *Artemia* nauplii enriched with low concentrations of α-tocopherol acetate (Huo et al.,1999), and greater than those of nauplii fed with microalgae (31 to 47 µg g^{-1}; Vismara et al., 2003).

Table 19. Vitamin C and E composition of enriched rotifers collected in Arno Bay in 2009.

Sample information			Vitamin concentration (µg/g)			
			Ascorbic acid	Tocopherol		Total
				α–	δ–	tocopherol
24/03/2009	Large - enriched	Mean	862	368	0	368
		± SD	-	-	-	-
8/04/2009	Small - enriched	Mean	468	51	3	54
		± SD	-	-	-	-
2/09/2009	Large - enriched	Mean	155	247	9	256
		± SD	48	51.0	0.2	51.2
12/09/2009	Large - enriched	Mean	948	237	5	242
		± SD	166	2	1	2
20/09/2009	Large - enriched	Mean	1157	839	17	856
		± SD	76	30	1	31

Table 20. Vitamin C and E composition of enriched *Artemia* collected in Arno Bay in 2009.

Sample information			Vitamin concentration (µg/g)			
			Ascorbic acid	Tocopherol		Total
				α–	δ–	tocopherol
8/04/2009	enriched	Mean	186	125	2	126
		± SD	-	-	-	-
24/09/2009	enriched	Mean	282	168	0	168
		± SD	84	0	0	0
12/09/2009	enriched	Mean	172	203	2	206
		± SD	93	106	3	110
15/09/2009	enriched	Mean	240	204	2	206
		± SD	-	-	-	-
18/09/2009	enriched	Mean	247	205	1	206
		± SD	176	5	2	7
Overall	**enriched**	**Mean**	**228**	**185**	**1**	**187**
		± SD	**94**	**50**	**2**	**51**

3.4 Histopathology

The final reports from DPIPWE, Fish Health Unit from two batches of Yellowtail Kingfish larvae and juveniles (n = 160 cassettes each from 2008 and 2009) were provided to CST in October 2009 and June 2010, respectively (Appendix 4). The reports confirmed non-inflation of swimbladders by histology in groups of larvae which were known to have low inflation rates based on microscopic evaluation of whole larvae. The appearance of non-inflated swimbladders was distinctive and showed a collapsed lumen, with infiltration of cuboidal epithelial cells and folding of the epithelium (Figure 14), similar to observations by Trotter et al. (2001) in Striped Trumpeter.

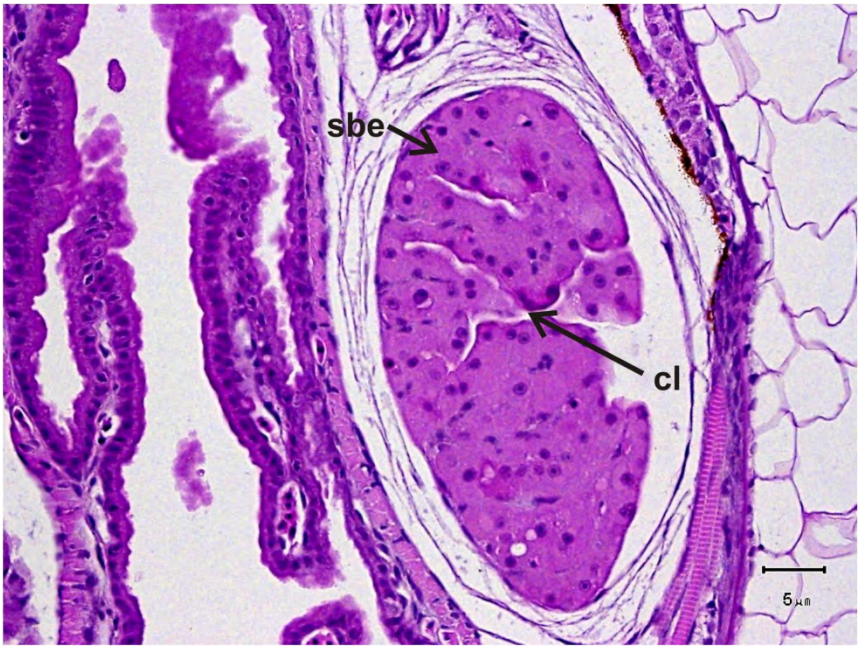


Figure 14. Histopathology section of YTK larva from the SARDI rotifer density experiment (Nov 2008) at 13 DPH, with a non-inflated swimbladder. Abbreviations: cl, collapsed lumen; sbe, swimbladder epithelium. Scale bar is 5 µm. Image provided by Graeme Knowles, DPIPWE, Fish Health Unit.

In 2008 samples (Table 21), minor changes in the liver of larvae were observed as diffuse feathery vacuolation of hepatocytes, most likely indicative of glycogen deposition. Non-inflated swimbladders were detected throughout all tanks and ages of larvae to varying proportion. The lowest inflation rates occurred in the SARDI 2008 rotifer density experiment where only one out of 39 sectioned fish was inflated. There were some changes in pyloric caecae (including low numbers of sloughed cells, and mild diffuse microvesiculation of the epithelium), proximate intestine (including mild

diffuse microvesiculation of epithelium), and distal intestine (a small amount of engulfed material and bacteria only observed in Port Augusta Run 1 at 20 DPH). The microvesiculation of intestinal epithelium is likely accumulation of lipid from digested diet, prior to transportation away from the gut. Some changes were seen in larval gills (short and fine secondary lamellar), that were potentially due to the early developmental stage of these structures. In juveniles in 2008, there were no differences between hatcheries. The changes observed were moderate feathery vacuolation in the hepatocytes and congestion in the liver, and also congestion in the brain tissue. In one fish of five from each hatchery, there were mild multifocal melanomacrophage foci in the spleen.

In 2009 samples (Table 22), similar observations were made to 2008, with the addition of changes to the gills in Arno Bay Run 1, where short gill filaments and no lamellae were seen in larvae sampled from tanks L1 and L2. The implications of these observations are not known.

Table 21. 2008 YTK larvae histopathology results from both CST hatcheries and SARDI facility. Average histopathology score in different production runs with larvae of different ages, for different tissues. Scoring system 0= normal; 1 = mild changes; 2 = moderate changes; 3 = marked / significant changes. For swimbladder, 0 = inflated (normal), and 1 = non-inflated.

