Final Report

AQUAFIN CRC PROJECT 1B3: INCREASING THE PROFITABILITY OF SNAPPER FARMING BY IMPROVING HATCHERY PRACTICES AND DIETS
VOLUME 3: FINGERLING PRODUCTION AND HEALTH

D. Stewart Fielder, Ashley Roberts-Thomson, Mark A. Booth, Geoff L. Allan and Robert Adlard

September 2008

FRDC Project No. 2001/208

National Library of Australia Cataloguing-in-Publication Entry

ISBN 1441-8487

Published by*:* NSW Department of Primary Industries (now incorporating NSW Fisheries)

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Aquafin CRC is established and supported under the Australian Government's Cooperative Research Centres Program

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LIST OF ACRONYMS

ADC	Apparent digestibility coefficient
ANOVA	Analysis of variance
CHO	Carbohydrates
CP	Crude protein
DE	Digestible energy
DNA	Deoxy ribonucleic acid
DO	Dissolved oxygen
DP	Digestible protein
FCR	Food conversion ratio
GE	Gross energy
GTT	Glucose Tolerance Test
ITS	Internal transcribed spacer region
LSU	Large subunit ribosomal region
OM	Organic matter
PCR	Polymerase chain reaction
PSFC	Port Stephens Fisheries Centre
rDNA	Ribosomal deoxy ribonucleic acid
SARDI	South Australian Research and Development Institute
SSU	Small subunit ribosomal region
UV	Ultraviolet

ACKNOWLEDGEMENTS

This work formed part of a project of Aquafin CRC, and received funds from the Australian Government's CRCs Program, the Fisheries R&D Corporation and CRC Participants.

We thank the staff of the Port Stephens Fisheries Centre for their assistance during this project. We especially thank Luke Cheviot, Luke Vandenberg, Paul Beevers, Geraldine McMahon, Ben Kearney, Debra Ballagh, Bill Bardsley and Brad Tucker for their significant technical expertise and support. Dr's Wayne O'Connor and Michael Dove provided valuable editorial comments during preparation of this report. We also gratefully acknowledge Mrs Helena Heasman for her tireless and good humoured assistance with preparation, compilation and editing of this report.

For assistance with the health component of this volume we thank Professor Bob Lester and Dr Andy Barnes for their guidance and Ms Jessica Worthington Wilmer, Stephen Wesche, Justice Baiano, Tim Green, Jess Hill and other staff at the CMS Laboratories. We also thank the staff of the Marine Parisitology Laboratory of the University of Queensland.

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EXECUTIVE SUMMARY

Australian snapper (*Pagrus auratus)*, known as red sea bream in Japan, is a premium table fish that fetches high market prices in eastern Australia. Commercial culture of this species has been constrained by the high costs of feeds and feeding and fingerlings. The work described in this report (in three volumes) details results of research to address these constraints. This research has increased our knowledge of the nutritional requirements of Australian snapper *Pagrus auratus* and provided information on the potential of Australian feed ingredients to reduce the level of fishmeal in diets for this species. To meet the general and specific aims of this study, a research strategy based on determination of apparent digestibility coefficients to evaluate the potential for a range of potential feed ingredients, establishment of requirements for digestible energy and protein, and an assessment of how well different nutrients from key ingredients was adopted. Nutritional requirements are presented here as are details of the value of different feed ingredients. Trials were conducted that showed that fishmeal could be reduced to approximately 16% provided digestible energy and protein contents were maintained.

Farmed snapper are generally darker and less red in appearance than those obtained from the wild harvest, leading to lower market prices. This issue was raised as a priority among pioneer snapper farmers in Australia. The research described here has increased our knowledge of the factors that affect skin colour and developed practical methods to improve the appearance of farmed snapper. The combination of culturing snapper in light coloured tanks and feeding diets high in the natural pigment, astaxanthin, produced 'pink" coloured snapper that met consumer preferences.

Before this project started, snapper larvae were cultured in hatcheries using intensive techniques and while several hatcheries had successfully produced fingerlings, production costs were high. During this project, new protocols for intensive production were developed for managing environmental variables such as light intensity, photoperiod and temperature and live and inert feeds and feeding regimes. These new protocols allowed greatly increased hatchery production and lower costs. In addition, prior to this project there was no information on the potential or procedures to culture larvae extensively in outdoor, fertilised ponds. Results presented here have provided an understanding of zooplankton production in ponds and allowed strategic stocking of snapper larvae. High quality juvenile snapper were successfully produced in large numbers in fertilised ponds, demonstrating that extensive larval rearing of snapper is a viable alternative to traditional intensive culture.

Snapper, in common with other marine fish, occasionally suffer from infestation of the ectoparasite, *Amyloodinium ocellatum.* During this project, this parasite was genetically characterised and an understanding was made of the methods in which infestation can take place within hatcheries. A variety of control methods including chemotherapeutics were evaluated and recommendations for hatchery biosecurity were made.

This research has been transferred to industry through publications and workshops. Three PhD projects were completed during the project, two have been awarded and the last one will be submitted soon.

Although snapper was the focus of early marine finfish farming operations in New South Wales, Western Australia, South Australia and Queensland, more recently, emphasis has shifted to other marine species, mulloway (Argyrosomus japonicus) and yellowtail kingfish (Seriola lalandi). Much of the research described in this series of reports is relevant to all temperate species of marine fish and research methods used are generally applicable for a range of aquaculture species facing similar constraints.

NON-TECHNICAL SUMMARY

2001/208	Aquafin CRC – Increasing the profitability of snapper farming by improving hatchery practices and diets

PRINCIPAL INVESTIGATOR: Dr Geoff Allan

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OBJECTIVES:

(1) Improve production of snapper fingerlings by developing extensive, fertilised-pond rearing techniques for the advanced production of snapper juveniles.

(2) Improve production of snapper fingerlings by developing larval feeding strategies to reduce the use of live feeds, in particular Artemia, by weaning larvae at an early age onto commercial and/or experimental artificial diets.

(3) Improve production of snapper fingerlings by developing methods to reduce and/or treat the incidence of parasite infestation.

(4) Improve the skin colour of farmed snapper by reducing melanisation and improving skin pigmentation.

(5) Determine digestibility for, and ability of fish to utilize, new ingredients with potential for use in low-polluting snapper diets.

(6) Evaluate ability of snapper to utilize carbohydrate and lipid sources for energy.

(7) Determine optimum protein:energy ratio for fish grown at one favourable temperature.

(8) Provide recommendations for feeding strategies to minimise overfeeding and maximise fish production.

This final report is published in three volumes. Objectives 5, 6 & 7 pertain to Volume 1: Diet Development; Objective 4 pertains to Volume 2: Skin Colour; **Objectives 1, 2, 3 and 8** pertain to **Volume 3: Fingerling Production and Health** (this volume).

The Executive Summary, Background, Need, Objectives, Benefits and Adoption, Further Development and Planned Outcomes are common to all three volumes.

NON-TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

Before this project started, snapper larvae were cultured in hatcheries using intensive techniques and no methods had been developed to culture larvae extensively in outdoor, fertilised ponds. A systematic research approach provided an understanding of zooplankton production in ponds and allowed strategic stocking of snapper larvae. High quality juvenile snapper were successfully produced in large numbers in fertilised ponds, demonstrating that extensive larval rearing of snapper is a viable alternative to traditional intensive culture.

The replacement of the live feed *Artemia* with extensively cultured copepods and artificial weaning pellets for culture of snapper larvae was successfully demonstrated in experiment and commercial-scale trials. Commercially available weaning pellets were also evaluated and new, best-practice rearing regimes for snapper culture are recommended.

The ectoparasite, *Amyloodinium ocellatum*, was genetically characterised and an understanding was made of the methods in which infestation can take place within hatcheries. A variety of control methods including chemotherapeutics were evaluated and recommendations for hatchery biosecurity were made in addition to control if infestation occurs.

The research in this volume has contributed to the the Aquafin CRC contract outcomes: Support for new and emerging industry sectors of finfish aquaculture; Quality fingerlings for farm stocking at an affordable price.

Fingerling Production Component

Objectives and summary of progress against objectives are:

1. Improve production of snapper fingerlings by developing extensive, fertilised-pond rearing techniques for the advanced production of snapper juveniles.

This objective was achieved by conducting a series of experiments designed to understand the dynamics and management of zooplankton production in ponds, determine optimal methods to transfer larvae from hatchery tanks to ponds and the effects of covering ponds with a polyhouse on juvenile snapper production.

The production of suitable size zooplankton at optimal densities for survival and growth of fish larvae varies from site to site, fertilisation regime, and is influenced by pond construction including earthen or plastic-lined pond bottoms. The ponds at PSFC are plastic-lined and development of optimal zooplankton blooms takes between 19-22 days after initial filling with estuarine water and fertilisation. Ponds should therefore be setup and managed approximately three weeks before stocking of snapper larvae. Fertilised ponds can also be used for production of zooplankton which can be harvested and used to feed larvae cultured in intensive hatchery tanks. Based on hatchery production data at PSFC, 3 x 350m^3 ponds, each set up at 7 d interval can produce enough live feeds to culture 200,000 juvenile snapper.

Stocking of newly-hatched snapper larvae into fertilised ponds was unsuccessful in preliminary experiments however when advanced larvae (16 and 23 days after hatching, dah) were stocked into ponds, larvae survived well (40 to 88.4%) and grew rapidly. Particular attention was made to understanding the effect of acclimatisation of larvae during transfer from the hatchery to stocking into ponds but there was no observable benefit if the water quality parameters of hatchery and pond water were similar. Mortality of larvae occurred due to handling and counting prior to stocking into ponds and new methods need to be developed to maximise production.

Polyhouses were highly effective in increasing mean pond water temperature and reducing the range in daily temperature fluctuation. Water temperature in covered ponds was generally 5°C higher with less variability (range 16.2 to 20.3°C) than in uncovered ponds (range 10.4 to 17.7°C). High-quality juvenile snapper were produced in both uncovered and covered ponds but snapper from covered ponds were more developed and 4.2 times heavier than fish cultured in ambient ponds.

Phytoplankton and zooplankton blooms in ponds occasionally "crashed" for unexplained reasons demonstrating the potential risk in extensive pond culture. In order to offset total mortality of snapper larvae due to starvation in the event that zooplankton densities are low, the effect of addition of *Artemia* to ponds as a live food source was investigated. When copepods of suitable size were present in the ponds there was no benefit in adding *Artemia*.

2. Improve production of snapper fingerlings by developing larval feeding strategies to reduce the use of live feeds, in particular *Artemia*, by weaning larvae at an early age onto commercial and/or experimental artificial diets.

This objective was achieved by conducting a series of experiments designed to evaluate the efficacy of replacing the live food, *Artemia*, with copepod or weaning pellet diets. Experiments done in small, 100-L tanks showed clearly that *Artemia* can be completely replaced with either live copepods of similar size to *Artemia* or with high quality weaning pellet diet (ML, Nippai, Japan) with no reduction in snapper larval growth, survival or size variation within the population. The optimal age of larvae for the commencement of feeding artificial pellet diet was identified at 25 dah. These results were validated in a commercial-scale trial at an independent hatchery

Comparison of three commercial weaning diets, ML-powered (Nippai, Japan), Proton (Inve, Belgium) and Gemma (Skretting, France) was done to identify an optimal diet in terms of product quality, commercial availability, growth performance of snapper larvae and cost of fingerling production. All diets were imported to Australia as there is no commercial production of marine fish weaning diets in Australia. The diets varied widely in price (range $39/kg to $352/kg) and the ease with which the diets could be imported e.g. AQIS restrictions, also varied. There was no difference in the performance of snapper larvae fed any of the diets, however the physical attributes of the diets were different, especially buoyancy and clumping of pellets, which necessitated development of different techniques to feed the pellets to larvae. Based on these results we recommend Proton pellets as the most cost-effective pellets for weaning of snapper larvae in Australia.

We conclude that an optimal diet, exclusive of *Artemia*, for intensive rearing of snapper larvae is: rotifers from first feeding (3 dah) to 35 dah; pellet weaning diet started at 25 dah. However, a range of suitable larval feeding strategies is available to hatchery operators thus allowing for personal preferences and scope for successful hatchery operation.

3 Provide recommendations for feeding strategies to minimise overfeeding and maximise fish production.

Identification of optimal feeding strategies of juvenile snapper was achieved by conducting experiments to determine the effects of photoperiod, feeding frequency and size of fish on feed intake, food conversion, fish growth and survival, and variation in fish size within a population. For small, post-weaned fish (range 1 g to 60 g) changes in previous best-practice were observed and incorporated into nursery culture procedures. A photoperiod of 18 h light:6 h dark was optimal for all sizes of juvenile fish tested but the optimal frequency of feeding changed as fish grew larger; small fish require feeding more than 8 times/d while larger fish require only two feeds/d to optimise growth and to reduce the variation in size within a population of fish.

Health Component

Few scientists will question that aquaculture production losses due to parasitic infections are significant on a global scale. With diminishing wild populations of many commercial fish species, aquaculture production is becoming a more important source of protein for human consumption. With progressive advances in technology of aquaculture systems, productivity is assessed and managed at an increasingly finer scale, thereby exposing previously hidden limitations in an attempt to fully maximise harvest. Nonetheless, significant diseases that have been present for many years can often receive relatively little research effort even though their level of their impact may be high.

Since its description by Brown (1931), *Amyloodinium ocellatum*, has been identified and implicated in mortality events in aquaria and aquaculture facilities spanning tropical and sub-tropical regions. A range of tropical aquaculture species are affected by the parasite. Despite its common occurrence, management of *A. ocellatum* has not changed in over two decades, in large part due to deficiencies in knowledge of the basic biology of the parasite.

This report focuses on existing knowledge of *A. ocellatum* and how it affects aquaculture production, with the intention of identifying those information gaps that this study addresses and to explore their potential to better manage this parasite.

Management of *A. ocellatum* infections in marine hatcheries relies on monitoring fish health rigorously and early, accurate diagnosis and treatment are essential. This study has increased our understanding of:

- Routes of infection (aerosol)
- The efficacy of treatment with chemotherapeutics
- Early detection using PCR assay

Most importantly it will have a significant impact on limiting the debilitating effects of this pathogen in marine aquaculture.

This study has enabled the following recommendations for managing outbreaks of *A. ocellatum* in marine hatcheries:

- Strict biosecurity, achieved through minimising equipment transfers, is important to limit entry of the parasite into the facility and its spread.
- New developments should include in their design, physical barriers that minimise impact of aerosols, and implement strategies to minimise aerosol transfer within the facility (e.g. alteration of airflow, covering tanks, situating ponds more than 3m apart); pre existing developments should try to implement these as much as possible.
- Given the persistence of the parasite within facilites once present, prophylaxis, in the form of weekly chemotherapeutic treatments (either as fresh water baths or hydrogen peroxide baths dependant on host tolerances), is essential to maintain infection at sub-clinical levels.
- Monitoring for the presence of infection is paramount to early treatment and minimisation of reactionary measures, weekly testing for the presence of *A. ocellatum* particularly using newly developed PCR based diagnostic procedures will help to achieve this.
- Use of water sterilisation techniques where possible to treat influent water and further limit entry of the parasite to the facility. Additionally, sterilisation procedures such as UV exposure, may help to limit the spread of *A. ocellatum* in recirculation systems..
- Use of chemotherapeutics that are suitable to the culture species and cost effective to the hatchery operation, e.g. freshwater for euryhaline species or hydrogen peroxide for all species.

KEYWORDS:
Pagrus auratus; Snapper, extensive pond culture, polyhouses, early weaning, *Amyloodinium ocellatum*

1. BACKGROUND

This project formed part of the Research Program of the CRC for Sustainable Aquaculture of Finfish ("Aquafin CRC"), and employed funds invested out of the CRC's Commonwealth grant and by FRDC and other participants of the CRC.

When this project was conceived aquaculture of snapper, *Pagrus auratus*, in Australia is developing and commercial farms now operate in NSW and SA (Fielder, unpublished data). The potential for snapper aquaculture is demonstrated by the huge (approximately 50 000 t/yr) industry for this species in Japan. Although technology used in Japan formed the basis for early investigation of snapper culture in Australia, development of this infant industry, especially in NSW, has only been possible following research conducted by NSW Fisheries, FRDC ("Potential of snapper *Chrysophrys auratus* for aquaculture" (1989-1992)) and the CRC for Aquaculture (Project No. C4.2) to develop technology for broodstock management, intensive larval rearing, and evaluation of seacages and inland saline ponds for growout, and diet development.

Some key differences between the industry in Australia and Japan have influenced technology transfer and the need for research described in this application. Most importantly, the market price for fish in Japan is approximately three times that received in Australia and that lessens the need to reduce production costs that is so important if snapper aquaculture is to reach its potential in Australia. Secondly, the hatchery sector in Japan is based on many generations of domestication. We found that just one generation of domestication with Australian snapper conferred major advantages in inducing spawning by manipulating phototherm regimes and expect that as subsequent generations are reared, advantages will compound. In general, hatchery production in Japan is based on greenwater systems. This is economically viable in Japan and even though extensive pond larval culture may be more cost-effective, many hatcheries in Japan have limited access to sufficient land for extensive ponds (land in general is very expensive in many areas in Japan where red sea bream hatcheries are located).

The red sea bream industry in Japan was built on feeding trash fish. It wasn't until the bait-fish industry in Japanese waters collapsed in the late 1980's and early 1990's that farmers were forced to replace trash fish. As late as 1995, commercial practices were still to blend frozen whole fish with a formulated premix. Diet development research in Japan since then has been conducted primarily by private feed companies and is generally not available in the technical or scientific literature.

In Australia, it is now possible to maintain captive broodstock snapper and to spawn high quality eggs on-demand, year-round. Also, intensive rearing of snapper larvae has been improved by identifying the optimum physical parameters such as photoperiod, salinity and temperature (Fielder and Allan, unpublished data). As a consequence, the time to rear snapper larvae to fully-weaned, metamorphosed fish has been reduced by approximately 2 weeks compared with the time taken using previous best-practice techniques. However, intensive larval fish rearing requires high capital and labour inputs as well as operation of facilities to culture live feeds such as rotifers and *Artemia*. These ancillary culture systems can be unreliable and expensive to operate. There is also a world shortage of *Artemia* cysts and purchase price has tripled in the last 12 months. Current industry estimates the cost of production of snapper at $1.00 /fingerling. In comparison, industry costs for producing other marine fingerlings are 34c for barramundi (Lobegeiger, 2001) and about 46c for bass. To improve profitability, there is an obvious need to develop techniques to reduce the cost and improve the vigour of fingerlings. Alternative hatchery methods or live foods and their replacement with artificial diets therefore needed to be identified.

Scientists at PSFC linked with counterparts at SARDI who regularly breed large numbers of snapper for industry. Advances in larval rearing techniques have been validated at SARDI

facilities. This has been an important conduit for technology transfer for South Australian hatcheries.

Extensive larval rearing, where ponds are fertilised to promote zooplankton populations (McCarty, Geiger, Sturmer, Gregg & Rutledge, 1986; Fielder, Bardsley & Allan, 1999), has been used to rear large numbers of several marine fish species such as red drum *Sciaenops ocellatus* (McCarty *et al.* 1986), barramundi *Lates calcarifer* (Rutledge & Rimmer, 1991), Australian bass *Macquaria novemaculeata* (Battaglene, Talbot & Allan, 1992) and mulloway *Argyrosomus japonicus* (Fielder *et al.*, 1999). Growth and quality of fish can be high from fertilised ponds and cost of fingerling production can be low due to the need for relatively unsophisticated facilities and low operating costs. However, survival of larvae can be variable due to sub-optimal environmental conditions. In the successful FRDC Project No. 95/148, survival of mulloway larvae in fertilised ponds increased as the age of larvae that were stocked increased (Fielder *et al.*, 1999). These results indicated that a combination of initial intensive rearing from hatch to consumption of *Artemia* (~14 d), when larvae are vulnerable to fluctuations in environmental conditions, followed by on-growing in fertilised ponds can optimise fingerling production. Preliminary trials during the CRC for Aquaculture project C4.2 demonstrated that snapper juveniles could be reared in commercial larval fish ponds; however, survival was low and the power of experiments was very low due to low or no replication. These techniques may be suitable for large-scale production of cheap, high quality, healthy juvenile snapper but this must be verified in rigorous, replicated experiments.

Development of methods to reduce the reliance on *Artemia* as a live feed in intensive culture of juvenile snapper may also decrease the cost of production. Preliminary larval snapper rearing trials have demonstrated that *Artemia* can be replaced completely by extending the period of rotifer feeding and early addition of high quality imported weaning diets (Fielder, unpublished data). Indeed replacement of *Artemia* in aquaculture in Australia is viewed as a priority (McKinnon, Rimmer & Kolkovski,, 2000) and FRDC have funded a project (2001-220) "Aquaculture Nutrition Subprogram: development of marine fish larval diets to replace *Artemia*" (PI Dr Sagiv Kolkovski), to investigate production of artificial microdiets for marine fish larvae. This technique has been evaluated in terms of fish quality, survival, reliability and cost of production. Also, replacement of *Artemia* with cultured copepods for marine fish larval rearing has recently attracted significant attention by larval fish culturists and was highlighted as a priority research area at the recent FRDC sponsored Live Feeds Workshop. As an alternative to intensive production of copepods (Rippingale, 1994), it may be possible to produce sustainable cultures of copepods in outdoor ponds, which could then be harvested and fed to snapper larvae in intensive tanks. Hundreds of thousands of juvenile Australian bass have been produced at the Port Stephens Fisheries Centre (PSFC) using this method, resulting in complete replacement of both rotifers and *Artemia*.

The research proposed in this application to decrease the cost of fingerling snapper has relevance to many other species of marine finfish. Feeding marine fish larvae is expensive and reducing mortality remains an international priority. Technologies developed and refined here will assist in efforts to reduce fingerling production costs for other species. Facilities at PSFC are unique as they include both large-scale production facilities and replicated smaller-scale facilities for larval rearing (intensive, semi-intensive and extensive). These type of facilities will be especially critical in the pursuit of successful tuna propagation and fingerling production.

New health management strategies are required to minimise the impact of disease in hatcheries. Disease outbreaks reduce vigour of fish and periodically cause excessive mortality resulting in increased cost of fingerling production. Infestations of the ectoparasite Amyloodinium sp. in particular have caused significant mortality in Australian snapper hatcheries (Fielder & Allan, unpublished data) and is a major problem in overseas marine fish hatcheries (Paperna, 1983). Strategies to minimise losses include management to exclude or reduce the prevalence of disease, enhancement of fish resistance, and application of new methods of control.

A post-graduate Certificate student at the University of Queensland, Mr James Stopford, recently developed an excellent method to produce large number of Amyloodinium parasites in the laboratory. He operated his cultures for at least 10 generations and used the product to conduct preliminary investigations on the parasite. His work provided an excellent foundation for this project.

The cost of purchasing and delivering feeds is the single highest operating cost for most types of fish culture. To farm fish profitably, there is an obvious economic imperative to develop high-performance diets and feeding systems which are cost-effective. The diets also need to produce fish with desirable marketing traits. These traits include skin and flesh colour and flesh composition. To fetch premium prices, snapper need to have pink skin and white flesh. Large deposits of intestinal fat are perceived to be a marketing disadvantage. Fish are marketed as a "healthy" product, largely because fish fat has relatively high contents of the omega-3 highly unsaturated fatty acids. However, while replacing fish meal and fish oil in fish diets may reduce diet cost, and may also reduce P content in the effluent from farms, it will also reduce these health benefits.

There is also an important environmental imperative to minimise the amounts of nutrients and organic matter that are discharged from fish farms. To achieve these goals, diets need to satisfy but not oversupply essential nutrients and be made from high quality, highly digestible, readily obtainable ingredients. Diets also need to stimulate maximum consumption and deliver optimal feed conversion efficiency.

Excretion of nitrogen (N), phosphorus (P) and organic matter (mainly carbon, C) are the major pollutants from fish farms and their release is regulated by environmental protection agencies in many states in Australia. Feed is the sole source of these potential pollutants. To minimise N loss, N (protein) digestibility and retention must be increased. The recommended approach is to optimise protein quality and minimise diet protein content. This might be achieved by optimising the protein:energy ratio and using as much non-protein energy as possible. Strategies to minimise P loss include: selecting ingredients with high P bioavailability and selecting P supplements with high P absorbtion and low water solubility. To reduce organic matter pollution, diets must be highly digestible, promote maximum feed consumption and feed conversion efficiency. Minimising feed wastage through ensuring optimal pellet stability and determining the best feeding frequencies and feeding rates are critical factors in reducing pollution from fish farms.

FRDC currently fund snapper diet development research through project 99/323 "Aquaculture Diet Development (ADD) Subprogram: rapid development of diets for Australian snapper." Research under this project has determined:

1. Effects of shading and dietary astaxanthin source on skin colour. Results have demonstrated that although shading will reduce "skin blackness", shaded, farmed fish are still darker than wild fish and although shading is a viable option for some smaller, inshore fish farms, it is very difficult for large, offshore farms. To harvest genuinely "light" fish, farmers will need to find better ways to prevent melanisation. Addition of astaxanthin in either the free or esterified form increased the intensity of the "red" colour in the skin but this colour was not the highly desired "pink" of wild fish. More needs to be done to evaluate combinations of commercially available pigments to make fish pink and to reduce skin melanisation.

2. Identification of the best available commercial diet for snapper. Previous research under the ADD Subprogram for barramundi demonstrated the cost-effectiveness of nutrient-dense diets. It was expected that these would also benefit snapper but this has not been apparent in results from this commercial diet evaluation. Clearly, snapper have different nutrient requirements to barramundi, indicating that determination of their protein:energy requirements and ability to utilize carbohydrates for energy warrant independent investigation.

3. Protein:energy requirements will be determined at one temperature. This research will commence as soon as digestibility of experimental ingredients is completed.

4. Digestibility and utilization of key ingredients with potential to replace fishmeal and be incorporated into lower cost, high performance diets for snapper. This research is underway and will allow full investigation of digestibility of up to 16 ingredients and utilization of up to 8 ingredients.

5. Preliminary experiment to determine whether ionic deficiencies in inland saline water can be overcome through nutrition supplements. This research is planned to commence within six months.

The nutrition component of of this research was fully integrated with FRDC 99/323. Already, the current FRDC project 99/323 addressed important questions and allowed formulation of better diets. However, diet development is an ongoing process. Increased funding for poultry and pig diet development is being allocated each year in recognition of the ongoing importance of feed and feeding to animal husbandry. This additional research on snapper diet development has proven to be a good investment for industry. The research on skin colour has identified the best mix of pigments and reduction of melanisation, beyond reduction possible using shading. Farmers have claimed they could receive up to an extra $3/kg for light and pink snapper. Research on alternative ingredients and on protein:energy requirements has been completed, in light of the apparent dissimilarity of snapper nutritional requirements to those of barramundi.

Research has also focussed on eco-friendly feeds that minimise concentration of N, P and C from uneaten or poorly utilized diets. One of the only ways to reduce N is to provide as much non-protein dietary energy as fish can utilize. The two non-protein dietary energy sources are lipid and carbohydrate. Utilization of these ingredients have been investigated and effects on body composition and taste determined.

Finally, recommendations have been made to optimize feeding strategies to minimize over-feeding.

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2. NEED

This project extended previous work, which demonstrated the feasibility of snapper farming in both marine and inland saline waters. It sought to reduce production costs by improving fingerling survival and growth and reducing input (feed) costs.

A reliable supply of cheap, high quality, healthy fingerlings is essential for development of viable snapper farming. Currently, industry estimates the cost of production of snapper at $1.00 per fingerling. This compares with under 35¢ for barramundi fingerlings and about 46¢ for bass. To improve profitability, there is a need to reduce the cost and improve the vigour of fingerlings and to develop cost-effective high-performance diets and feeding systems for both hatchery and grow-out. This need has been recognised through the FRDC sponsored Hatchery Feeds R&D Plan (McKinnon, Rimmer & Kolkovski, 2000: http://www.aims.gov.au/hatchery-feeds), This project improved hatchery methods and replaced live feeds, such as brine shrimp (*Artemia*) whose supply and quality are unreliable, with alternative live feeds or artificial feeds. The project also developed better strategies for combining intensive and extensive rearing methods so as to optimise fingerling survival and quality. Research will have application for other species, including tuna.

Grow-out diets need to produce fish with desirable marketing traits, including colour. Fish are marketed as a "healthy" product, largely because fish fat has relatively high contents of the omega-3 highly unsaturated fatty acids. However, while replacing fishmeal and fish oil in fish diets may reduce diet costs, it will also reduce these health benefits. Minimising feed wastage through ensuring optimal pellet stability and determining the best feeding frequencies and feeding rates are critical factors in reducing pollution from fish farms. To achieve these goals, diets have been developed that satisfy but not oversupply essential nutrients and that are made from high quality, highly digestible, readily obtainable ingredients. Diets have been designed to stimulate maximum consumption and deliver optimal feed conversion efficiency. This research has built on successful results with snapper diet development under the previous FRDC ADD Subprogram snapper diet development project. The nutrition component of this project was fully integrated with the FRDC snapper diet development project.

Finally, the project sought to reduce disease-induced mortality by developing treatment methods for common parasites and establishing a foundation for immunological approaches to fish skin diseases.

3. OBJECTIVES

1. **Improve production of snapper fingerlings by developing extensive, fertilised-pond rearing techniques for the advanced production of snapper juveniles.**

2. **Improve production of snapper fingerlings by developing larval feeding strategies to reduce the use of live feeds, in particular Artemia, by weaning larvae at an early age onto commercial and/or experimental artificial diets.**

3. **Improve production of snapper fingerlings by developing methods to reduce and/or treat the incidence of parasite infestation.**

4. Improve the skin colour of farmed snapper by reducing melanisation and improving skin pigmentation.

5. Determine digestibility for, and ability of fish to utilize, new ingredients with potential for use in low-polluting snapper diets.

6. Evaluate ability of snapper to utilize carbohydrate and lipid sources for energy. (Diet Development Component Vol. 1)

7. Determine optimum protein:energy ratio for fish grown at one favourable temperature. (Diet Development Component Vol. 1)

8. **Provide recommendations for feeding strategies to minimise overfeeding and maximise fish production.**

This final report is published in three volumes. Objectives 5, 6 & 7 pertain to Volume 1: Diet Development; Objective 4 pertains to Volume 2: Skin Colour; **Objectives 1, 2, 3 and 8** pertain to **Volume 3: Fingerling Production and Health** (this volume).

4. RESULTS AND DISCUSSION

4.1 Replacement of *Artemia* in the larval rearing diets of Australian snapper, *Pagrus auratus*

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1. INTRODUCTION

Culture of Australian snapper, *Pagrus auratus*, has received much interest in Australia since the mid 1980s, however industry has been slow to grow (Battaglene & Fielder, 1997; Fielder, Bardsley & Allan, 2001, 2002; Tucker, Booth, Allan, Booth & Fielder, 2006). Reliable spawning and larval rearing techniques for snapper have been developed but a major constraint to industry development has been the cost of fingerling production (Fielder, Allan & Battaglene, 1999).

Consequently, research has been targeted at reducing costs of fingerling production including identification of optimal salinity, temperature and photoperiod for larval rearing (Fielder, Bardsley, Allan & Pankhurst, 2002; Fielder, Allan & Pankhurst, 2005; Fielder, Allan & Pankhurst, 2008). Behind labour, the use of live foods including *Artemia* and rotifers to feed larvae represents significant costs to production of snapper fingerlings.

Artemia are used extensively in marine fish hatcheries throughout the world as a primary food source for developing larvae. *Artemia* are expensive to buy and their use as a live feed can represent up to 17% of the total hatchery operating costs, second only to total labour costs (35%) (Candreva, Dhert, Novelli & Brissi, 1996). *Artemia* availability is also prone to anomalies in the wild harvest fisheries with consequent swings in purchase price. In addition, *Artemia* culture in hatcheries requires dedicated facilities and labour and *Artemia* must be enhanced nutritionally before feeding to fish larvae.