2008				Average score for tissue/organ															
Hatchery	Run/Expt	Fish Age	Tank	Eye	Brain	Liver	Heart	Pancreas	Swimbladder	Stomach	Pyloric caeca	Prox Intestine	Dist. Intestine	Muscle	Cartilage/bone	Gills	Skin	Spleen	Kidney
AB	Pre Run 1	5		0.0	0.0	0.1	0.0	0.0	0.2		0.0	0.0		0.0	0.0		0.0		0.0
		10		0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0	1.0	0.0		0.0
	Pre Run 1 Total			**0.0**	**0.0**	**0.1**	**0.0**	**0.0**	**0.1**		**0.0**	**0.0**		**0.0**	**0.0**	**1.0**	**0.0**		**0.0**
AB	Run 1	5		0.0	0.0	0.0	0.0	0.0	0.1		0.0	0.0		0.0	0.0		0.0		0.0
		10		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
		15		0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.4	0.5	0.0	0.0	0.0	1.0	0.0		0.0
		20		0.0	0.0	0.5	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0		0.0
		Juveniles		0.0	1.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.3	0.0
	Run 1 Total			**0.0**	**0.1**	**0.3**	**0.0**	**0.0**	**0.1**	**0.0**	**0.1**	**0.1**	**0.0**	**0.0**	**0.0**	**0.7**	**0.0**	**0.3**	**0.0**
AB	Run 2	5		0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0		0.0		0.0
		10		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
		15		0.0	0.0	0.3	0.0	0.0	0.2		0.4	0.0		0.0	0.0	0.9	0.0		0.0
		20		0.0	0.0	0.5	0.0	0.0	0.1		0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Run 2 Total			**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.2**	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.6**	**0.0**	**0.0**	**0.0**
AB	Run 3	5		0.0	0.0	0.0	0.0	0.0	0.1		0.0	0.0		0.0	0.0		0.0		0.0
		10		0.0	0.0	0.0	0.0	0.0	0.3		0.0	0.0		0.0	0.0	1.0	0.0		0.0
		15		0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0
		20		0.0	0.0	0.7	0.0	0.0	0.0		1.0	0.0		0.0	0.0	0.0	0.0		0.0
	Run 3 Total			**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.2**	**0.0**	**0.2**	**0.0**		**0.0**	**0.0**	**0.3**	**0.0**		**0.0**
AB Total				**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.2**	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.7**	**0.0**	**0.1**	**0.0**
PA	Run 1	5		0.0	0.0	0.1		0.0	0.3	0.0	0.1	0.0		0.0	0.0		0.0		0.0
		10		0.0	0.0	0.1	0.0	0.0	0.3	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
		15		0.0	0.0	0.2	0.0	0.0	0.4	0.0	0.4	0.0		0.0	0.0	1.0	0.0		0.1
		20		0.0	0.0	0.8	0.0	0.0	0.2	0.0	0.7	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
		Juveniles		0.0	1.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.2	0.0	0.3	0.0
	Run 1 Total			**0.0**	**0.0**	**0.4**	**0.0**	**0.0**	**0.3**	**0.0**	**0.3**	**0.0**	**0.5**	**0.0**	**0.0**	**0.6**	**0.0**	**0.1**	**0.0**
PA	Run 2	5		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.5	0.0	0.0	0.0
		10		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0	0.0	0.0
		15		0.0	0.0	0.3	0.0	0.0	0.2	0.0	0.3	0.1		0.0	0.0	0.1	0.0		0.0
		20		0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.8	0.0		0.0	0.0	0.3	0.0		0.0
	Run 2 Total			**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	**0.3**	**0.0**		**0.0**	**0.0**	**0.4**	**0.0**	**0.0**	**0.0**
PA Total				**0.0**	**0.0**	**0.3**	**0.0**	**0.0**	**0.1**	**0.0**	**0.3**	**0.0**	**0.5**	**0.0**	**0.0**	**0.5**	**0.0**	**0.1**	**0.0**
SARDI	Rot Nov08	13		0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
SARDI Total				**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.0**	**0.0**		**0.0**
Grand Total				**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.2**	**0.0**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	**0.6**	**0.0**	**0.1**	**0.0**

Table 22. 2009. YTK larvae histopathology results from both CST hatcheries. Average histopathology score in different production runs and with larvae of different ages, for different tissues. Scoring system 0= normal; 1 = mild changes; 2 = moderate changes; 3 = marked / significant changes. For swimbladder, 0 = inflated (normal), and 1 = non-inflated.

2009				Average score for tissue/organ																
Hatchery	Run/Expt	Fish Age	Tank	Eye	Brain	Liver	Heart	Pancreas	Swimbladder	Stomach	Pyloric caeca	Prox Intestine	Dist. Intestine	Muscle	Cartilage/bone	Gills	Skin	Spleen	Kidney	Yolk sac
AB	1	1		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0
		5		0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0		0.0	0.0
		10		0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0		0.0	
		15		0.0	0.0	0.4	0.0	0.0	0.4	0.0	0.7	0.7	0.6	0.0	0.0	0.1	0.0		0.0	
		20		0.0	0.0	0.7	0.0	0.0	0.3	0.0	0.8	0.8	0.8	0.0	0.0	0.0	0.0		0.0	
AB	**1 Total**			**0.0**	**0.0**	**0.3**	**0.0**	**0.0**	**0.3**	**0.0**	**0.4**	**0.4**	**0.4**	**0.0**	**0.0**	**0.1**	**0.0**		**0.0**	**0.0**
AB	2	1		0.0	0.0	0.0	0.0	0.0	0.3	0.0				0.0			0.0			0.0
		2		0.0	0.0					0.0				0.0			0.0			0.0
		5		0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		10		0.0	0.0	0.2	0.0	0.0	0.5	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0		0.0	
		15		0.0	0.0	0.9	0.0	0.0	0.3	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
		20		0.0	0.0	0.6	0.0	0.0	0.4	0.0	0.8	0.8	0.8	0.0	0.0	0.0	0.0		0.0	
AB	**2 Total**			**0.0**	**0.0**	**0.4**	**0.0**	**0.0**	**0.4**	**0.0**	**0.5**	**0.5**	**0.5**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	**0.0**
AB	3	1		0.0	0.0					0.0				0.0			0.0			0.0
		5		0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		10		0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		15		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.3	0.3	0.3	0.0	0.0	0.0	0.0		0.0	
		20		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2	0.2	0.2	0.0	0.0	0.0	0.0		0.0	
		21		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0		0.0	
AB	**3 Total**			**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.2**	**0.0**	**0.1**	**0.1**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	**0.0**
AB Total				**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.3**	**0.0**	**0.3**	**0.3**	**0.3**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	**0.0**
PA	1	27		0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
PA Total				**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	
Grand Total				**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.3**	**0.0**	**0.3**	**0.3**	**0.3**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	**0.0**

4. Further Development

Clearly, within the constraints of a commercial hatchery it can be difficult to setup and run controlled replicated trials. That was demonstrated by the limitations of experiments and analyses reported in this project.

Whilst most of the trials reported in this project were confounded by changes in more than one variable, and/or had no appropriate control(s), the data did throw light on several critical areas of hatchery practice. These were:

- The use, training in and quality control checks of accurate sampling methods for estimating mortality and deformity rates – the key performance criteria for the hatchery. Not only should external collaborators be used to benchmark existing practices and procedures, but internally there should be a level of cross-checking of data, especially in areas where staff turnover is high. This emphasises the need for 'living' SOPs that are routinely used by hatchery staff.
- Swimbladder inflation is an area that needs additional work to improve the understanding of when and how this occurs. This will allow hatchery staff to better anticipate the needs of the larvae at this time and to provide the optimal conditions that allow it to happen successfully.
- Abiotic conditions do appear to affect larval survival and deformity rates. Of the factors that were varied in this project the two that appear to be most interesting are tank hydrodynamics (method and purpose), and illumination. Tank wall colour did not seem to be as important, although it is possible that it could become important if, and when, the hydrodynamics and illumination conditions are better resolved.

5. Conclusions

Malformations that arise during larval rearing in Yellowtail Kingfish remain a major impediment and cost to the production of farmed fish. There is now three years' detailed data on which to better understand the nature of the problem, using the differences among hatcheries and different runs within and between seasons as a basis to identify the factor(s) that correlate with either good or poor production outcomes.