Methods to reduce the reliance on *Artemia* as a live feed in intensive culture of marine fish is viewed as a priority for marine fish hatcheries (McKinnon, Rimmer & Kolkovski, 2000) and must be developed for juvenile snapper to potentially decrease the cost of production. Preliminary larval snapper rearing trials demonstrated that *Artemia* can be replaced completely by extending the period of rotifer feeding and early addition of high quality imported weaning diets (Fielder, unpublished data). However, this technique needs to be evaluated in terms of fish quality, survival, reliability and cost of production. Also, replacement of *Artemia* with cultured copepods for marine fish larval rearing has recently attracted significant attention by larval fish culturists and was highlighted as a priority research area in Australia (FRDC Live Feeds Workshop 2004).

As an alternative to intensive production of copepods (Rippingale, 1994), it may be possible to produce sustainable cultures of copepods in outdoor ponds, which could then be harvested and fed to snapper larvae in intensive tanks. Hundreds of thousands of juvenile Australian bass have been produced at the Port Stephens Fisheries Centre (PSFC) using this method, resulting in complete replacement of both rotifers and *Artemia*.

The aim of the study was to determine if snapper larvae could be reared intensively using diets without *Artemia* or where *Artemia* was reduced. Artificial pellet diets or pond-cultured copepods were evaluated in experiments.

2. MATERIALS AND METHODS

Three experiments were done to evaluate the efficacy of replacing *Artemia* with pellet or copepod diets. The first experiment investigated whether snapper larvae can be weaned from live feed to pellet feeds at an early age and if *Artemia* can be replaced as a live feed by either extending the period of rotifer feeding and/or feeding pellet. The second experiment was done to validate the results of Experiment 1 in large-scale, commercial tanks. The third experiment was done to evaluate the suitability of pond grown zooplankton for rearing of advanced snapper larvae.

2.1 Experiment 1. Effect of replacing Artemia with artificial pellet diet.

An experiment was completed to evaluate the effect of different combinations of live feed (rotifers and *Artemia*) and formulated pellet diet (ML-powered, Nippai, Japan) on performance of snapper larvae. The experiment treatments are described in Table 1.

TABLE 1

Experiment feeding regimes

Treatment	Rotifers (Days after hatch)	Artemia (Days after hatch)	ML diet (200 um) (Days after hatch)
1	10-35	20-33 (full ration)	25-35
2	10-35	20-33 (50% ration)	25-35
3	10-35	Nil	25-35
4	10-35	20-33 (full ration)	15-35
5	10-35	20-33 (50% ration)	15-35
6	10-35	Nil	15-35

First generation hatchery-reared broodstock snapper were induced to spawn spontaneously after manipulation of water temperature and photoperiod. Fertilised eggs were then placed into a 2000-L recirculation larval rearing tank and reared for 10 days under optimal salinity, temperature and photoperiod conditions until larval swimbladders had inflated (Fielder *et al*., 2002). After 10 days, larvae were harvested from the rearing tank and distributed evenly to 30, 100-L experiment tanks (see Fielder *et al*., 2002) to provide 2238 ± 798 larvae/tank. Physical parameters including salinity 25‰, temperature 24^{o}C and photoperiod 18 h light: 6 h dark were maintained for the duration of the 33 day experiment. Tank water was exchanged through biofilters approximately 400-600%/d and 100% new seawater was exchanged each day.

Treatment feeding regimes were instigated after larvae were stocked. Rotifers and *Artemia* were fed four times/d between 0900 and 1700h and ML pellet diet (Nippai, Japan) was fed by hand 5-6 times/d in similar size portions to provide excess feed. Uneaten feed was removed by vacuum daily from each tank.

There were five randomly selected tanks for each treatment. Larval performance was determined in terms of growth (final total length, wet weight, dry weight), survival, and time to weaning from live feed to pellet diet.

2.2 Experiment 2. Validation of results in commercial-scale tanks

An experiment was completed at the South Australian Research & Development Institute, West Beach to evaluate in commercial-scale tanks the performance of snapper larvae fed a diet of rotifers/pellet compared with a diet of rotifers/*Artemia*/pellet.

2.3 Stocking

Eight larval rearing tanks of 1300L where each stocked with approximately 33,500 snapper larvae. A total of 21,100 larvae originated from hand stripped eggs from F1 broodstock which had been induced to spawn using slow release intramuscular pellet hormone (25 ug/kg bw) LHRHa implants, and 12,400 larvae originated from wild-caught broodstock which spawned spontaneously in tanks. Quality of eggs from both spawns was considered high according to the positive buoyancy of the eggs.

2.4 Feeding

Four replicate tanks were allocated at random to each of two feeding treatments.

Treatment 1. With *Artemia*.

Standard snapper larval rearing protocol incorporating the use of *Artemia* nauplii.

Treatment 2. Without *Artemia*.

Modified snapper larval rearing protocol totally replacing the use of *Artemia* with an agglomerated particulate diet formulated for marine finfish.

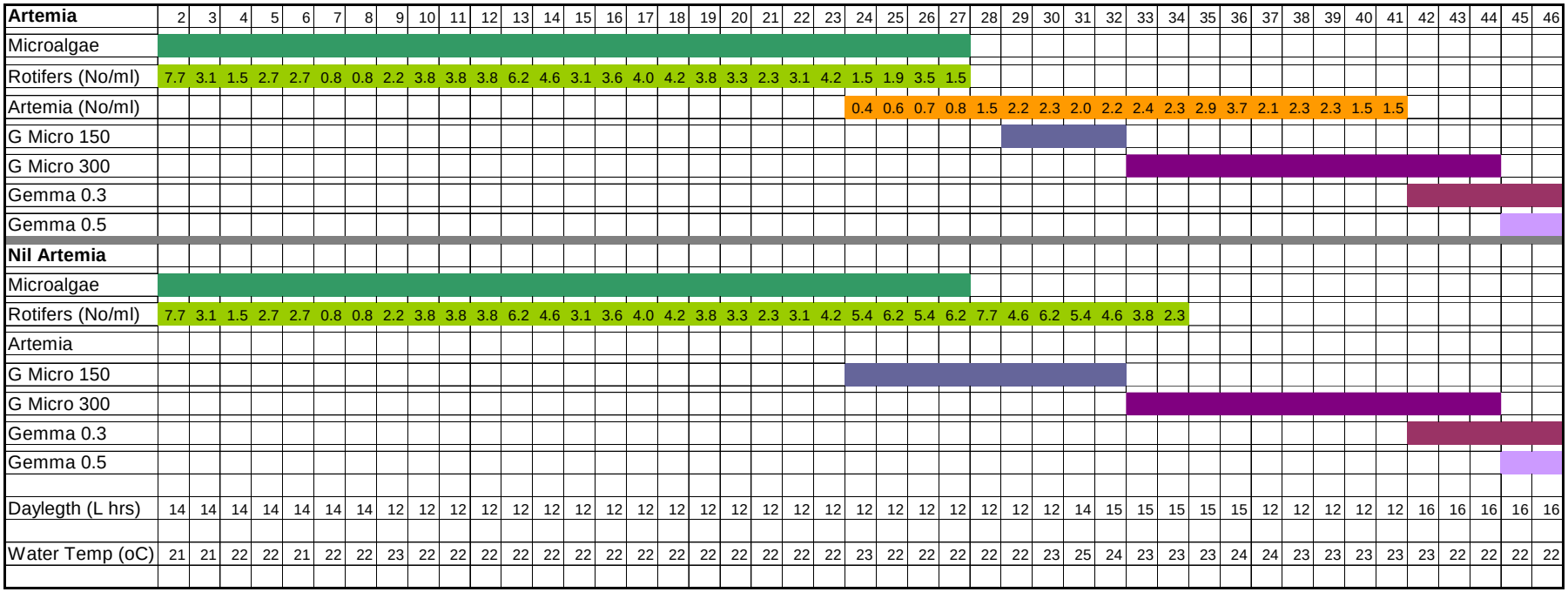
Both feeding treatments (Figure 1) followed those used in Experiment 1 with the exception that the pellet diet was produced by a different manufacturer. The agglomerated particulate diets used were the Gemma range provided by Skretting Australia and included Gemma Micro 150™, Gemma Micro 300™, Gemma 0.3™ and Gemma 0.5™. Transitions between feed sizes were based upon the ability of larvae to easily ingest particles offered.

All tanks were provided with a belt feeder for distribution of the marine starter feeds. The belt feeder was positioned above the water inlet that had been modified to provide a small surface jet of water to assist feed distribution. After 30dph supplemental hand feeding was conducted periodically to assess feeding activity.

The trial was conducted over the first 46 days of larval rearing with differentiation between treatments commencing from 24 dph when either enriched (DHA Selco™) second stage *Artemia* nauplii or Gemma Micro™ 150 was introduced. The trial was terminated 5 days (46 dph) after the *Artemia* fed treatment had completed weaning. Throughout the trial larvae from each tank of each treatment were randomly sampled (n = 10 – 20) two times each week and measured using image analyses (Sigma Scan Pro) to provide growth data.

FIGURE 1

Feeding schedule used for experimental treatments *Artemia* and Nil *Artemia*



Artemia	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
Microalgae																																														
Rotifers (No/ml)	7.7	3.1	1.5	2.7	2.7	0.8	0.8	2.2	3.8	3.8	3.8	6.2	4.6	3.1	3.6	4.0	4.2	3.8	3.3	2.3	3.1	4.2	1.5	1.9	3.5	1.5																			
Artemia (No/ml)																							0.4	0.6	0.7	0.8	1.5	2.2	2.3	2.0	2.2	2.4	2.3	2.9	3.7	2.1	2.3	2.3	1.5	1.5					
G Micro 150																																												
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Gemma 0.3																																												
Gemma 0.5																																												
Nil Artemia																																												
Microalgae																																												
Rotifers (No/ml)	7.7	3.1	1.5	2.7	2.7	0.8	0.8	2.2	3.8	3.8	3.8	6.2	4.6	3.1	3.6	4.0	4.2	3.8	3.3	2.3	3.1	4.2	5.4	6.2	5.4	6.2	7.7	4.6	6.2	5.4	4.6	3.8	2.3											
Artemia																																												
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Daylegth (L hrs)	14	14	14	14	14	14	14	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	14	15	15	15	15	15	12	12	12	12	12	16	16	16	16	16
Water Temp (oC)	21	21	22	22	21	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	23	25	24	23	23	23	24	24	23	23	23	23	23	22	22	22	22

2.5 Larval rearing

All larval rearing tanks were operated using a "brown water" culture protocol until 27dph while rotifers were being fed. *Isochrysis* sp Tahitian strain (T. *Iso*) or *Pavlova lutheri* was added each morning and evening and if necessary this was supplemented with a concentrate of *Nannochloropsis oculata* (Nanno 3600™, Reed Mariculture).

As water exchange rate was increased the system was transformed from a brown water system to a "clear water" system as *Artemia* or micro-feeds were introduced. Water exchange to all tanks was provided from three recirculating water treatment systems that were joined to share the same water. Water exchange in each system was 100% per day. Salinity was maintained at 35-37 ppt. Other environmental parameters (i.e. water temperature, day-length) for larval rearing were modified from optimal parameters provided by NSW Fisheries due to limitations with rotifer supply and the need to reduce the feeding period (during light hours) (Figure 1).

Throughout early larval rearing, supply of rotifers was problematic as microalgal cultures used to culture the rotifers failed regularly. Daily rations available for feeding to larvae were consequently compromised and only 25% of the daily standing stock could be used for feeding larvae. On several occasions new batches of rotifers were purchased in an attempt to remedy this situation however this problem persisted throughout the trial.

2.6 Experiment 3: Effect of replacing artemia with pond-cultured zooplankton

An experiment was completed to evaluate the suitability of pond grown zooplankton for rearing of advanced snapper larvae. Feeding regimes included combinations of rotifers, Nippai ML pellet, enriched *Artemia* and pond grown zooplankton (principally copepods) (Table 1).

TABLE 2

Experiment 3 feeding regimes

Treatment	Rotifers (Days after hatch)	Artemia	Copepods	ML Pellet diet (200 um) (Days after hatch)
1	25-35	nil	nil	25-42
2	25-35	25-35 dah (full ration)	nil	25-42
3	25-35	nil	25-35 dah (full ration)	25-42
4	25-35	25-35 dah (half ration)	25-35 dah (half ration)	25-42

First generation hatchery-reared broodstock snapper were induced to spawn using hormone induction (LHRH-a at 50ug/kg fish). Fertilised eggs were than placed into a 10,000-L greenwater larval rearing tank and reared for 25 days under optimal salinity, temperature and photoperiod conditions until larval swimbladders had inflated (Fielder *et al*., 2002). After 25 days larvae were harvested from the rearing tank and 175 larvae were placed into each of 16, 100-L experiment tanks (see Fielder *et al*., 2002). Physical parameters including salinity 25-35‰, temperature 24^{o}C and photoperiod 18 h light: 6 h dark were maintained for the duration of the 17 day experiment. Tank water was exchanged on a flow-through system with UV sterilised seawater at approximately 100% new seawater was exchanged each day.

Live feeds were fed up to four times/d between 0900 and 1700 h to provide treatment densities (Table 2). Zooplankton was produced in ponds by applying organic and inorganic fertilisers and harvested by pumping through a 150 um mesh screen. Uneaten feed and detritus was removed by vacuum daily from each tank. There were four randomly selected tanks for each treatment. Larval

performance was determined in terms of growth (final total length, wet weight, dry weight) and survival.

3. RESULTS

3.1 Experiment 1

Mean survival of snapper larvae from 10 days after hatch (dah) to 43 dah was high in all treatments (73-98%) and was not affected by feeding regime. Growth of snapper larvae was also not significantly different (P>0.05) between feeding treatments (Figure 2). Larvae were observed to be eating ML pellet in the water column and off the water surface within 2-3 days of it being introduced in the feeding regime but larvae increased their vigour of pellet consumption with time. There was no observable difference in larval feeding behaviour when feeding of rotifers was stopped at 35 dah.

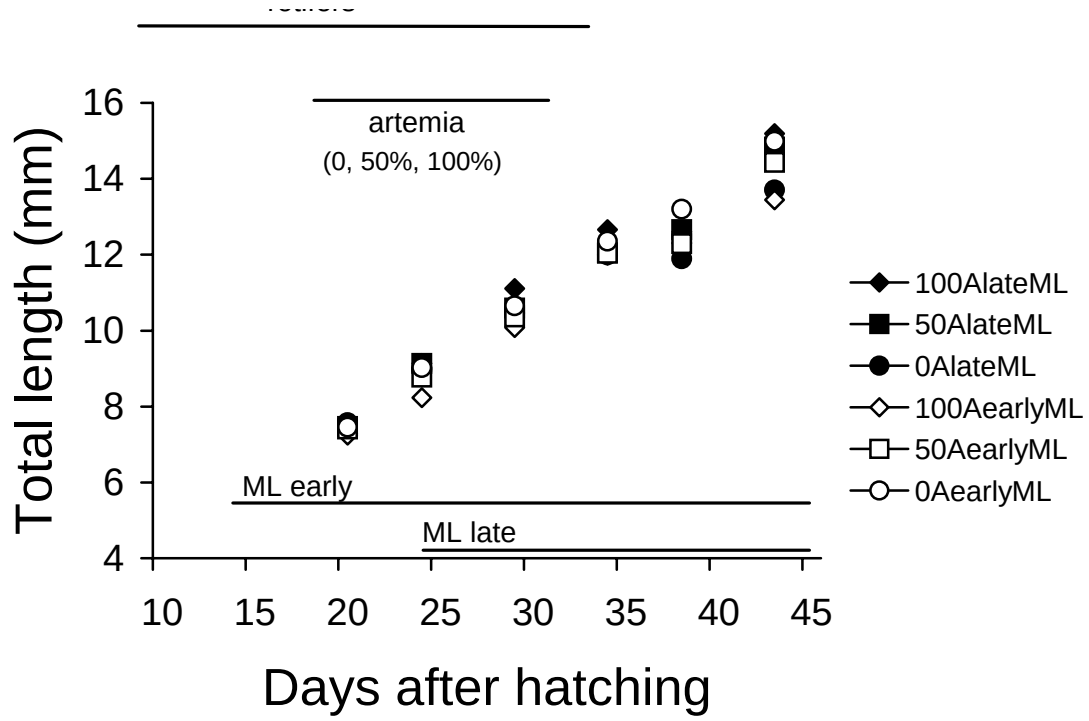

FIGURE 2

Total length of snapper larvae fed different combinations of *Artemia* and ML diet

3.2 Experiment 2

Mean growth (Figure 3) and survival of larvae from both treatments (7.4 ± 3.0%, *Artemia*; 4.62 ± 3.39%, no *Artemia* were not significantly different (P>0.05) at 46 dph.