While the implementation of treatments to improve fish quality by a better understanding of those contributing factors have, to date (2011), been largely unsuccessful, there have been many mitigating factors which have made analysis difficult. First, there are possible genetic and/or egg quality factors involved, and for practical and logistical reasons the broodstock has varied over the course of the project. Secondly, changes in staffing levels and workload expectations (such as decisions to running both the nursery and outdoor tanks simultaneously) between seasons has resulted in an overall increase in hatchery production, but stretched physical and man power resources possibly leading to poorer quality fish.
The level of data collection and attention to detail has dramatically improved at CST in part due to working with scientists on the collection of data. Both hatcheries have skilled and dedicated staff working under, at times, difficult conditions.

One issue which has not yet been completely resolved, has been that major changes to standard larval rearing protocols (SOPs) have been undertaken with little, if any, prior experimentation or trials. These included adopting the use of algal paste and probionts in 2008 and the large-scale use of formalin in 2009. Larval rearing is a complex process and it would be highly desirable to test changes to protocols before the production season. It is unlikely that any one change is going to solve the current production issues of low survival and high malformation rates. An approach where one factor is tested at a time is needed. Testing should occur in a minimum of 2 tanks in two runs and be compared with parallel tanks run using standard protocols.

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Appendix 1 – Intellectual property

There was no new intellectual property arising from this project.

Appendix 2 – Staff

IMAS

A/Prof Stephen Battaglene	Project funded 15%
Dr Jennifer Cobcroft	Project funded 5%
Ms Melanie Evans	Project funded 100% FTE (1 year only)
Mr Ross Goldsmid	In-kind 5%
Ms Anna Overweter	In-kind 5%
Mr Tom Litjens	In-kind 5%

CST

Dr Craig Foster	In-kind 2%
Mr Morten Deichmann	In-kind 2%
Mr Adrian McIntyre	In-kind 2%
Mr Travis Dymmott	In-kind 2%
Mr Alex Czypionka	
Mr Konrad Czypionka	

Collaborators

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Assoc Prof Jian Qin

Mr Zhenhua Ma (PhD student)

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Darwin Aquaculture Centre

Jerome Bosmans

Glenn Schipp

Chad Mumme

Damon Gore

Appendix 3 - TAFI 4th Trip report

24th to 30th November 2009 to Arno Bay and Port Lincoln
Jennifer Cobcroft and Melanie Evans

[Edited – bacteriology section only]

Bacteriology
We took samples from two different rotifer culture systems to demonstrate a method to assess culturable bacteria per rotifer, as well as comparing plating methods of CST and TAFI for the rotifer culture water.

In brief, the ad hoc method used by CST did not provide usable results and could be improved by the routine use of serial dilutions. The aseptic techniques used at CST were good, indicated by no colonies on the control plates.

Materials & Methods
TAFI (Jenny)
A 50 mL sample of culture water including rotifers was sampled in clean sample jars from 2 tanks:

Tank	Culture method	Rotifer density (no./mL)	Volume for bacteriology	No. rotifers homogenised
P2	440L recirculation, Culture Selco and yeast	2500	3 mL	7500
Eco	900L recirculation, Algae paste (Nanno)	1500	5 mL	7500

The samples were stored in the lab at room temperature (~25-28°C) for about 2 hours before plating. Best practise would be to keep the samples cool and plate immediately after collection (or within 4 hours) as prolonged storage will distort the number of bacteria.

Procedure

1. Remove the plunger of a sterile 10mL syringe and fit the sterile barrel to the top of a sterile 13mm filter unit (with 5µm nitrocellulose filter); keep the plunger.
2. Collect the required volume of rotifer culture with a sterile disposable transfer pipette and load it into the syringe barrel.
3. Aseptically replace the syringe plunger and slowly push the sample through the filter.
4. Collect the supernatant (filtered water) in an eppendorf tube (rotifer culture 'water' sample)
5. Disassemble the filter unit and with sterile forceps remove the filter membrane with collected rotifers and place in a sterile eppendorf microfuge tube.
6. With a sterile pestle and motorised driver, macerate the membrane and rotifers for no more than 30 seconds. Ensure that membrane is pushed well down to the bottom of the tube.
7. Add 500µL of sterile seawater to rotifer tube and pulse mix with the pestle for ~5s.
8. Pulse centrifuge the microfuge tubes for 40s at 10,000rpm (Minispin) to sediment gross material.

9. Prepare dilutions of the supernatant ('rotifer sample') and discard sediment.

Dilutions

Note: This method used small volumes due to <500µL available of raw rotifer sample, however larger volumes (1-5mL) are preferred for water samples. Alex is familiar with the larger volume technique.

10. In a sterile 96-well plate, add 270µL of sterile seawater to 5 consecutive wells, leaving the first well empty, as follows:

well 'label'	**neat**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**
volume sterile seawater (µL)		270	270	270	270	270
volume rotifer supernatant (µL)	300	30				
volume from previous well (µL)			30	30	30	30

11. Transfer 300µL of 'neat' rotifer supernatant to 1st well.
12. Transfer 30µL of neat sample to the next well (10^{-1}).
13. Change pipette tip and mix well by aspirating 5 times, then transfer 30µL to the next well (10^{-2}).
14. Continue with above method from one well to the next until 10^{-5} is completed.
15. Rotifer culture 'water' sample – prepare dilutions as above for 10^{-1} and 10^{-2}

Plating

Note: All plates were air dried at room temperature for approx 30 min before use, to avoid issues with condensation when they were removed from the fridge.

16. With a fresh pipette tip, aspirate to mix the 10^{-5} dilution and transfer 100µL to each of two 90mm agar plates, one TCBS (selective for *Vibrio*) and ZMA (for marine bacteria).
17. Use a sterile glass 'hockey stick' to spread. Replace lid of petri dish and leave to dry for 10 min before turning upside down to incubate.
18. With a fresh tip (only needed because the pipette was put down while spreading previous plates), mix and plate the 10^{-4} dilution as above.
19. Repeat for 10^{-3} and 10^{-1} dilution. Note – Plated 10^{-1}, 10^{-3}, 10^{-4}, and 10^{-5} as a range finder to determine best dilution to plate. If this is done regularly only 1 or 2 dilutions need to be plated.
20. Rotifer culture 'water' sample – plate neat, 10^{-1} and 10^{-2} dilutions, as above
21. Seawater control – sterile seawater was passed through the filter membrane as above for rotifers, the membrane was homogenised and 500µL sterile seawater added mixed and centrifuged as above. The supernatant of this water was plated as the seawater control.
22. All plates were incubated at lab room temperature for 24 h.
23. Colonies were counted on the plates were the number was ≤ 360 CFU (colony forming units). Note: it is preferable to count between 30 and 300 colonies and adjust dilutions to achieve this.

CST (Alex)

Water samples were collected from all rotifer culture tanks and rotifer recirculation system sumps using current CST methods, except for the algae paste system (Eco). The samples were stored in the lab at room temperature (~25-28°C) for about 3 hours before plating, but they would normally be plated immediately.