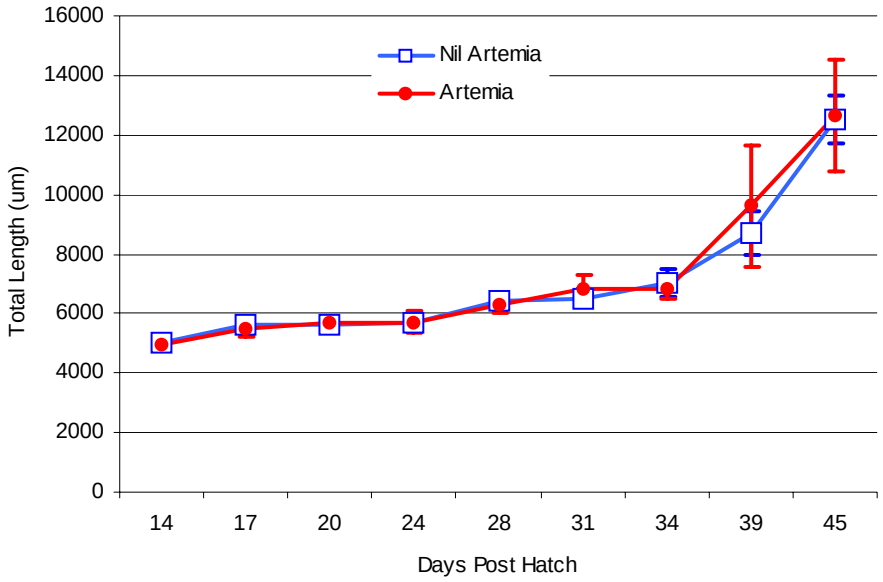

FIGURE 3

Growth (TL) of snapper fingerlings from 14 to 46 days post hatch using feeding schedules with *Artemia* and without *Artemia*.

3.3 Experiment 3

Survival, growth and coefficient of variation of final total length of snapper were not affected by the feeding regime (Table 3).

TABLE 3

Feeding schedule for intensive snapper larval rearing

		Larvae age (dah)													
		25	26	27	28	29	30	31	32	33	34	35	36	37	38
Treatment 1	rotifers(/ml)	20	20	20	20	20	20	20	20	20	20	20			
control	artemia/	0	0	0	0	0	0	0	0	0	0	0			
	copepods	0	0	0	0	0	0	0	0	0	0	0			
	ML (g/ta	0.2	0.3	0.3	0.3	0.4	0.4	0.5	0.6	0.6	0.7	0.8	0.9	1.0	1.1
Treatment 2	rotifers(/ml)	20	20	20	20	20	20	20	20	20	20	20			
previous best	artemia/	1.2	1.6	2	1.6	1.2	0.8	0.6	0.4	0.2	0.1	0.1			
practice	copepods														
	ML (g/ta	0.2	0.3	0.3	0.3	0.4	0.4	0.5	0.6	0.6	0.7	0.8	0.9	1.0	1.1
Treatment 3	rotifers(/ml)	20	20	20	20	20	20	20	20	20	20	20			
half ration	artemia/	0.6	0.8	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.05			
half ration	copepods	0.6	0.8	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.05			
	ML (g/ta	0.2	0.3	0.3	0.3	0.4	0.4	0.5	0.6	0.6	0.7	0.8	0.9	1.0	1.1
Treatment 4	rotifers(/ml)	20	20	20	20	20	20	20	20	20	20	20			
	artemia/	0	0	0	0	0	0	0	0	0	0	0			
	copepods	1.2	1.6	2	1.6	1.2	0.8	0.6	0.4	0.2	0.1	0.1			
	ML (g/ta	0.6	0.7	0.8	1.0	1.1	1.2	1.4	1.6	1.8	2.0	2.2	2.5	2.8	3.0

TABLE 4

Performance of advanced snapper larvae fed different combinations of rotifers, atremia, copepods and weaning pellet diet from 25-42 days after hatching. Data are means ± S.E. (n=4 tanks). Data in each column are not significantly different (P>0.05).

Treatment	Survival (%)	Final wet weight (mg)	Final dry weight (mg)	Final total length (mm)	CV of total length (%)
1	29.6 ± 4.8	31.2 ± 3.3	6.0 ± 0.7	13.0 ± 0.6	14.2 ± 1.1
2	41.0 ± 4.9	39.2 ± 6.1	7.8 ± 1.3	13.4 ± 0.8	18.4 ± 3.8
3	41.7 ± 4.3	40.9 ± 8.8	8.1 ± 1.8	13.8 ± 1.1	15.5 ± 3.4
4	40.9 ± 4.3	43.5 ± 11.6	8.6 ± 2.4	13.7 ± 1.1	17.9 ± 2.3

4. DISCUSSION

Artemia were successfully excluded from the diet of snapper larvae in small, experiment tanks and this result was confirmed in commercial-scale intensive, clearwater tanks which were operated at a production facility independent of the experiment facility. Provided rotifers, copepods and/or high quality weaning pellet diets were provided to larvae, survival and growth of snapper larvae from post swimbladder inflation to metamorphosis was similar to a diet which included enriched *Artemia* metanauplii.

Artemia cysts are expensive to buy and hatching, ongrowing and nutritional boosting of *Artemia* requires specialised facilities as well as labour. Therefore exclusion of *Artemia* from snapper larval diets and replacement with alternatives will reduce the cost of intensive larval rearing. Although early age snapper larvae (15+ dah) were observed consuming ML pellet there was no apparent difference in growth and survival of larvae to metamorphosis when ML pellet was offered at a later stage (25+ dah). Excess feeding with pellet diet can decrease water quality in larval tanks as well as increase the labour costs associated with tank maintenance. We therefore conclude that a satisfactory diet, exclusive of *Artemia*, for intensive larval rearing of snapper larvae is: rotifers from first feeding (3 dah) to 35 dah; ML or Gemma pellet (200 um) started at 25 dah.

A range of suitable snapper larval feeding strategies is available to hatchery operators thus providing scope for successful hatchery operation.

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4.2 Comparison of commercial weaning diets for larval rearing of Australian snapper, *Pagrus auratus*

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1. INTRODUCTION

Marine fish larvae must be weaned from live feeds including rotifers and *Artemia* onto commercial, formulated diets. In Australia, marine fish weaning diets are not manufactured by local feed mills and consequently must be imported from Japanese and European manufacturers. The diets manufactured overseas have been developed for Japanese and European fish species and are not necessarily ideal for Australian species including snapper. In addition, some weaning diets are easier to access than others due to import regulations from the Australian Quarantine Inspection Service (AQIS) and the diets vary greatly in purchase price.

The aim of the experiment was to evaluate the suitability of three commercial marine fish weaning diets for early weaning of snapper larvae.

2. MATERIALS AND METHODS

Three commercial marine fish weaning diets, ML-powered (Nippai, Japan), Proton (Inve, Belgium) and Gemma (Skretting, France) were selected for the experiment. The age of snapper larvae and combination of live feed/pellet diet used in the experiment were based on previous experiment results: where the optimal age of snapper larvae (25 days after hatch) and live/artificial feed combination (rotifers/ML pellet) were identified for early weaning of intensively reared snapper. The experiment treatments are described in Table 1.

TABLE 1

Live and artificial diet feeding schedule for weaning Australian snapper larvae.

Treatment	Rotifers (Days after hatch)	Pellet diet (200 um) (Days after hatch)
1	10-35	ML-powered (25-44)
2	10-35	Gemma (25-44)
3	10-35	Proton (25-44)

First generation hatchery-reared broodstock snapper were induced to spawn spontaneously after manipulation of water temperature and photoperiod. Fertilised eggs were than placed into a 2000-L recirculation larval rearing tank and reared for 23 days under optimal salinity, temperature and photoperiod conditions until larval swimbladders had inflated (Fielder, Bardsley, Allan & Pankhurst, 2002). After 23 days larvae were harvested from the rearing tank and 800 larvae were placed into each of 24, 100-L experiment tanks (see Fielder *et al*., 2002). Physical parameters including salinity 25‰, temperature 24^{o}C and photoperiod 18 h light: 6 h dark were maintained for the duration of the 21 day experiment. Tank water was exchanged through biofilters approximately 400-600%/d and 100% new seawater was exchanged each day.

Rotifers were fed up to four times/d between 0900 and 1700 h to provide a target density of 20 rotifers/ml and pellet diets were fed in similar size portions to provide excess feed by hand 6 times/d until 41 dah and 8 times/d from 41-44 dah. Uneaten feed was removed by vacuum daily from each tank.

There were eight randomly selected tanks for each treatment. Larval performance was determined in terms of growth (final total length, wet weight, dry weight) and survival. Physical characteristics of pellets including sinking speed and associated water quality were also observed.

3. RESULTS

Mean survival of snapper larvae from 23 to 44 dah was high in all treatments and was not affected by feeding regime ($P>0.05$) (Figure 1). Wet weight increase of snapper larvae was also not significantly different ($P>0.05$) between pellet treatments (Figure 2). Larvae were observed to be eating each pellet type in the water column and off the water surface within 1-2 days of it being introduced in the feeding regime but larvae increased their vigour of pellet consumption with time. The dynamics of the pellets after introduction to the larval rearing tanks varied between brand of pellet. The Nippai and Proton pellets spread evenly over the water surface after introduction to the tank but the Gemma pellet tended to form small clumps and sank to the bottom faster than Nippai and Proton pellets. The Nippai pellets appeared to sink more slowly than the Proton pellet, however this needs to be confirmed in a replicated, controlled experiment.

FIGURE 1

Survival of snapper larvae from 23 to 44 days after hatchery fed three different weaning diets. Data are means ±S.E. ($n=8$). Bars are not significantly different ($P>0.05$).

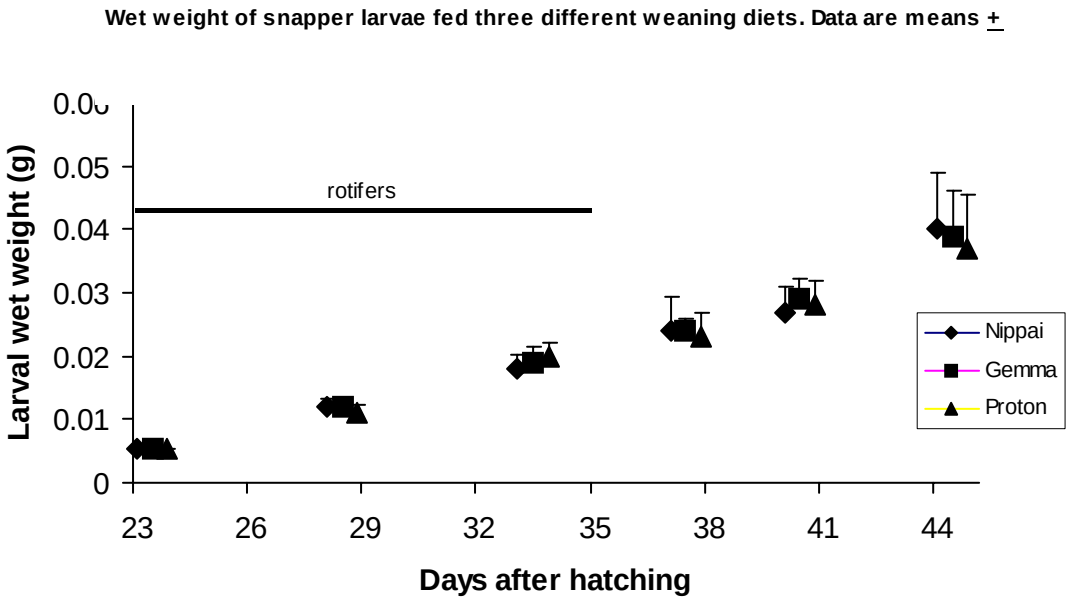

FIGURE 2

Wet weight of snapper larvae fed three different weaning diets. Data are means ±S.D. Points are not significantly different at each sampling time (P>0.05)

4. DISCUSSION

In terms of larval growth and survival of snapper larvae, there was no difference between the commercial weaning pellet diets tested in experiment tanks. However the pellet dynamics varied between brands and this may have ramifications for application in commercial tanks. Snapper larvae and post-metamorphosis juveniles tend to feed on pellet from the surface and in the water column, therefore fish have more opportunity to consume pellets if the pellet sinks slowly. Fast sinking pellets can foul the tank bottom, potentially requiring more diligent tank siphoning (=labour) than that for slow sinking pellets. The clumping aspect of the Gemma diet may cause problems in commercial tanks where pellet is often introduced to the larval tanks by automatic, belt feeders.

The cost to purchase each pellet varied significantly: Gemma (A$352/kg)>Nippai (A$125/kg)>Proton (A$39/kg). In addition, the availability of the pellets in Australia varied. The Nippai pellet required acquisition of an import permit and each shipment of pellet from Japan was inspected and tested by AQIS, especially for inclusion of meat products. The cost of the testing was borne by the importers. On the other-hand both Proton and Gemma diets were purchased directly from the manufacturers or importers (who are likely to have borne the import costs already and at more cost effective rates for large volumes of product).

We therefore suggest that Proton pellets are the most cost-effective pellets for weaning of snapper in Australia.

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4.3 Effect of polyhouse covers and addition of Artemia on larval rearing of snapper in fertilised ponds.

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1. INTRODUCTION

Preliminary experiments done at the Port Stephens Fisheries Centre (PSFC) demonstrated that snapper larvae can be reared successfully in ponds filled with estuarine seawater and fertilised to promote blooms of zooplankton. Ponds however, are generally exposed to ambient conditions and the environmental parameters including water temperature, pH, dissolved oxygen (DO) and salinity can vary during the course of larval culture.

Snapper larvae require specific temperature and salinity ranges of 18-24^{o}C and 20-35 ppt, respectively for optimal growth and survival (Fielder, Bardsley, Allan & Pankhurst, 2005). Therefore for pond rearing of snapper larvae to be successful and importantly, reliable, management of these parameters is essential. The period during the year that ambient conditions are suitable for snapper larval culture in ponds is also restricted to 2-3 months. Growout farms require a reliable supply of fingerlings, and preferably should be available for stocking when seawater temperatures are increasing in spring. In order for this to occur, hatchery operators must spawn and rear larvae during winter when water temperatures are likely to be suboptimal.

Polyhouses are used widely in the horticulture industry to improve environmental air conditions for plant growth. In particular air temperature and humidity are increased above ambient minima during cool periods of the day and season. Preliminary research at PSFC also showed that polyhouses can increase pond water temperatures above daily minima and to reduce the daily temperature fluctuation. In addition, polyhouse covers prevent rainwater from entering the ponds and thus salinity fluctuation could be managed.

Reliable production of high quality fingerlings is essential for viable hatchery and growout fish farms. Management of risk of failure to produce fingerlings is therefore vital. Extensive, fertilised ponds can occasionally “crash” unexpectedly and phyto- and zooplankton blooms die. Often there are no obvious reasons for the crash and adjacent ponds can behave differently despite ponds being treated similarly. Once the natural plankton blooms are depleted in a pond the stocked fish larvae are vulnerable to starvation. Supplemental feeding of larvae by adding *Artemia* may therefore reduce the risk of culture failure.

The aim of the study was to determine if snapper larvae could be reared in polyhouse-covered fertilised ponds and if the addition of *Artemia* to ponds improved success of larval rearing.

2. MATERIALS AND METHODS

Three experiments were done to evaluate the efficacy of polyhouses constructed over plastic-lined ponds and supplementing natural zooplankton with *Artemia* on the survival and growth of snapper larvae. Experiments 1 and 2 investigated whether polyhouses improved water quality including temperature to enhance larval snapper performance compared with that in ambient, uncovered ponds. The third experiment investigated whether adding extra *Artemia* to the natural zooplankton produced in fertilised ponds improved survival and growth of snapper larvae.

2.1 Experiment 1. Effect of polyhouses on larval snapper performance

An experiment was completed to evaluate the effect of polyhouses constructed over plastic-lined ponds on water quality and performance of snapper larvae.

2.2 Larval rearing

On 7 December 2004 a 6.1 kg captive G1 snapper broodstock was induced to spawn using 50 ug/kg of LHRHa. After 2 days fertilised eggs were obtained and stocked into a 2000-L hatchery tank. Larvae were reared until 17 dah using methods described in Fielder *et al.* (2004).