Procedure

1. 1 mL samples were collected from within the culture tanks (at approx. 9:45am). A 70µm screen was held just under the surface and 1 mL sample was extracted from inside the screen (no rotifers) with a micropipette & fresh pipette tip. The first 1 mL was discarded to rinse the tip and the second transferred to a 1.5 mL eppendorf tube and the lid was closed.
2. Water samples were collected from P2, P4, C1, C5, C sump and P sump.
3. All samples were plated 'neat' as above, with 100µL inoculated onto both TCBS and ZMA. The plates were spread with a sterile 'blue loop' (Fig. 4). Note – normally CST only plate TCBS.
4. Sterile seawater was plated as the control.

Figure 4. CST bacteriology plating. Spreading a 100 µL inoculum with a blue loop.

Results

The bacterial colony counts for rotifers and culture system water using serial dilutions were similar in the two different culture systems (P2 and Eco) (Table 3). There were approx 430 CFU/rotifer on marine agar and 116 CFU/rotifer on TCBS. There were 3.0×10^5 and 3.6×10^5 CFU/mL on ZMA and presumptive Vibrios on TCBS were higher in water from tank P2 (2.6×10^5) than the Eco tank (1.6×10^5). There were no colonies on the TAFI control plates.

All plates of rotifer culture and sump water from the CST method had too many colonies to count so that comparison of the two plating methods was not possible (Fig. 5). There were no colonies on the CST control plates.

Table 3. Bacterial counts from samples of rotifers and culture water from two different rotifer recirculation culture systems at Arno Bay.

Sample		No. Colony Forming Units (CFU) per mL		No. Colony Forming Units (CFU) per rotifer	
		ZMA	TCBS	ZMA	TCBS
rotifers	P2	6,400,000	1,700,000	427	113
	Eco	6,500,000	1,790,000	433	119
water	P2	360,000	262,000	na	na
	Eco	300,000	160,000	na	na

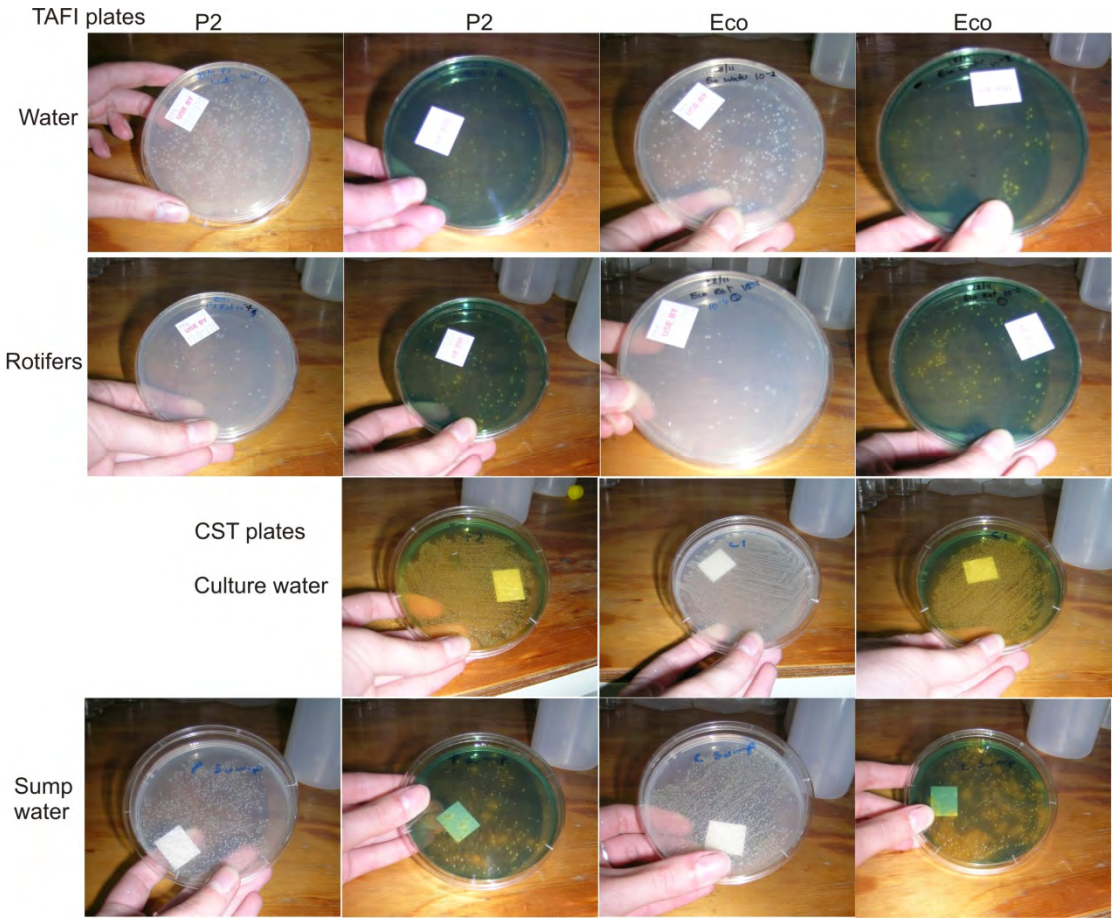

Figure 5. Examples of colonies on plates from TAFI and CST bacteriology plating of rotifer culture tank water, rotifers, and recirculation sump water.

Standard CST method

- Aseptic techniques were appropriate
- Currently an ad hoc regime for plating, only when requested by rotifer and larval technicians or managers
- Normally collect the requested samples and plate immediately, without dilution
- Observe colours of colonies as well as the number of colonies
- If there are too many colonies to count, then the sampling will be repeated the following day with a dilution series prepared (based on 100L of sample into 900L of sterile seawater), and 10^{-1} or 10^{-2} is plated
- Incubation is at lab room temperature for 24 h
- Control plates of sterile seawater and used to confirm aseptic techniques

Discussion

The TAFI method, particularly of homogenising rotifers was demonstrated as a potential tool for use at CST, but not recommended for routine assessment as it is time consuming. The serial dilution method for water samples is critical to obtain accurate results and we recommend should be routinely used when plating at CST. The current CST method is an inefficient use of technical effort and time if the colonies cannot be counted and the samples need to be plated again the following day. Monitoring bacterial counts in rotifer and larval culture routinely (e.g. 2-3 times a week) could be a useful addition to the increasing data set at CST of larval performance, but if no management actions are in place to respond to changes in values, then the need for this monitoring is questioned given the time involved to collect the data. Regular monitoring of existing water treatments systems would be worthwhile.

In comparison with previous trials, the colony counts for the rotifers themselves were more than twice as high as results using a similar technique at TAFI on enriched and rinsed rotifers, 192 CFU/rotifer on ZMA and 53 CFU/rotifer on a *Vibrio* selective agar (BTBT). However, this difference could be due to the prior rinsing of rotifers in the TAFI study. Further plating at CST would be required, if this was deemed to be valuable information, with rotifers rinsed in preparation to feed to larval culture tanks.

There was an indication of a higher *Vibrio* load in the traditional Culture Selco and yeast production system than in the new paste algae rotifer culture. Further plating would be required if this is considered important to verify.

Consider adopting a routine monitoring approach to water treatment into egg incubation and larval systems (i.e. before and after 5µm and UV disinfection).

We recommend serial dilutions are used at CST whenever plating is required. If the bacterial load is unknown, every second dilution can be plated (in the range of 'neat' to 10^{-5}) as a range finder, then only one or two dilution levels plated on subsequent occasions.

Appendix 4 - Histopathology reports

Final histopathology reports from Animal Health Laboratories, Dept of Primary Industries, Parks, Water and Environment, Mt Pleasant, Tasmania.

REPORT 1
(samples submitted 21 July 2009 – 2008 YTK samples)
Date: 28 August 2009

CLINICAL HISTORY
Specimen Advice Form – No.4113.

GROSS SUMMARY
Please refer to spreadsheet for slide numbers correlated to samples

HISTOPATHOLOGY DESCRIPTION
Please refer to spreadsheet

DIAGNOSIS
Please refer to comments

PATHOLOGIST'S COMMENTS:
A brief explanation of the comments:
Twelve levels are cut for each larvae (except for STJC 161 and STJC 0049 which only needed 2-3 levels). This was the most thorough method to examine all organs, as best as possible.

Grading:
0 = normal
1= mild changes
2= moderate changes
3 = marked / significant changes

The comments below refer to your specific questions (your comments are in bold italic type):

Level of vacuolation in the liver – indicative of glycogen deposition
There is mild glycogen deposition (feathery vacuolation) in the livers of some larvae.

Gut/intestine and swimbladder condition/pathology, and any signs of bacterial infection, although an overview of everything would be great.

There are low numbers of larvae (see STJC 161 and STJC 0049) with mild focal inflammation in the muscle of the upper jaw. On Gram and PAS stains microorganisms are not evident.

There are variable numbers of larvae with non-inflated swim bladders. This, in some cases, is correlated with lack of ingesta in the alimentary tract.

In some fish there are diffuse clear microvesiculation (most likely lipid vacuoles) in the epithelium. These are sometimes correlated with feathery vacuolation of the liver (glycogen deposition).

In low numbers of fish (see STJC 161 and STJC 0049) there is a mild increase in the numbers of melanomacrophages suggesting an increased turnover of cells.

The intestinal content of fish varies and includes absent content (no comment in spreadsheet), engulfed material (no food material just wispy fine fragments) or ingesta / food (multicellular organisms).

Some larvae have sloughed cells in the gut lumen. This is either an artefact due to delayed fixing in formalin or suggests inappetence.

These fish are from different hatcheries that were transferred to nursery systems of different salinities. At hatchery PA they move from 35 ppt in larval culture to 42-47 ppt in the nursery, and at hatchery AB, salinity remains similar about 36-37 ppt.

We are most interested in changes associated with salinity change/stress. I assume this would be most apparent in the gills and intestine

The chloride cells generally appear within normal limits. In some fish there is separation of the epithelium from the secondary gill lamellae. Because there is no protein pooling under the epithelium or other inflammatory or degenerative changes the separation is most likely artefact.

REPORT 2
(samples submitted 24 March 2010 – 2009 YTK samples)
Date: 1 June 2010

CLINICAL HISTORY
Yellowtail Kingfish

SPECIMENS SUBMITTED
160 formalin pots with fry placed in cassettes

GROSS SUMMARY
Slides labelled 1-160 correspond to pots labelled 1-160. 6 levels are cut for all fish to ensure as many organs as possible are checked.

HISTOPATHOLOGY FINDINGS
Please refer to the spread sheet completed for this report.

On the spread sheet the slide number, your record of a) fish age, b) hatchery and c) run/expt, tank ID and individual fish ID (allocated by pathologist reviewing slides) are recorded.

Histopathological findings are graded:
0 = within normal limits
1 = mild changes
2 = moderate changes
3 = severe changes
And comments are recorded for any histopathological changes

DIAGNOSIS
Please refer to spread sheet

COMMENT
The most notable finding is the variable inflation of the swim bladder in fish.

There are physiological changes including variable glycogen stores in the liver and vacuolation of epithelium lining the intestinal tract. These may reflect the diet fed to fish.

There were no changes in any fish consistent with inflammation.

The stomach, pyloric caeca, proximal and distal intestines in all fish (except for those still with yolk sac) contained moderate to abundant ingesta, including multicellular organisms.

Please note that in some fish not all organs could be included in the multiple levels cut because of their very small size. If a cell is left blank in the spread sheet the organ was not seen.

If you have any further questions regarding this submission please contact me by email on Graeme.Knowles@dpipwe.tas.gov.au or telephone 63365461.

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Table A1. 2008. Average histopathology score for different tissues of larval and juvenile Yellowtail Kingfish.