2.3 Pond preparation and management

Extensive rearing was undertaken in four plastic lined 350,000 L ponds (38 x 10.5 m, with filled depth of 1.4 m at the deeper end). Ponds were battered at a gradient of around 3:1 and a fall of 0.3 m over their length allowed ponds to drain into a concrete sump with a volume of 2.15 x 0.92 x 0.3 m. Effluent water exited the sump via a 150 mm diameter pipe. Pond depth was dictated by an external standpipe, which facilitated water flow through. A frame (1.67 x 0.9 x 0.95 m) covered in sandfly netting (approximately 1000 µm) fitted in the sump prevented the escape of larvae during flushing and draining. Influent estuarine water was screened through a 500 µm mesh bag.

Two larval rearing ponds were covered with bird exclusion mesh but exposed to ambient conditions and two other ponds were covered by twin-domed polyhouse structures. To allow some thermal regulation, the greenhouses were elevated 1.45 m above the pond batters and the side walls were enclosed with polyhouse curtains capable of being winched up or down to allow ventilation. This capacity was utilised to attempt to stabilise water temperatures, sides being lowered on cooler days to trap heated air, and elevated on warmer days to expel heated air.

An additional three 500,000-L plastic lined ponds were used for live food production. This entailed filling the ponds with filtered (500 µm) estuarine seawater and fertilising with organic and inorganic fertilisers to promote phyto- and zooplankton blooms. All ponds including food production and experiment ponds were fertilised by adding organic (lucerne chaff and dynamic lifter) and inorganic (Liquifert P, Liquifert N) fertiliser. The initial dose of organic fertiliser was lucerne chaff at 300 L/ha. Thereafter, organic fertiliser was administered three times per week, alternating between dosing dynamic lifter at 60 kg/ha and lucerne chaff at 300 L/ha. The initial dose of inorganic fertiliser consisted of a mix of Liquifert N, calculated to provide 1 ppm of nitrogen, and Liquifert P, to provide 0.25 ppm of phosphorous. Thereafter, inorganic fertilisation was administered (typically weekly) in quarter doses, as dictated by phytoplankton dynamics.

On the day of stocking, one of the 500,000-L food production ponds was pumped equally into the four 350,000-L larval rearing ponds. This provided standard live feed conditions in each pond at the start of the experiment and removed the need for individual pond acclimation of larvae. After larvae were stocked the ponds were filled to capacity with estuarine water.

Temperature, dissolved oxygen, pH, and salinity were monitored twice daily.

Zooplankton densities were monitored by siphoning 150 L of pond water through a 53 µm box screen. Concentrated plankton was harvested into a 1-L beaker. Aliquots were used to infer total plankton densities.

2.4 Larval stocking into ponds

Larvae were stocked into the extensive rearing ponds at 17 dah (total length 7.1 ± 0.6 mm). Larvae were concentrated in the hatchery tank by draining the tank and placed by bucket into a 150 L tank which was filled to a volume of 50-L. Larvae were then homogenised in the water column by hand-stirring, and

three 1L aliquots were used to estimate the total number of larvae. The tank was covered with black plastic to exclude light and moved to an area close to the ponds. Larvae were then transferred to a 250 L tank filled with water from the hatchery tank. A submersible pump was used to exchange pond water in the tank for 1.5 hours, and the larvae were then drain harvested into a 53 μm box screen. Larvae were divided evenly into each of four experiment ponds.

The experiment was run for 21 days (larvae 38 dah) when ponds were drained and snapper harvested to provide estimates of survival and growth.

2.5 Experiment 2. Effect of polyhouses on larval snapper performance - repeat

An experiment was done to determine the effects of polyhouse covers over ponds on performance of snapper larvae. This experiment repeated the aims of Experiment 1 which failed to produce any juvenile snapper.

The effect of greenhouse covers on growth and survival of snapper larvae during winter was evaluated from May 17 to 1 July 2005 at the Port Stephens Fisheries Centre. Two uncovered (ambient) and two polyhouse covered, 500m^2 plastic-lined ponds were each filled with fertilised estuarine water and stocked with approximately 12,000, 21 dah (mean wet weight 1.41 ± 0.03 mg; n=30) snapper larvae. Ponds were managed using techniques described in Experiment 1.

2.6 Experiment 3. Effect of supplementing wild zooplankton with Artemia on production of juvenile snapper in ponds

An experiment was completed to determine the effect of supplementing pond zooplankton with *Artemia* on production of juvenile snapper in ponds.

Fertilised snapper eggs were obtained from first generation hatchery-reared (G1) broodstock held in 17,000-L recirculation tanks at the PSFC. Snapper larvae were produced after a 5.3kg female snapper was induced to spawn using an intramuscular pellet implant containing 115 μg LHRHa.

Fertilised eggs were collected from the collection sump on two consecutive days and were transferred to 2000-L conical-bottomed tanks with black sides and white bases filled with sterilized seawater.

Larvae were held in these incubation tanks until 9 and 10 days after hatch at a photoperiod of 12L:12D with temperatures between 19.9-22.8°C and 19.8-23.0°C respectively. Larvae were fed wild zooplankton (rotifers and copepods) enriched with the micro-algae *Pavlova lutheri* and Tahitian *Isochrysis* aff. *galbana*, and Algamac 2000 (Aquafauna Bio Marine, California). Wild zooplankton was harvested continually from food production ponds described in Experiment 1.

All larvae were then combined into a single 10,000-L concrete flow-through seawater tank which was supplied with wild zooplankton harvested from the food production ponds.

Experiment ponds and management of plankton were the same as those described in Experiment 1.

Before the trial began water was pumped between ponds in order to achieve similar plankton populations at the time the trial started (Figures 1 and 2). Zooplankton samples were taken at regular intervals before the trial started and throughout the trial by either siphoning 130-L of water from a 50 mm hose suspended mid-water in each pond or by throwing a 20-L bucket tethered to a rope into the water column where it was allowed to sink and was then retrieved. The water collected was poured through a 54μm screen and reduced to a known volume (1-5-L). One ml aliquots of each sample were examined and concentrations of zooplankton (copepods, rotifers and *Artemia*) were determined.

2.7 Experiment treatments

Two treatments were established. Treatment 1 had an average of 27.5 x 10^6 *Artemia* nauplii (target of 0.1 nauplii/ml) added every afternoon to the pond in addition to the zooplankton bloom already present, while Treatment 2 remained dependent on the zooplankton blooms alone. There were three replicate ponds for each treatment with one replicate of each treatment located in ponds situated within a greenhouse (sides were raised to reduce the effect of the greenhouse on water temperature.

The experiment began on the 12th March, 2005 when 10,000 larvae (=200,000 larvae/ha) (19 dah) were stocked into each pond. The experiment ran for 24-26 days with one replicate from each treatment being harvested on each of three successive days. Water quality parameters (salinity, temperature, pH, dissolved oxygen) were measured every morning and afternoon to the nearest 0.1‰, 0.1°C, 0.1 pH unit, and 0.1 mg l^{-1}, respectively, using a water quality meter (Horiba U-10, Horiba, Japan).

At the end of the experiment each pond was drained and the fish were collected in a mesh harvester situated within an outside pond sump. Fifty fish were sampled from each pond to provide final wet weight (g) and total length (mm). All fish from each pond were counted to estimate survival (%).

3. RESULTS

3.1 Experiment 1. Effect of polyhouses on larval snapper performance

No snapper were harvested from any of the treatment ponds. With the exception of pond water temperature, mean DO, salinity and pH were similar and within the suitable range for snapper larvae in uncovered and covered ponds for the duration of the experiment (Table 1) (Fielder, Bardsley, Allan & Pankhurst, 2005). Density of the live feeds copepods and rotifers varied between ponds and with time but was always greater than 1/ml. Pond water temperature however was consistently 2 to 3°C higher in the greenhouse covered ponds compared with uncovered ponds in the morning and afternoon (Figure 1; Table 1).

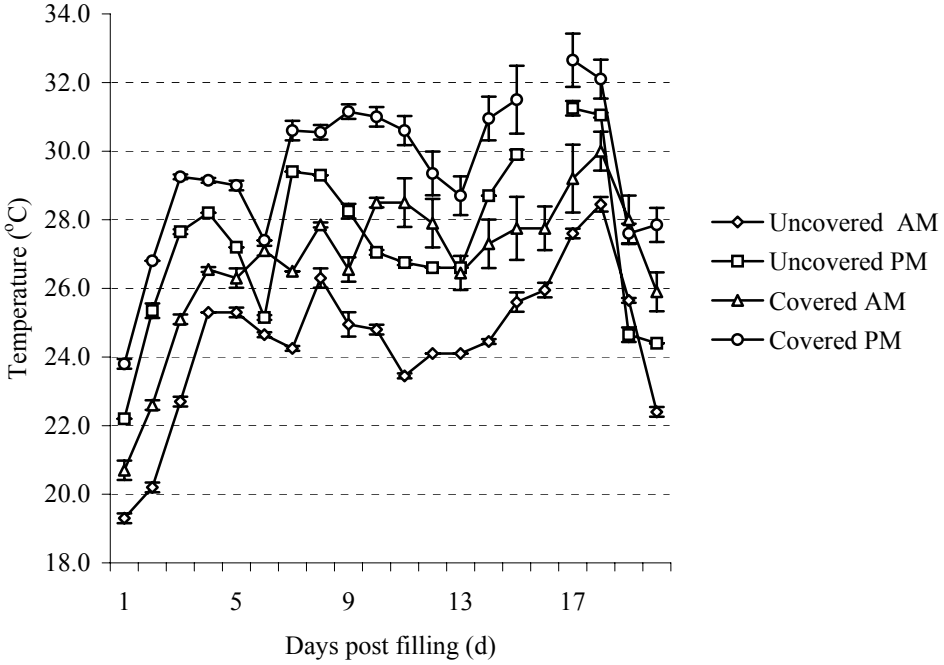

FIGURE 1

Average morning and afternoon temperatures in covered and uncovered 350,000 L extensive larval rearing ponds at PSFC. Points represent the means ± standard deviation (*n*=2 ponds)

TABLE 1

Mean DO, Temperature, Salinity and pH of greenhouse covered and uncovered plastic-lined ponds at PSFC from December 2004 to January 2005.

		DO (ppm)		Temperature (°C)		Salinity (ppt)		pH	
		AM	PM	AM	PM	AM	PM	AM	PM
Covered	Av. ± SD	6.8±0.9	11.3±2.5	26.8±2.1	29.5±2.1	34.3±2.4	34.3±2.1	8.7±0.27	8.9±0.3
	Max.	8.6	17.3	30.4	33.2	37.1	37.1	9.1	9.5
	Min.	4.9	6.8	20.5	23.7	26.5	29.3	8.1	8.2
Un-covered	Av. ± SD	7.4±0.8	11.1±1.7	24.5±2.1	27.4±2.3	34.3±2.4	34.2±2.5	8.7±0.2	8.9±0.3
	Max.	8.9	16.1	28.6	31.4	37.2	37.4	9.0	8.2
	Min.	5.4	8.3	19.2	22.2	28.4	27.8	8.2	9.2

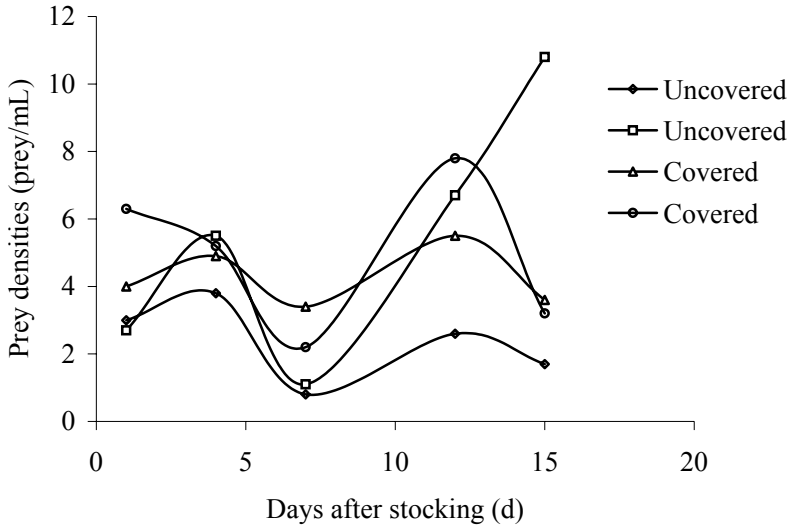

FIGURE 2

Prey densities during an extensive larval rearing trial at PSFC. Points represent the means of three aliquot counts (n=3)

3.2 Experiment 2. Effect of polyhouses on larval snapper performance - repeat

After 7 days, one pond from each treatment "crashed" for unexplained reasons and the phytoplankton and zooplankton blooms dropped out of suspension. After 45 days all ponds were drained and surviving fish harvested, counted and a sample of fish (n=50) weighed. No fish were harvested from either of the covered or uncovered ponds which crashed, however a total of 2066 (17.2% survival) and 2792 (23.3% survival) larvae were harvested from the remaining uncovered and covered ponds, respectively. After 45 days snapper cultured in the covered pond were significantly more developed and 4.2-fold heavier than fish cultured in the ambient pond (Figure 3).

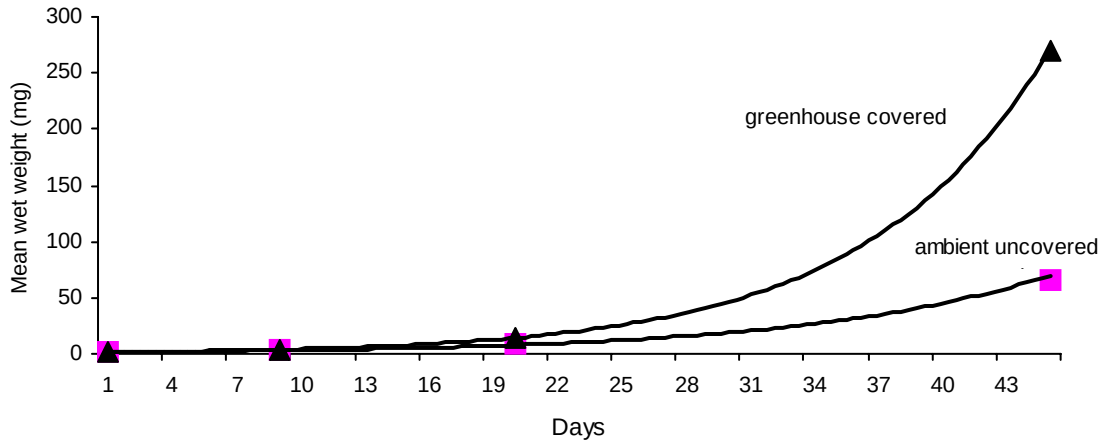

FIGURE 3

Mean wet weight (mg) of snapper grown in covered or uncovered ponds from 21 to 65 dah

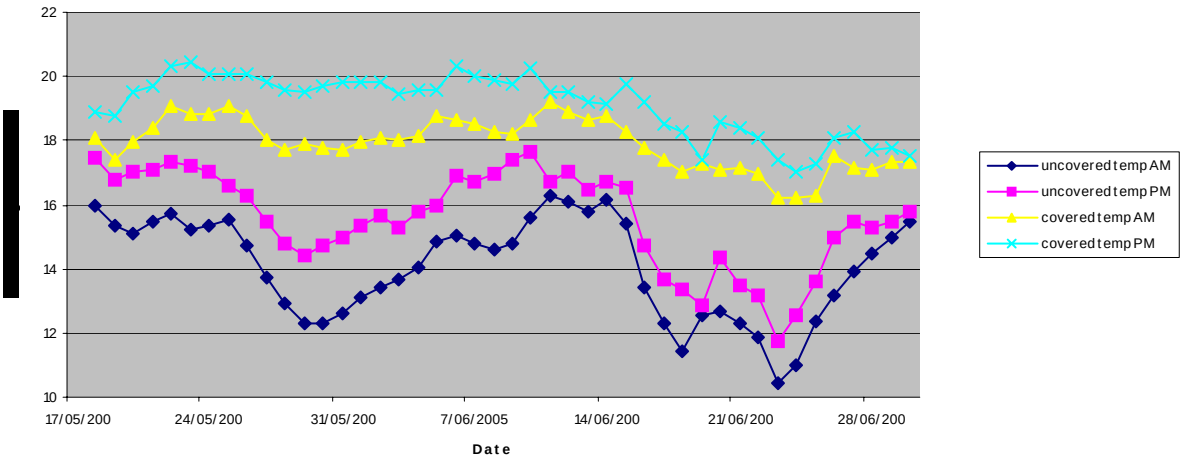

FIGURE 4

Mean am and pm water temperature for greenhouse covered and uncovered ponds at PSFC. Data are means (n=2 ponds)

3.3 Experiment 3. Effect of supplementing wild zooplankton with Artemia on production of juvenile snapper in ponds

The addition of *Artemia* nauplii to fertilized ponds did not improve survival, growth or the coefficient of variation of total length of juvenile snapper grown in ponds from 19 to 44 dah (Table 2) and stocked initially at 200,000 larvae/ha. Natural zooplankton was present at high densities in all ponds for the duration of the culture period (Figures 5 & 6). Rotifers were dominant in the zooplankton initially but decreased to approximately 1 rotifer/ml after 18 days from filling (Figure 5). Copepods increased in density from approximately 0.05 copepods/ml initially to 0.5-1.0 copepods/ml at the end of the culture period (Figure 6).

TABLE 2

Performance of advanced snapper larvae from 19-44 dah stocked into fertilised ponds with or without supplemental atremia. Data are means ± S.E. (*n*=3 ponds). Data in each column are not significantly different (*P*>0.05)

Treatment	Survival (%)	Final wet weight (g)	Final total length (mm)	CV of total length (%)
with artemia	42.2 ± 5.3	0.51 ± 0.1	26.1 ± 1.2	16.1 ± 1.8
without artemia	36.2 ± 2.0	0.47 ± 0.1	25.7 ± 1.5	20.1 ± 5.4

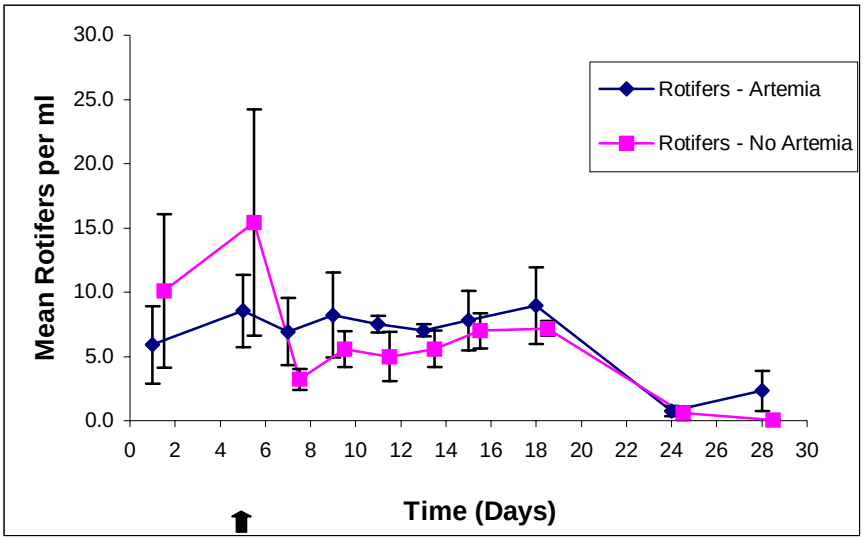

FIGURE 5

Mean number of rotifers in treatment ponds. Snapper larvae were stocked into ponds 6 days after filling. Data are means ± S.D (*n*=3 ponds).

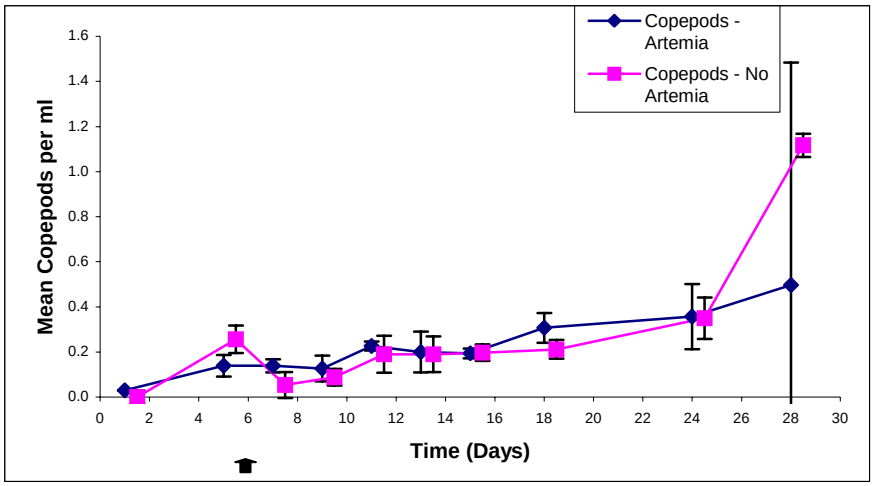

FIGURE 6

Mean number of copepods in treatment ponds. Snapper larvae were stocked into ponds 6 days after filling. Data are means ± S.D (*n*=3 ponds).

4. DISCUSSION

In Experiment 1, no larvae survived and this is likely due to excessively high pond temperatures in both ambient and polyhouse-covered ponds. Fielder *et al.* (2005) showed that all early age snapper larvae died following transfer from 21°C to 30°C and 33°C in controlled clearwater experiments. At the start of this experiment, pond water temperature in both treatments was 18-24°C, which is optimal for snapper growth and survival (Fielder *et al.* 2005). However, water temperatures rose steadily in both covered and uncovered ponds (Figure 1) and maximum temperatures were 33.2°C and 31.4°C for covered and uncovered ponds, respectively. We did not observe dead fish at any stage during the experiment however dead larvae decompose rapidly and it is therefore difficult to correlate a mortality event with water quality parameters.

Although inconclusive, our results for Experiment 1 suggest that high summer water temperatures are likely to be problematic for snapper larval rearing in outdoor ponds. The efficacy of greenhouse covers to maintain optimal water temperatures and to extend the possible pond rearing season for snapper larvae needs to be done during autumn or spring when ambient maximum temperatures are below 30°C. This was tested in Experiment 2 when an attempt to rear snapper larvae during Autumn/Winter was done.

Juvenile snapper were successfully reared in both ambient and polyhouse-covered ponds but fish from covered ponds were significantly larger than those grown in uncovered, ambient ponds. The improved growth of snapper cultured in covered ponds compared with uncovered ponds is likely due to elevated water temperatures in the ponds (Figure 4). Water temperature in covered ponds was generally 5°C higher with less variability (range 16.2 – 20.3°C) than in uncovered ponds (range 10.4-17.7°C) (Figure 3). Results of this trial demonstrate that greenhouse covers provide suitable improvement in water quality, especially temperature, during winter to allow viable culture of snapper fingerlings. However, the failure of two ponds to produce any juvenile snapper following plankton "crashes" highlights the fact that pond culture can be more unpredictable and therefore have higher risk than clearwater, indoor larval culture .

The addition of *Artemia* to our fertilised ponds did not improve survival and growth juvenile snapper. This suggests that zooplankton production was sustainable when ponds were stocked with advanced snapper larvae at 200,000 larvae/ha and that optimal or maximum stocking density of larvae was not

identified. Optimal larvae stocking density needs to be determined in order to maximise should be identified in future experiments. The efficacy of supplementing natural zooplankton with *Artemia* may also become evident when larvae are stocked at higher densities.

Growth, in terms of larval wet weight, was 3.5-fold greater in ponds than growth experienced using the best practice intensive rearing methods (Fielder & Allan, 2003). Snapper juveniles harvested from ponds also weaned onto cheap pellet diet as soon as the pellet was offered to the fish in tanks. Improved performance of snapper larvae in ponds may be due to lower stocking density resulting in less competition for food and/or less interaction between conspecifics and access to a more diverse and nutritionally balanced diet than available in intensive tank culture.

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4.4 Determination of the optimal age of larvae and protocols for stocking snapper *Pagrus auratus* larvae in fertilised ponds

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1. INTRODUCTION

Rearing of snapper larvae using extensive methods in fertilised ponds is not common practice as most hatcheries use intensive, clearwater methods. Intensive hatcheries are expensive to operate and require sophisticated facilities and highly-skilled labour. Extensive larval rearing methods on the other hand require less degree of sophistication and can produce cheaply, extremely high quality fingerlings. Success of rearing can however be unreliable due to factors including variable and uncontrolled environmental conditions during the culture period, mortality of larvae following transfer from incubation or hatchery tanks, and stocking of inappropriately age/sized larvae i.e. too small and sensitive to tolerate the pond environment.

Research done at the Port Stephens Fisheries Centre with mulloway demonstrated that survival of larvae stocked into fertilised ponds increased as the age of larvae at stocking was increased (Fielder, Allan & Battaglene, 1999). This was presumably because older larvae were able to feed on a larger variety of different size zooplankton compared with small larvae which were restricted to feeding on commensurately small zooplankton. Also, large larvae were potentially more tolerant of variable environmental conditions in the pond than were small larvae.

Preliminary pond experiments with snapper also showed that potential mortality of snapper larvae may have occurred soon after stocking into ponds. Success or failure of pond production of fingerlings may therefore be influenced directly by the initial survival of larvae at stocking. Stocking of larvae from the hatchery to ponds entails harvest from hatchery tanks, transport to the ponds and then placement into the ponds. In addition to physical handling and crowding of larvae in transport vessels, the physical environment including light intensity, water pH, dissolved oxygen, temperature and salinity can vary significantly between clear water hatchery tanks and turbid, fertilised ponds.

The aims of this study were to determine (1) the effect of age of snapper larvae at stocking on survival and growth in fertilised ponds and (2) the optimal protocols for stocking larvae into fertilised ponds.

2. MATERIALS AND METHODS

Two experiments were done to determine the optimal strategy for stocking snapper larvae into fertilised ponds. The first experiment investigated whether age of snapper larvae (16 and 23 days after hatching, dah) improved growth and survival of fish when stocked and cultured in fertilised ponds. The second experiment investigated whether the method and timing of transfer of snapper larvae from hatchery tanks to ponds affected growth and survival of fish.

2.1 Experiment 1. Optimum age of snapper larvae for stocking

An experiment was completed to determine the effect of larval snapper age on growth and survival when stocked into fertilised, seawater ponds. Snapper were stocked at two ages: 16 dah (treatment 1) and 23 dah (treatment 2).

Snapper eggs were obtained from spontaneously spawning broodstock fish and larvae were reared in a 2000-L recirculation tank using methods described in Fielder, Bardsley, Allan & Pankhurst, (2002). For each stocking age treatment, 500m^2 ponds (n=3 ponds per treatment) were each filled with 200 um filtered estuarine seawater and fertilised with nitrogen and phosphorous at 1 mg L^{-1}, respectively and an organic carbon source (lucerne hay at 900 kg/ha) at 16 days prior to stocking with larvae. In addition, approximately 20% of the total pond volume was filled from adjacent 0.1 ha ponds which had established zooplankton blooms.

For each treatment, larvae were transferred by bucket from the stock larval rearing tank into 200-L hemispherical, acclimation tanks filled with water from the stock tank (n=3 tanks per treatment). A total of 4000 larvae were counted into each acclimation tank by carefully pouring the larvae from 600-ml containers. Acclimation tanks were kept in darkness to reduce larval stress. Prior to stocking larvae from the acclimation tanks into ponds, larvae were acclimated to each recipient pond by exchanging 500-L of water from each pond into a randomly selected acclimation tank (1 pond per acclimation tank) over a 12 h period. Live larvae were then stocked into treatment ponds at night to prevent larval stress due to a rapid change in light intensity from the indoor hatchery to the outdoor ponds. Larvae, which had died during acclimation were siphoned from the tanks and counted.

During the production period, inorganic fertiliser was added as necessary to maintain phytoplankton blooms. Water quality parameters (salinity, temperature, pH, dissolved oxygen) were measured daily in each pond. Treatment 1 and 2 ponds were harvested when snapper were 38 and 39 days after hatch, respectively. Snapper were harvested from each pond by draining the pond and concentrating the fish in a perforated tank. The total number of harvested fish from each pond was estimated by measuring the total biomass of snapper and dividing by the mean fish weight. Mean fish weight was estimated by weighing 5 randomly selected samples (n= 50 fish per sample) of fish from each pond. Final mean total length and wet and dry weights of snapper were estimated using methods described in Fielder *et al.* (2002). Data were analysed using one-way ANOVA (P<0.05). All harvested fish were transferred to 2, 10,000-L flow-through tanks for subjective determination of fish quality and ability to wean onto artificial pellet diets.

2.2 Experiment 2. Effect of acclimation and time of stocking into ponds on post-stocking survival of hatchery-reared snapper larvae

A fully orthogonal two-factor experiment was designed to determine the interaction between stocking method and time of day that larvae were transferred from the indoor hatchery to an outdoor pond.

Snapper larvae were reared in a 2000-L clear water hatchery tank until 21 dah (6.5 mm total length) and were then used in the acclimation experiment.

Stocking methods were:

1. Direct transfer, where larvae were captured by bucket from a hatchery tank and poured straight into 10-L buckets filled with water from the fertilised pond and floating within the pond (n=100 larvae/bucket; 3 buckets/treatment).
2. Acclimation transfer, where larvae were captured by bucket from the hatchery tank 24 h before treatment 1 and placed into an insulated 600-L tank with lid which was filled with water collected from the hatchery tank and placed outdoors next to the fertilised pond. Pond water was then pumped from the pond to the insulated tank to exchange approximately 100% of the volume every 6 hours (1.7 L/min). After 24 h, larvae were collected by beaker and placed into

10-L buckets filled with water from the fertilised pond and floating within the pond (n=100 larvae/bucket; 3 buckets/treatment).

3. Control, where larvae were captured by bucket from a hatchery tank and poured straight into 10-L buckets filled with water from the hatchery tank (n=100 larvae/bucket; 3 buckets per treatment). Treatment buckets remained in the hatchery.
4. Time of transfer. For each stocking method, larvae were stocked into treatment buckets at 0900 h, 1300 h and 1700 h.

After 24 h from stocking into treatment buckets the number of surviving larvae in the treatment buckets was determined. Water quality parameters including temperature, pH, dissolved oxygen and salinity were monitored by multiprobe Horiba U-10 meter.

3. RESULTS

3.1 Experiment 1. Optimum age of snapper larvae for stocking

Some larvae of both age groups died in the acclimation tanks after transfer from the stock rearing tank. A total of 29.6±21.2% and 1.3±1.0% of larvae died initially in 16 dah and 23 dah larvae groups, respectively but there was no significant difference in mortality between age groups.

Survival of larvae to 38 and 39 dah was high and did not differ between treatments (Table 1) and ranged from 40.0 to 88.4%. However, the final total length, wet weight and dry weights of snapper stocked at16 dah were significantly greater than snapper stocked at 23 dah (Table 1). After harvest from ponds, juvenile snapper were held in 10,000-L tanks and offered Nippai weaning pellet. No snapper died as a consequence of handling and snapper were in excellent general health and vigour. Snapper from both treatment groups fed immediately on a pellet diet and did not require any addition of live feeds including *Artemia*.

TABLE 1

Mean number of larvae stocked, final total length, wet and dry weights of snapper stocked into fertilised ponds at 16 and 23 days after hatcherying (dah) and ongrown until 38 and 39 dah, respectively`

Age at stocking	Mean number larvae stocked	Mean final survival (%)	Mean Total Length (mm)	Mean wet weight (g)	Mean dry weight (g)
16	2814±490[a]	70.6±8.9[a]	31.7±0.8[a]	0.57±0.04[a]	0.12±0.01[a]
23	3961±26[a]	54.9±10.5[a]	26.0±0.2[b]	0.33±0.02[b]	0.06±0.004[b]

Data are means ± SE. Means in each column with a different superscript are significantly different (P<0.05).

3.2 Experiment 2. Effect of acclimation and time of stocking into ponds on post-stocking survival of hatchery-reared snapper larvae

Stocking method significantly ($P<0.05$) affected survival of transferred snapper larvae (Figure 1) but time of transfer did not affect survival. Larvae, which were harvested from the hatchery tank and held in buckets within the hatchery overnight had approximately 10% higher survival (97±2.2%) than larvae transferred directly from the hatchery (88.3±2.2%) and after acclimation to pond water (90.6%±2.2%), which did not differ. Water temperature, pH, DO and salinity were similar and within acceptable limits at the time of stocking for all treatments and differences were within 0.7°C, 0.2 pH units, 3mg/l and 2.1 g/L, respectively.