				Average score for tissue/organ																
Hatchery	**Run/Expt**	**Fish Age**	**Tank**	**Eye**	**Brain**	**Liver**	**Heart**	**Pancreas**	**Swimbladder**	**Stomach**	**Pyloric caeca**	**Prox Intestine**	**Dist. Intestine**	**Musde**	**Cartilage/bone**	**Gills**	**Skin**	**Spleen**	**Kidney**
AB	Pre Run 1	5	L1	0.0	0.0	0.8		0.0	0.0		0.0	0.0		0.0	0.0		0.0		0.0
			L2	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0		
			L3 (26/7)	0.0	0.0	0.0		0.0	0.3		0.0	0.0		0.0	0.0		0.0		
			L3 (3/8)	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0		0.0
			L4	0.0	0.0	0.0		0.0	0.5		0.0	0.0		0.0	0.0		0.0		
			L5	0.0	0.0	0.0	0.0	0.0	0.7		0.0	0.0		0.0	0.0		0.0		0.0
		5 Total		0.0	0.0	0.1	0.0	0.0	0.2		0.0	0.0		0.0	0.0		0.0		0.0
		10	L1	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L2	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L4	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L5	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0	1.0	0.0		0.0
		10 Total		0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0	1.0	0.0		0.0
	Pre Run 1 Total			**0.0**	**0.0**	**0.1**	**0.0**	**0.0**	**0.1**		**0.0**	**0.0**		**0.0**	**0.0**	**1.0**	**0.0**		**0.0**
	Run 1	5	L1	0.0	0.0			0.0	0.3		0.0	0.0		0.0	0.0		0.0		
			L2	0.0	0.0	0.0	0.0	0.0	0.1		0.0	0.0		0.0	0.0		0.0		0.0
			L3	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0		
			L4	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0		
			L5	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0		
		5 Total		0.0	0.0	0.0	0.0	0.0	0.1		0.0	0.0		0.0	0.0		0.0		0.0
		10	L1	0.0	0.0	0.0		0.0	0.4		0.0	0.0		0.0	0.0	1.0	0.0		
			L2	0.0	0.0	0.0		0.0	0.2	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L3	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L4	0.0	0.0	0.0		0.0	0.0		0.0			0.0	0.0	1.0	0.0		0.0
			L5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
		10 Total		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
		15	L3	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.8	0.8	0.0	0.0	0.0	1.0	0.0		0.0
			L4	0.0	0.0	0.2	0.0	0.0	0.4	0.0	0.0	0.2	0.0	0.0	0.0	1.0	0.0		0.0
			L5	0.0	0.0	0.6	0.0	0.0	0.4	0.0	0.5	0.4	0.0	0.0	0.0	1.0	0.0		0.0
		15 Total		0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.4	0.5	0.0	0.0	0.0	1.0	0.0		0.0
		20	L1	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0
			L2	0.0	0.0	0.2	0.0	0.0	0.5	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0		0.0
			L4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0
			L5	0.0	0.0	1.0		0.0	0.0	0.0	0.2	0.0		0.0	0.0	0.0	0.0		0.0
		20 Total		0.0	0.0	0.5	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0		0.0
		Juveniles	(blank)	0.0	1.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.3	0.0
		Juveniles	Total	0.0	1.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.3	0.0
	Run 1 Total			**0.0**	**0.1**	**0.3**	**0.0**	**0.0**	**0.1**	**0.0**	**0.1**	**0.1**	**0.0**	**0.0**	**0.0**	**0.7**	**0.0**	**0.3**	**0.0**
	Run 2	5	L1	0.0	0.0	0.0		0.0	0.5		0.0	0.0		0.0	0.0		0.0		
			L2	0.0	0.0	0.0			0.2		0.0	0.0		0.0	0.0		0.0		
			L3	0.0	0.0	0.0		0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0		0.0		0.0
			L4	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0		0.0
			L5	0.0	0.0	0.0		0.0	0.8		0.3			0.0	0.0		0.0		0.0
			L6	0.0	0.0	0.0		0.0	0.8		0.0	0.0		0.0	0.0		0.0		0.0
			L7	0.0	0.0	0.0	0.0	0.0	1.0		0.0	0.0		0.0	0.0		0.0		
		5 Total		0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0		0.0		0.0
		10	L1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			L2	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			L3	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			L4	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			L5	0.0	0.0	0.0		0.0	0.0		0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			L6	0.0	0.0	0.0		0.0	0.4		0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L7	0.0	0.0	0.0		0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
		10 Total		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
		15	L1	0.0	0.0	0.0	0.0	0.0	0.2		0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L2	0.0	0.0	1.0	0.0	0.0	0.0		1.0	0.0		0.0	0.0	1.0	0.0		0.0
			L3	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L4	0.0	0.0	0.2		0.0	0.2		0.2	0.0		0.0	0.0	1.0	0.0		0.0
			L5	0.0	0.0	0.0	0.0	0.0	1.0		0.6	0.0		0.0	0.0	1.0	0.0		0.0
			L6	0.0	0.0	0.8	0.0	0.0	0.0		0.8	0.0		0.0	0.0	0.0	0.0		0.0
			L7	0.0	0.0	0.2	0.0	0.0	0.0		0.0	0.0		0.0	0.0	1.0	0.0		0.0
		15 Total		0.0	0.0	0.3	0.0	0.0	0.2		0.4	0.0		0.0	0.0	0.9	0.0		0.0
		20	L1	0.0	0.0	0.6	0.0	0.0	0.0		0.6	0.0		0.0	0.0	0.0	0.0		0.0
			L2	0.0	0.0	0.8	0.0	0.0	0.0		0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L3	0.0	0.0	0.4	0.0	0.0	0.0		0.4	0.0		0.0	0.0	0.0	0.0		0.0
			L4	0.0	0.0	0.0		0.0	0.2		0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L5	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
			L6	0.0	0.0	1.0	0.0	0.0	0.2		0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L7	0.0	0.0	1.0	0.0	0.0	0.2		0.0	0.0		0.0	0.0	0.0	0.0		0.0
		20 Total		0.0	0.0	0.5	0.0	0.0	0.1		0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Run 2 Total			**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.2**	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.6**	**0.0**	**0.0**	**0.0**
	Run 3	5	L1	0.0	0.0	0.0		0.0	0.1		0.0	0.0		0.0	0.0		0.0		0.0
			L2	0.0	0.0	0.0	0.0	0.0	0.1		0.0	0.0		0.0	0.0		0.0		0.0
		5 Total		0.0	0.0	0.0	0.0	0.0	0.1		0.0	0.0		0.0	0.0		0.0		0.0
		10	L1	0.0	0.0	0.0	0.0	0.0	0.2		0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L2	0.0	0.0	0.0	0.0	0.0	0.4		0.0	0.0		0.0	0.0	1.0	0.0		0.0
		10 Total		0.0	0.0	0.0	0.0	0.0	0.3		0.0	0.0		0.0	0.0	1.0	0.0		0.0
		15	L1	0.0	0.0	0.6		0.0	0.6		0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0
		15 Total		0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0
		20	L1	0.0	0.0	0.4		0.0	0.0		2.0	0.0		0.0	0.0	0.0	0.0		0.0
			L2	0.0	0.0	1.0	0.0	0.0	0.0		0.0	0.0		0.0	0.0	0.0	0.0		0.0
		20 Total		0.0	0.0	0.7	0.0	0.0	0.0		1.0	0.0		0.0	0.0	0.0	0.0		0.0
	Run 3 Total			**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.2**	**0.0**	**0.2**	**0.0**		**0.0**	**0.0**	**0.3**	**0.0**		**0.0**
AB Total				**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.2**	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.7**	**0.0**	**0.1**	**0.0**

Table A1. (continued) 2008. Average histopathology score for different tissues of larval and juvenile Yellowtail Kingfish.