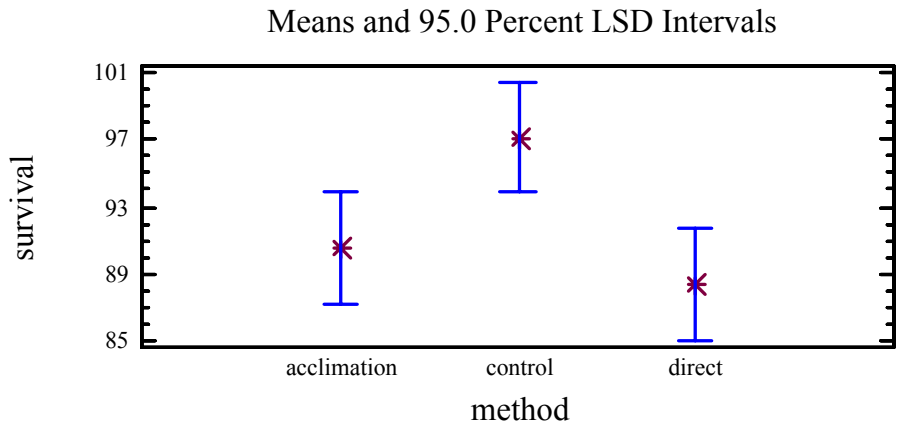

FIGURE 1

Mean survival of 21 dah snapper larvae transferred from an intensive hatchery to fertilised pond water using a range of acclimation procedures ($n=9$).

4. DISCUSSION

Despite there being no significance between treatment mortality in Experiment 1, 16 dah snapper larvae appear to be more susceptible than 23 dah larvae to stress of transfer from hatchery to acclimation tanks. Causes of the mortality are not clear however rapid exposure of larvae to slight differences in environmental conditions between the stock rearing hatchery and acclimation tanks may have increased larval stress and subsequent mortality. The hatchery has low intensity artificial light compared with a combination of brighter artificial and filtered sunlight in the acclimation tanks. It was also necessary to count the larvae by slowly pouring larvae from aliquots into the acclimation tank. Physical handling stress may have contributed to larvae mortality.

Growth of fish larvae in fertilised ponds can be much greater than that of larvae in intensive tanks due to reduced larval densities and greater availability of live feeds (Fielder *et al.*, 1999). However age of stocking larvae can have a significant effect on larval survival. Survival of snapper was low when newly hatched (3-5 dah) larvae were stocked into fertilised ponds (Fielder & Allan, 2003). In Experiment 1, both 16 and 23 dah snapper larvae performed well in fertilised ponds but based on growth the optimal age of snapper for stocking into fertilised ponds is approximately 16 dah.

In Experiment 2, although water quality parameters were similar for all treatments at the time of transfer, larvae which were stocked to treatment tanks in the pond had a lower survival than larvae which remained in the hatchery. Acclimation of larvae to pond water conditions did not improve survival compared with direct transfer from the hatchery to the pond nor did time of day of transfer influence survival suggesting that handling in buckets from the hatchery to the pond site (about 2 minutes) may influence survival. This result confirmed that of Experiment 1 and suggests that new methods for handling and counting need to be developed to reduce the incidence of initial transfer mortality

Provided water quality parameters are similar between hatchery tanks and ponds, stocking of larvae directly from hatchery to ponds is a suitable transfer method, however at least a 10% initial mortality should be accounted for in initial stocking numbers of larvae.

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4.5 Evaluation of seawater zooplankton production in plastic-lined ponds for extensive and intensive culture of snapper larvae

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1. INTRODUCTION

Many species of larval freshwater fish and some species of marine fish can be successfully reared extensively in fertilized ponds. Pond culture can have major benefit for larval fish culture including cheaper establishment and operating costs of facilities, requirement for less skilled labour, and production of cheaper, high quality fingerlings compared with that of intensive culture. Snapper larvae are generally cultured using intensive hatchery techniques and there is an opportunity to develop techniques for reliable culture of fingerlings in fertilized ponds.

Production of fish larvae in ponds is done by promoting the growth of natural zooplankton on which fish larvae feed by adding a combination of inorganic and organic fertilizers to seawater. The seawater is usually coarsely filtered through 500um filters to remove potentially deleterious pond inhabitants such as fish eggs and jellyfish which can consume the stocked larvae, but allows naturally occurring phytoplankton and zooplankton including rotifers and copepods to pass into the ponds. The fertilizers promote the growth of phytoplankton and bacteria on which the zooplankton feed and ultimately reproduce and increase in density.

One of the main factors which can determine the success of pond larval culture however is the availability of suitable density and size of zooplankton. It is therefore essential to understand the population dynamics of the zooplankton in ponds to develop pond management protocols and larval stocking strategies to optimize the success of fingerling culture.

The aim of the study was to determine the population structure and succession of zooplankton over 22 days in fertilized, plastic-lined ponds at the Port Stephens Fisheries Centre. This information was then used to predict optimal time of stocking snapper larvae after fertilization and also to determine if production and harvest of wild zooplankton in ponds was feasible for supply of live feeds for intensive culture of snapper larve.

2. MATERIALS AND METHODS

An experiment was done to determine the productivity of natural zooplankton in plastic-lined ponds filled with fertilised seawater. The experiment investigated the population structure, density and succession of zooplankton species for three weeks in ponds following fertilisation. Data from this detailed study was combined with results of several other experiments to produce snapper larvae in ponds (see Chapters 4.1. and 4.4) to evaluate potential for large-scale production of wild zooplankton in ponds for intensive larval snapper culture.

2.1 Experiment 1: Population structure and succession of fertilised ponds

Three 500 m^2 ponds were filled with 200 μm filtered estuarine water and fertilised with nitrogen and phosphorous at 1 mg l^{-1}, respectively and an organic carbon source (lucerne hay at 900 kg/ha). Water quality parameters (salinity, temperature, pH, dissolved oxygen) were measured daily in each pond. Every three days samples of plankton were collected from each pond by siphoning 150 L of water from a 50 mm diameter hose which was suspended in mid-water in each pond. The collected water was then sieved through a 50 μm screen to concentrate the zooplankton. Zooplankton samples were then stored in

50 ml of buffered formalin and archived for later analyses. The trial ended after 22 days. Zooplankton samples were then assessed to estimate the frequency, size and species identification of zooplankton. Each 50 ml zooplankton concentrate was mixed well with aeration and 3, 1 ml samples were taken randomly and placed onto a Sedgewick Rafter Slide. Zooplankton were then identified to at least Order, counted and measured using a stereo microscope (40-100x mag; total length; maximum of n=20).

3. RESULTS

Productivity of zooplankton was highly variable between ponds but in general the total number of zooplankton in fertilised ponds was low for the first 7 days and then increased steadily until 19 days after fertilisation when mean densities of zooplankton were approximately 500/L (Figure 1). The dominant zooplankton at 19 days after fertilisation were crustacean nauplii, calanoid copepods and rotifers (Figure 2). At 22 days after fertilisation the total number of zooplankton/L increased 4-fold and was due to a rapid increase in the number of rotifers (Figures 1 and 2). The initial total length (TL) of zooplankton ranged from approximately 70 to 330 um but by 7 days after fertilisation the dominant calanoid copepods had moulted to their adult size and were approximately 550 um TL (Figure 3). At 19 and 22 days after fertilisation the size of crustacean nauplii, rotifers and calanoid copepods was approximately 150, 180 and 550 µm, respectively. At the same time, chironomid larvae of 1800 to 3900 µm TL were observed in low densities (0.1-0.3 larvae/L).

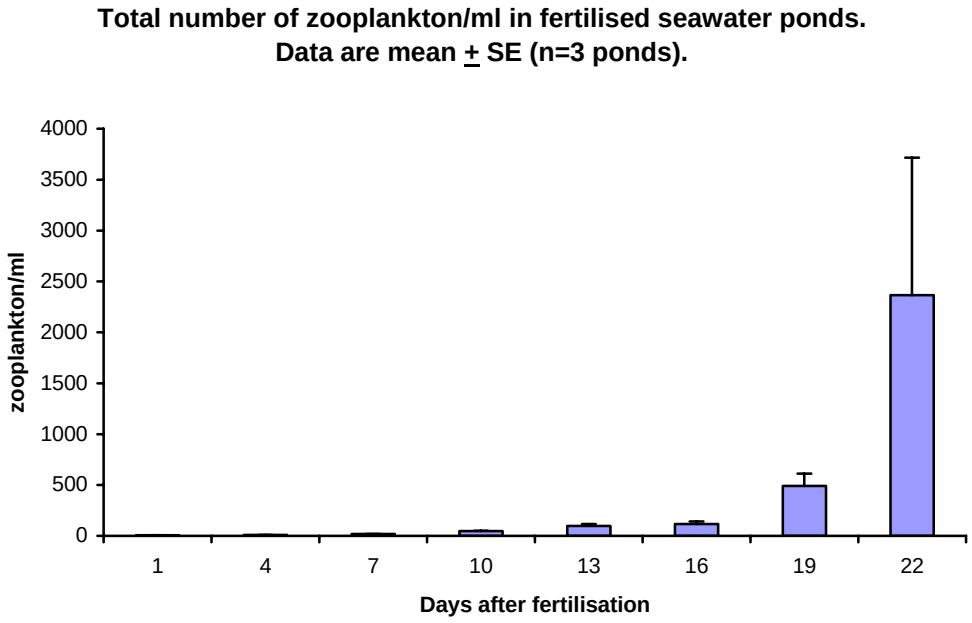

FIGURE 1

Total density of zooplankton in plastic-lined ponds filled with seawater and fertilised

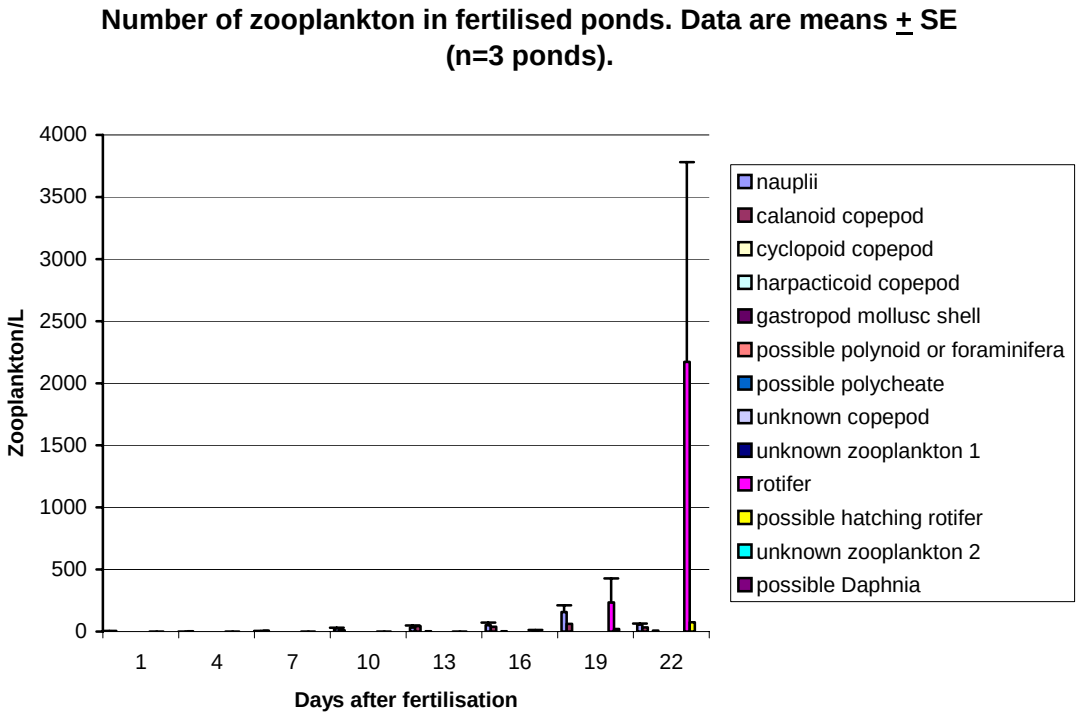

FIGURE 2

Population structure and density of wild zooplankton in plastic-lined ponds filled with seawater and fertilized

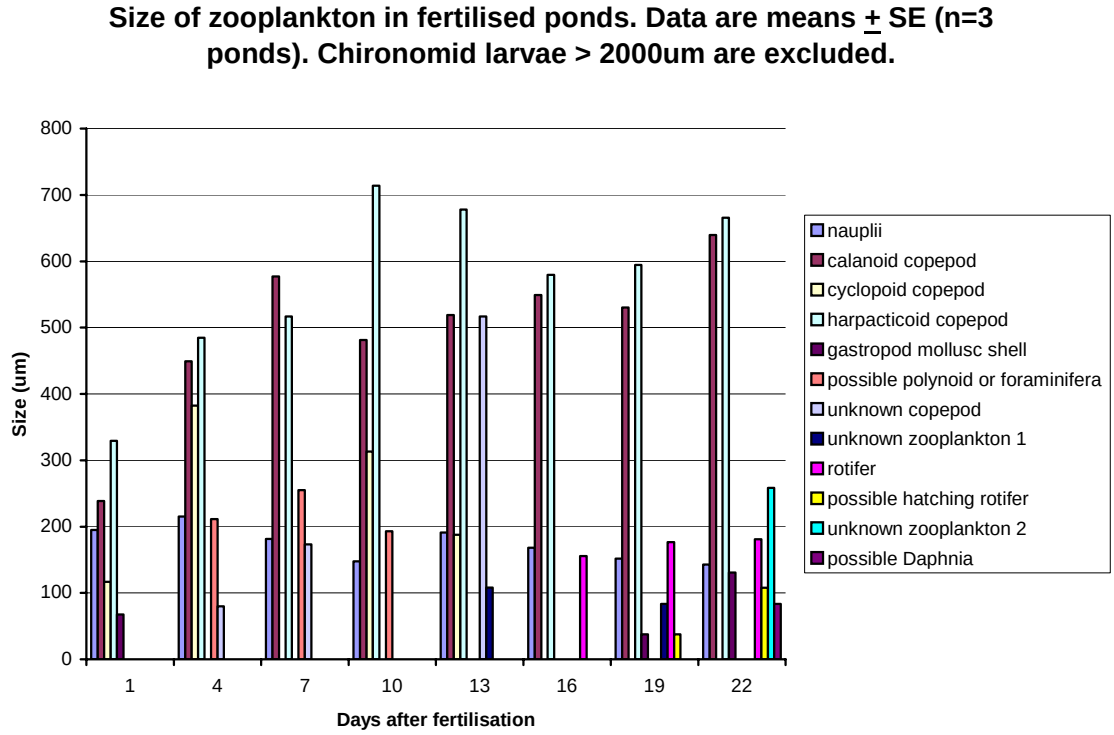

FIGURE 3

Size of zooplankton in plastic-lined ponds filled with seawater and fertilised

4. DISCUSSION

One of the major factors affecting the success of rearing fish larvae in ponds is the availability of suitable size and density of live feeds. Therefore the timing of pond preparation and management before stocking with larvae is critical to ensure that stocked larvae will have access to optimal live feed population. Productivity of zooplankton in fertilised, plastic-lined ponds at PSFC can vary between ponds however the zooplankton succession is typically dominated by rotifers initially followed by copepods. Advanced snapper larvae (>14 days after hatching to pre-metamorphosis) are capable of consuming live feeds to approximately 1000 µm TL, therefore in general, suitable numbers of zooplankton for stocking larvae directly into ponds or for harvesting to feed intensive tanks are achieved after 19-22 days after filling and fertilisation.