				Average score for tissue/organ																
Hatchery	Run/Expt	Fish Age	Tank	Eye	Brain	Liver	Heart	Pancreas	Swimbladder	Stomach	Pyloric caeca	Prox Intestine	Dist. Intestine	Musde	Cartilage/bone	Gills	Skin	Spleen	Kidney
PA	Run 1	5	L1	0.0	0.0	0.4		0.0	1.0		0.0			0.0	0.0		0.0		
			L2	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0		
			L3	0.0	0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0		
			L4	0.0	0.0					0.0	0.0	0.0		0.0	0.0		0.0		
			L5	0.0	0.0				0.0	0.0	0.0	0.0		0.0	0.0		0.0		
			L6	0.0	0.0	0.0		0.0	0.5	0.0		0.0		0.0	0.0		0.0		0.0
			L7	0.0	0.0	0.0		0.0	0.6	0.0	0.4	0.0		0.0	0.0		0.0		
			L8	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0		
		5 Total		0.0	0.0	0.1		0.0	0.3	0.0	0.1	0.0		0.0	0.0		0.0		0.0
		10	L1	0.0	0.0	0.4		0.0	0.2	0.0	0.0	0.0		0.0	0.0	1.0	0.0		
			L2	0.0	0.0	0.0		0.0	0.2	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L3	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0		
			L4	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0		
			L5	0.0	0.0	0.0		0.0	1.0	0.0	0.0	0.0		0.0	0.0		0.0		
			L6	0.0	0.0	0.0		0.0	0.7	0.0	0.0	0.0		0.0	0.0	1.0	0.0		
			L7	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L8	0.0	0.0	0.0		0.0	0.3		0.0	0.0		0.0	0.0	1.0	0.0		
		10 Total		0.0	0.0	0.1	0.0	0.0	0.3	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
		15	L1	0.0	0.0	0.2		0.0	0.6	0.0	0.2	0.0		0.0	0.0	1.0	0.0		0.0
			L2	0.0	0.0	0.0		0.0	0.8	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L3	0.0	0.0	0.2		0.0	0.4	0.0	0.2	0.0		0.0	0.0	1.0	0.0		
			L4	0.0	0.0	0.2		0.0	0.3	0.0	0.3	0.0		0.0	0.0	1.0	0.0		
			L5	0.0	0.0	0.4		0.0	0.0	0.0	0.4	0.0		0.0	0.0	1.0	0.0		
			L6	0.0	0.0	0.0		0.0	1.0	0.0	0.6	0.0		0.0	0.0	1.0	0.0		1.0
			L7	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.4	0.0		0.0	0.0	1.0	0.0		0.0
			L8	0.0	0.0	0.2	0.0	0.0	0.3	0.0	0.6	0.0		0.0	0.0	1.0	0.0		0.0
		15 Total		0.0	0.0	0.2	0.0	0.0	0.4	0.0	0.4	0.0		0.0	0.0	1.0	0.0		0.1
		20	L1	0.0	0.0	0.5		0.0	0.0	0.0	0.2	0.0		0.0	0.0	0.0	0.0		0.0
			L2	0.0	0.0	0.6		0.0	0.3	0.0	0.6	0.0		0.0	0.0	0.0	0.0		0.0
			L3	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.2	0.0		0.0	0.0	0.0	0.0		0.0
			L4	0.0	0.0	1.0	0.0	0.0	0.0		1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
			L5	0.0	0.0	1.0	0.0	0.0	0.4	0.0	0.4	0.0		0.0	0.0	0.0	0.0		0.0
			L6	0.0	0.0	1.0		0.0	0.6	0.0	1.0	0.0		0.0	0.0	0.0	0.0		0.0
			L7	0.0	0.0	1.0		0.0	0.0	0.0	1.0	0.0		0.0	0.0	0.0	0.0		0.0
			L8	0.0	0.0	1.0		0.0	0.0	0.0	1.0	0.0		0.0	0.0	0.0	0.0		0.0
		20 Total		0.0	0.0	0.8	0.0	0.0	0.2	0.0	0.7	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
		Juveniles	(blank)	0.0	1.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.2	0.0	0.3	0.0
		Juveniles	Total	0.0	1.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.2	0.0	0.3	0.0
	Run 1 Total			**0.0**	**0.0**	**0.4**	**0.0**	**0.0**	**0.3**	**0.0**	**0.3**	**0.0**	**0.5**	**0.0**	**0.0**	**0.6**	**0.0**	**0.1**	**0.0**
	Run 2	5	L1	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L2	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0		0.0
			L3	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0		0.0
			L4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0	0.0	0.0
			L5	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0		0.0
			L6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0		
			L7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0		
			L8	0.0	0.0	0.0		0.0	0.2	0.0	0.0	0.0		0.0	0.0		0.0		
		5 Total		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.5	0.0	0.0	0.0
		10	L1	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0	0.0	0.0
			L2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L4	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0		
			L6																
			L7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0		0.0
			L8	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0	0.0	1.0	0.0		0.0
		10 Total		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	1.0	0.0	0.0	0.0
		15	L1	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L2	0.0	0.0	1.0	0.0	0.0	0.0		0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L3	0.0	0.0	0.2	0.0	0.0	0.8	0.0	0.0	0.0		0.0	0.0	0.8	0.0		0.0
			L4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L5	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L7	0.0	0.0	0.0		0.0	0.0		1.0	1.0		0.0	0.0	0.0	0.0		0.0
			L8	0.0	0.0	1.0	0.0	0.0	0.0		1.0	0.0		0.0	0.0	0.0	0.0		0.0
		15 Total		0.0	0.0	0.3	0.0	0.0	0.2	0.0	0.3	0.1		0.0	0.0	0.1	0.0		0.0
		20	L1	0.0	0.0	1.0	0.0	0.0	0.0		0.8	0.0		0.0	0.0	0.0	0.0		0.0
			L2	0.0	0.0	1.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0	0.0	0.0	0.0		0.0
			L3	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0
			L4	0.0	0.0	1.0		0.0	0.0	0.0	1.0	0.0		0.0	0.0	0.0	0.0		0.0
			L5	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.6	0.0		0.0	0.0	0.0	0.0		0.0
			L6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0	0.0	0.0	0.0		0.0
			L7	0.0	0.0	0.2	0.0	0.0	0.0	0.0	1.0	0.0		0.0	0.0	1.0	0.0		0.0
			L8	0.0	0.0	1.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0	0.0	1.0	0.0		0.0
		20 Total		0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.8	0.0		0.0	0.0	0.3	0.0		0.0
	Run 2 Total			**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	**0.3**	**0.0**		**0.0**	**0.0**	**0.4**	**0.0**	**0.0**	**0.0**
PA Total				**0.0**	**0.0**	**0.3**	**0.0**	**0.0**	**0.1**	**0.0**	**0.3**	**0.0**	**0.5**	**0.0**	**0.0**	**0.5**	**0.0**	**0.1**	**0.0**

Table A1. (continued) 2008. Average histopathology score for different tissues of larval and juvenile Yellowtail Kingfish.

				Average score for tissue/organ															
Hatchery	**Run/Expt**	**Fish Age**	**Tank**	**Eye**	**Brain**	**Liver**	**Heart**	**Pancreas**	**Swimbladder**	**Stomach**	**Pyloric caeca**	**Prox Intestine**	**Dist. Intestine**	**Muscle**	**Cartilage/bone**	**Gills**	**Skin**	**Spleen**	**Kidney**
SARDI	Rot Nov08	13	19	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			20	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			21	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			25	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			26	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			27	0.0	0.0	0.0	0.0	0.0	1.0	0.0			0.0	0.0	0.0	1.0	0.0		0.0
			31	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			32	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
			33	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
		13 Total		0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0
	Rot Nov08 Total			**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.0**	**0.0**		**0.0**
SARDI Total				**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.0**	**0.0**		**0.0**

Table A2. 2009. Average histopathology score for different tissues of larval and juvenile Yellowtail Kingfish.

				Average score for tissue/organ																	
Hatchery	Run/Expt	Fish Age	Tank	Eye	Brain	Liver	Heart	Pancreas	Swimbladder	Stomach	Pyloric caeca	Prox Intestine	Dist. Intestine	Musde	Cartilage/bone	Gills	Skin	Spleen	Kidney	Yolk sac
AB	1	1	L1	0.0	0.0					0.0				0.0	0.0		0.0			0.0
			L2	0.0	0.0					0.0		0.0	0.0	0.0	0.0		0.0			0.0
			L3	0.0	0.0					0.0				0.0	0.0		0.0			0.0
			L4	0.0	0.0					0.0				0.0	0.0		0.0			0.0
			L5	0.0	0.0					0.0				0.0	0.0		0.0		0.0	0.0
			L6	0.0	0.0					0.0				0.0	0.0		0.0		0.0	0.0
			L7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N1	0.0	0.0					0.0				0.0			0.0			0.0
			N2	0.0	0.0					0.0				0.0			0.0			0.0
			N3	0.0	0.0					0.0				0.0			0.0			0.0
			N4	0.0	0.0					0.0				0.0			0.0			0.0
			N5	0.0	0.0					0.0				0.0			0.0			0.0
			N6	0.0	0.0					0.0				0.0			0.0			0.0
		1 Total		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0
		5	L1	0.0	0.0	0.0		0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0	
			L2	0.0	0.0		0.0		0.5	0.0		0.0	0.0	0.0	0.0		0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L6	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0		0.0		0.0	
			L7	0.0	0.0					0.0				0.0			0.0			0.0
			N1	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0		0.0	
			N3	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N4	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0		0.0		0.0	
			N5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N6	0.0	0.0				0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		5 Total		0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0		0.0	0.0
		10	L1	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0		0.0	
			L2	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.3	0.3	0.3	0.0	0.0	1.0	0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0		0.0	
			L6	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L7	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N1	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N2	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N4	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N6	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		10 Total		0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0		0.0	
		15	L1	0.0	0.0	0.0	0.0	0.0	0.4	0.0	1.0	1.0	1.0	0.0	0.0	0.7	0.0		0.0	
			L2	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.2	0.2	0.2	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.3	0.3	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.4	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	0.7	0.0	0.0	1.0	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L6	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2	0.2	0.2	0.0	0.0	0.0	0.0		0.0	
			L7	0.0	0.0	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N1	0.0	0.0	1.0	0.0	0.0	0.2	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			N2	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0	1.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N3	0.0	0.0	1.0	0.0	0.0	0.1	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			N4	0.0	0.0	1.0	0.0	0.0	0.1	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			N5	0.0	0.0	1.0	0.0	0.0	0.4	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			N6	0.0	0.0	1.0	0.0	0.0	0.5	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
		15 Total		0.0	0.0	0.4	0.0	0.0	0.4	0.0	0.7	0.7	0.6	0.0	0.0	0.1	0.0		0.0	
		20	L1	0.0	0.0	1.0	0.0	0.0	0.3	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L2	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.3	0.3	0.3	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	1.0	0.0	0.0	0.7	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5	0.5	0.0	0.0	0.0	0.0		0.0	
			L7	0.0	0.0	1.0	0.0	0.0	0.0	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			N1	0.0	0.0	1.0	0.0	0.0	0.2	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			N2	0.0	0.0	1.0	0.0	0.0	1.0	0.0	1.0	1.0	0.0	0.0	0.0	0.0	0.0		0.0	
			N3	0.0	0.0	1.0	0.0	0.0	0.8	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			N4	0.0	0.0	1.0	0.0	0.0	0.5	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			N5	0.0	0.0	1.0	0.0	0.0	0.0	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			N6	0.0	0.0	1.0	0.0	0.0	0.2	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
		20 Total		0.0	0.0	0.7	0.0	0.0	0.3	0.0	0.8	0.8	0.8	0.0	0.0	0.0	0.0		0.0	
	1 Total			**0.0**	**0.0**	**0.3**	**0.0**	**0.0**	**0.3**	**0.0**	**0.4**	**0.4**	**0.4**	**0.0**	**0.0**	**0.1**	**0.0**		**0.0**	**0.0**

Table A2. (continued) 2009. Average histopathology score for different tissues of larval and juvenile Yellowtail Kingfish.

				Average score for tissue/organ																
Hatchery	Run/Expt	Fish Age	Tank	Eye	Brain	Liver	Heart	Pancreas	Swimbladder	Stomach	Pyloric caeca	Prox Intestine	Dist. Intestine	Muscle	Cartilage/bone	Gills	Skin	Spleen	Kidney	Yolk sac
AB	2	1	L4	0.0	0.0					0.0				0.0			0.0			0.0
			L5	0.0	0.0					0.0				0.0			0.0			0.0
			L6	0.0	0.0	0.0	0.0	0.0	0.3	0.0				0.0			0.0			0.0
			L7	0.0	0.0					0.0				0.0			0.0			0.0
		1 Total		0.0	0.0	0.0	0.0	0.0	0.3	0.0				0.0			0.0			0.0
		2	L1	0.0	0.0					0.0				0.0			0.0			0.0
			L2	0.0	0.0					0.0				0.0			0.0			0.0
			L3	0.0	0.0					0.0				0.0			0.0			0.0
		2 Total		0.0	0.0					0.0				0.0			0.0			0.0
		5	L1	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L2	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0		0.0		0.0	
			L5	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0		0.0		0.0	
			L6	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L7	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		5 Total		0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		10	L1	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L2	0.0	0.0	1.0	0.0	0.0	0.8	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	0.1	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L6	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L7	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		10 Total		0.0	0.0	0.2	0.0	0.0	0.5	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0		0.0	
		15	L1	0.0	0.0	1.0	0.0	0.0	0.3	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L2	0.0	0.0	1.0	0.0	0.0	0.4	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	1.0	0.0	0.0	0.6	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	1.0	0.0	0.0	0.4	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	1.0	0.0	0.0	0.3	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L6	0.0	0.0	1.0	0.0	0.0	0.2	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L7	0.0	0.0	0.0	0.0	0.0	0.1	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
		15 Total		0.0	0.0	0.9	0.0	0.0	0.3	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
		20	L1	0.0	0.0	1.0	0.0	0.0	0.2	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L2	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	1.0	0.0	0.0	0.7	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	1.0	0.0	0.0	0.6	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	1.0	0.0	0.0	0.2	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L6	0.0	0.0	0.0	0.0	0.0	0.8	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L7	0.0	0.0	0.0	0.0	0.0	0.2	0.0				0.0	0.0	0.0	0.0		0.0	
		20 Total		0.0	0.0	0.6	0.0	0.0	0.4	0.0	0.8	0.8	0.8	0.0	0.0	0.0	0.0		0.0	
	2 Total			**0.0**	**0.0**	**0.4**	**0.0**	**0.0**	**0.4**	**0.0**	**0.5**	**0.5**	**0.5**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	**0.0**
	3	1	L1	0.0	0.0					0.0				0.0			0.0			0.0
			L2	0.0	0.0					0.0				0.0			0.0			0.0
			L3	0.0	0.0					0.0				0.0			0.0			0.0
			L4	0.0	0.0					0.0				0.0			0.0			0.0
			L5	0.0	0.0					0.0				0.0			0.0			0.0
			L6	0.0	0.0					0.0				0.0			0.0			0.0
			L7	0.0	0.0					0.0				0.0			0.0			0.0
		1 Total		0.0	0.0					0.0				0.0			0.0			0.0
		5	L1	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L2	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L6	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L7	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		5 Total		0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		10	L1	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L2	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L6	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L7	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		10 Total		0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		15	L1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L2	0.0	0.0	0.0	0.0	0.0	0.2	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L6	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L7	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		15 Total		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.3	0.3	0.3	0.0	0.0	0.0	0.0		0.0	
		20	L2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		20 Total		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2	0.2	0.2	0.0	0.0	0.0	0.0		0.0	
		21	L1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0		0.0	
			L2	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		21 Total		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0		0.0	
	3 Total			**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.2**	**0.0**	**0.1**	**0.1**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	**0.0**
AB Total				**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.3**	**0.0**	**0.3**	**0.3**	**0.3**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	**0.0**

Table A2. (continued) 2009. Average histopathology score for different tissues of larval and juvenile Yellowtail Kingfish.

				Average score for tissue/organ																
Hatchery	**Run/Expt**	**Fish Age**	**Tank**	**Eye**	**Brain**	**Liver**	**Heart**	**Pancreas**	**Swimbladder**	**Stomach**	**Pyloric caeca**	**Prox Intestine**	**Dist. Intestine**	**Muscle**	**Cartilage/bone**	**Gills**	**Skin**	**Spleen**	**Kidney**	**Yolk sac**
PA	1	27	L1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L2	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L3	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L4	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
			L5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
		27 Total		0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	
	1 Total			**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	
PA Total				**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	
Grand Total				**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.3**	**0.0**	**0.3**	**0.3**	**0.3**	**0.0**	**0.0**	**0.0**	**0.0**		**0.0**	**0.0**