Wild zooplankton, especially copepods were demonstrated as suitable substitutes for *Artemia* for intensively-reared advanced snapper larvae (see 4.1) therefore large-scale, extensive production of copepods is a feasible method of food production for intensive rearing of snapper juveniles. Based on the production of copepods in our 350,000-L ponds at PSFC, and assuming an initial pond density of 0.5 copepods/ml, three ponds set-up in series 7 days apart can provide enough copepods for three weeks to feed 10,000-L of hatchery tanks at 2 copepods/ml/d and larvae stocked initially at a density of 100 larvae/L ($1x10^6$ snapper larvae). If survival is 20%, then a total of 200,000 snapper juveniles could be produced. A total of 38,000 L/d (=1583 L/h) of water would be harvested from the mature pond for seven days and live zooplankton filtered and retained for feeding. A high volume wild zooplankton harvester has been designed and is operational at PSFC using adaptations of commercial *Artemia* harvesting technology.

4.6 Effects of photoperiod and feeding frequency on performance of newly weaned Australian snapper *Pagrus auratus*

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ABSTRACT

An experiment was done to investigate the interactive effects of photoperiod (12L:12D or 18L:6D) and feeding frequency on the growth of newly weaned Australian snapper (mean weight = 0.14 g fish^{-1}). Feeding frequency was investigated over 4 levels with 2 feeds delivered during the first half of the daylight period (2FE), 2 feeds during the latter half of the daylight period (2FL), 4 (4F) or 8 (8F) evenly spaced feeds per daylight period. Each treatment combination was replicated in 6 tanks and each tank was stocked with a biomass of 15 g tank^{-1} (i.e. approximately 108 fish tank^{-1}). Snapper were fed a constant ration of 10% BW d^{-1} for 32 days, which was adjusted during the experiment according to frequent weight check procedures. Fish that died were counted but not replaced.

Photoperiod, feeding frequency and the interaction of these factors significantly affected the individual harvest weight and thermal growth coefficient (TGC) of snapper. Interactions were driven by an increase in the magnitude of individual weight and TGC in snapper fed the 4F and 8F treatments and reared under the 18L:6D photoperiod, compared to snapper fed at the same frequencies but reared under the 12L:12D regime. Weight gain and TGC were best in snapper reared under a 18L:6D photoperiod regime and fed 8 feeds d^{-1}, however, weight gain did not plateau, suggesting further increases in weight gain may be possible if feeding frequencies greater then 8F are employed. Survival and apparent feed conversion ratio (AFCR) were significantly affected by feeding frequency alone, with significant improvements in snapper fed more frequently and in snapper fed twice daily but later in the same photoperiod (2FE < 2FL < 4F < 8F). Size heterogeneity (measured by the coefficient of variation for individual harvest weight, CV_{hw}) was affected by photoperiod, and decreased significantly in snapper reared under the 18L:6D regime. Size heterogeneity was also affected by feeding frequency, however, only the CV_{hw} for snapper reared under the 8F feeding frequency was significantly lower than snapper fed at other rates (i.e. 2FE = 2FL = 4F < 8F).

Snapper fed later in a photoperiod regime generally performed better than snapper fed earlier. Results from this study indicate that in order to maximize weight gain, survival and AFCR and to reduce size heterogeneity, newly weaned snapper should be reared under a 18L:6D photoperiod and, for fish fed 10% BW d^{-1}, fed 8 times d^{-1}.

1. INTRODUCTION

The Australian snapper (*Pagrus auratus* = red sea bream *P. major*; Paulin, 2001) is one of several marine species being grown in small sea-cage operations in New South Wales (NSW) and South Australia (SA). The growth of the snapper industry has been constrained by several factors including the poor performance of snapper reared in the cooler waters of SA (Hutchinson, 2003), the high cost of imported and locally manufactured feeds and the low availability of high quality, competitively priced hatchery reared fingerlings. Fingerling supply and price is a particular problem for the snapper industry in NSW, where prices can range from $AUS 0.90-1.20 per 35 mm fingerling. These costs are between two to five times higher than prices paid for similar sized freshwater or diadromous fingerlings including silver perch and barramundi. The cost of snapper fingerlings in NSW is high because most marine fish hatcheries operate labour intensive rearing technologies and at the same time produce relatively small numbers of fish (Fielder, Booth & Allan, 2003).

The prospect of a major expansion in the snapper industry providing the economies of scale that might ultimately reduce the price of hatchery reared fingerlings is unlikely in the near future. However, improvements in rearing technology can dramatically increase production efficiency by improving the quality and quantity of hatchery reared juveniles. For example, Fielder, Allan & Battaglene (1999) improved the efficiency and quality of egg production in domesticated brood-stock snapper by applying truncated phototherms; effectively allowing year round production of eggs. In addition, Fielder, Bardsley, Allan & Pankhurst (2005), demonstrated that identification of optimal salinity and temperature regimes dramatically improved the growth of snapper larvae. Identification of improved weaning strategies for snapper has also dramatically improved weight gain and survival (Fielder, Bardsley, Allan & Pankhurst, 2002). Although many of the optimal rearing regimes for larval snapper (15-20 mg $fish^{-1}$) have been elucidated, there is little information on optimizing the performance of snapper during the nursery or intermediate phase of growth (Partridge, Jenkins & Frankish, 2003).

Two parameters under the immediate control of hatchery personnel that may affect the performance of nursery fish are photoperiod and feeding frequency (Boeuf & Le Bail, 1999; Dwyer, Brown, Parrish & Lall, 2002). Extended photoperiod regimes have induced faster growth rates in gilthead sea bream (Tandler & Helps, 1985), Atlantic halibut (Simensen, Jonassen, Imsland & Stefansson, 2000), greenback flounder (Hart, Hutchinson & Purser, 1996), sea bass (Barahona-Fernandes, 1979) and snapper larvae (Fielder *et al*., 2002). However, increased photoperiod does not always result in increased survival and increased photoperiod can sometimes have a deleterious effect (Barahona-Fernandes 1979; Hart *et al*., 1996). Optimal feeding strategies can also enhance growth, survival and feed conversion efficiency by minimizing feed wastage, improving water quality and reducing size variation within different cohorts (Dwyer *et al*., 2002). Poorly timed or sporadic feeding regimes may lead to increased hunger, intra-specific aggression and increased rates of cannibalism (Folkvord & Otterra, 1993); all problems that decrease the efficiency of production and ultimately increase labour input costs.

There is a paucity of scientifically evaluated technologies designed to increase the performance and efficiency of snapper reared during the nursery or intermediate phase of growth. Therefore, the aim of this experiment was to compare the weight gain and performance of newly weaned snapper reared under different photoperiods and subjected to different feeding frequencies.

2. MATERIALS AND METHODS

2.1 Experimental design

A factorial experiment was designed to investigate the interactive effects of photoperiod (12 light:12 dark or 18 light:6 dark; hereafter 12L:12D or 18L:6D, respectively) and feeding frequency (2 feeds early, 2 feeds late, 4 feeds or 8 feeds per day; hereafter 2FE, 2FL, 4F or 8F, respectively), on the weight gain and performance of newly weaned Australian snapper *Pagrus auratus*. These photoperiods were selected based on photoperiod recommendations for larval snapper made by Fielder *et al*. (2005). Feed was distributed at evenly spaced intervals within the daylight hours of each photoperiod regime (Table 1). Each of the experimental treatments was randomly assigned to 6 replicate experiment tanks and the experiment was run for 32 consecutive days.

2.2 Stocking, weight check and harvest procedures

Approximately 7000 newly weaned snapper (46 days after hatching; dah) were removed from weaning tanks at the Port Stephens Fisheries Centre (PSFC) and carefully transported to the rearing laboratory. Snapper were acclimated to general laboratory conditions for 3 days. Prior to stocking procedures, snapper were visually graded in order to reduce the initial size variation within replicate tanks and decrease the possibility of cannibalism. After grading, fish were stocked into each experiment tank in two rounds of 10 and 5 g per tank respectively, resulting in approximately 106 ± 4 (mean ± SD) fish being placed in each of the 48 tanks. This density is consistent with the recommendations of 2 to 4 fish l^{-1} made by Partridge *et al.* (2003). According to sub samples (n=8) taken during stocking procedures the individual weight (mean ± SD) of snapper was 0.14 ± 0.01 g.

Fish were weighed 12, 22 and 32 days after stocking the experiment in order to adjust feed ration. At these times, snapper were anaesthetized *in-situ* with 15-20 mg l^{-1} ethyl ρ-aminobenzoate, gently captured in a small dip net and bulk weighed before being returned to clean aquaria. At the completion of the experiment all fish were counted. In addition, the individual weight of fish from 3 randomly selected replicate tanks in each treatment was recorded. Any moribund fish or whole fish carcasses found during weight check procedures were included in bulk weight determinations. Mortality was recorded during the experiment but fish were not replaced.

2.3 Feeding protocols

Snapper were fed exclusively on an Atlantic salmon starter prior to and during experimental use (Skrettings Pty. Ltd., Nutreco; typical analysis 52% crude protein, 18 MJ gross energy kg^{-1}). Before the experiment, newly weaned snapper were fed a 200 µm dust. Four days before being transferred to the laboratory they were weaned to a particle size of 400 µm. After stocking, snapper were fed a fixed ration (10% tank biomass d^{-1}; 600 µm particle size) at regular intervals for 3 days in order to acclimate to tank based conditions and new photoperiod regimes. Following this period snapper were switched to their respective feeding frequency regimes and fed each day for the remainder of the experiment.

Feed particle size was gradually increased from 600 to 1000 µm over the course of the experiment to accommodate changes in fish size. To ensure that feed intake did not limit growth or performance, all tanks were fed an amount equivalent to 10% of their biomass day^{-1} based on feeding recommendations for red sea bream (= snapper; Koshio, 2002). The relative amount of feed offered to each tank was maintained throughout the experiment by adjusting feed rates according to the aforementioned weight checks. Small increments in daily feed allowance were based on predicted rates of growth calculated from the previous growth period. The measured quantity of feed was hand fed to each tank at the appropriate time over an approximately 10 min period.

Exact feed conversion calculations were not possible because snapper were fed a fixed ration and uneaten feed was not collected. Consequently, data are presented as apparent feed conversion ratio (AFCR).

2.4 Laboratory facilities

Individual experiment tanks (60 l rectangular clear acrylic) were adjusted to a volume of 40 l and supplied with continuously flowing (0.9 – 1.0 l min^{-1}), temperature controlled estuarine water. Before reaching experiment tanks, re-circulated water was filtered through a cartridge filter (nominal pore size 20 µm), then passed through a 2 m^3 trickling bio-filter and an ultra-violet sterilizer (Vf-9 Big Blue, Australian Ultra-Violet Products Pty.. Ltd., Seven Hills, NSW, Australia). Approximately 30% of water was exchanged each day and replaced with disinfected (chlorinated then de-chlorinated), pre-filtered estuarine water. Each tank was covered with a clear perspex lid and aerated with two air-stone diffusers. Individual fluorescent lighting was provided for each tank and was automatically controlled to provide either a 12L:12D (0700 h to 1900 h) or a 18L:6D (0700 h to 0100 h) photoperiod. The different photoperiods were established by dividing the laboratory into 2 distinct areas, each containing 24 experiment tanks. These areas were screened from each other using thick black plastic that prevented light from the longer photoperiod affecting fish held under the shorter photoperiod regime. Visible light intensity in experiment tanks was measured as photo synthetically active radiation (PAR 26 µmol s^{-1} m^{-2} ; 1580 lux) using a LI-188B Quantum/Radiometer/Photometer and a LI-192SB Underwater Quantum Sensor (LI-COR, Lincoln, Nebraska, USA).

Water temperature (range 24.0 to 27.1°C), dissolved oxygen (range 6.0 to 8.2 mg l^{-1}), pH (between 7.5 and 8.5) and salinity (range 28-33 ‰), were monitored regularly with a Model 611-Intelligent Water Quality Analyzer (Yeo-Kal Electronics Pty., Ltd., Brookvale, NSW, Australia). Total ammonia levels were monitored regularly using a rapid test kit procedure (E. Merck, Model 1.08024, Germany) and always remained < 0.6 mg l^{-1}.

2.5 Statistical analyses

Two-way analysis of variance (*ANOVA)* was used to investigate the interactive effects of photoperiod and feeding frequency on individual harvest weight, survival, apparent feed conversion ratio (AFCR) and thermal growth coefficient (TGC) of newly weaned snapper. Two-way *ANOVA* was also used to assess the interactive effects of the fixed factors on size heterogeneity in snapper by employing the coefficient of variation of harvest weight (CV_{hw}) as the response variable.

Prior to *ANOVA,* data were tested for homogeneity of variance using Cochran's C test. Angular transformation (i.e. arcsin (√(value / 100)) was required to remove heterogeneous variance's from data collected on individual harvest weight, survival, and AFCR. Transformation of TGC and CV_{hw} was not required. Where *ANOVA* tests proved significant, a Student-Newman-Keuls (SNK) multiple comparisons procedure was used to discriminate between marginal or cell means. All statistical tests were performed at the 95% confidence interval using GMAV5 for Windows (Marine Ecology Laboratory, University of Sydney, NSW, Australia).

3. RESULTS

The photoperiod and feeding frequencies selected for this experiment affected the weight gain and performance of newly weaned snapper. Generally, measures of weight gain increased in response to increased feeding frequency or to being fed later in the same photoperiod (i.e. 2FE vs 2FL), irrespective of the photoperiod employed. In addition, increasing the number of feeds from 2 to 8 feeds d^{-1} within each photoperiod regime improved survival of snapper from approximately 81 to 97%. Importantly, increased feeding frequency improved AFCR and reduced size heterogeneity in groups of snapper, as indicated by a decrease in the CV_{hw} (Table 2).

The interaction between photoperiod and feeding frequency significantly affected the individual harvest weight ($F_{3,40}$ = 3.72; P=0.0189) and TGC of snapper ($F_{3,40}$ = 3.51; P=0.028). Both the main effects for each of these response variables were also highly significant (P<0.0001). Interactions were synergistic and driven primarily by an increase in the magnitude of harvest weight or TGC in snapper fed the 4F and 8F treatments reared under the 18L:6D photoperiod, compared to snapper fed at the same frequencies but reared under the 12L:12D photoperiod (Table 2; Figure 1). Comparisons of simple main effects indicated there were significant differences (P<0.05) between all feeding frequencies within each photoperiod for each of these performance indices, with frequencies ranked 8F > 4F > 2FL > 2FE in both cases. In terms of photoperiod regime, significant differences (P<0.05) were found between the individual harvest weight of snapper fed the 4F and 8F treatments and between the TGC of snapper fed the 2FL, 4F and 8F treatments, respectively.

Survival, AFCR and CV_{hw} were not affected by the interaction of photoperiod and feeding frequency (P>0.05). Photoperiod regime did not significantly affect the survival or AFCR of newly weaned snapper (P>0.05), but these response variables were affected by feeding frequency (P<0.05). AFCR improved significantly as feeding frequency was increased or time of feeding varied such that 8F > 4F > 2FL > 2FE. Survival was significantly higher in snapper fed more frequently or in snapper fed twice daily but later in the same photoperiod (8F > 4F > 2FL > 2FE).

The CV_{hw} was significantly lower in snapper reared under the 18L:6D photoperiod compared to snapper subjected to the 12L:12D regime ($F_{1,16}$ = 9.71; P=0.0067), however, only the CV_{hw} for snapper reared under the 8F feeding frequency was significantly lower than other treatments (8F < 4F = 2FL = 2FE; $F_{3,16}$ = 8.59; P=0.0013), despite an apparent trend for the CV_{hw} to decrease as feeding frequency increased (Table 2).

4. DISCUSSION

The photoperiod regimes tested in this study did not affect the survival or the AFCR of newly weaned snapper, however, they did affect individual harvest weight, TGC and CV_{hw}. Our results reflect those

reported by Fielder *et al.* (2002), who showed that snapper larvae exhibited improved weight gain when reared under an 18L:6D photoperiod compared to larvae reared under a 12L:12D regime. However, we note that these improvements occurred only after larvae had inflated their swim-bladders. Prior to swim bladder inflation, a 12L:12D photoperiod was deemed optimal as a compromise between periods of light when larvae feed, and periods of darkness, when larvae inflate their swim-bladders. Similarly, many other species of a similar size to the snapper used in this experiment have shown improvements in growth from extended natural photoperiods including greenback flounder *Rhombosolea tapirina* (Hart *et al.*, 1996), gilthead sea bream *Sparus auratus* (Silva-Garcia, 1996), juvenile haddock *Melanogrammus aeglefinus* (Trippel & Neil 2003), striped trumpeter *Latris lineata* (Trotter, Battaglene & Pankhurst, 2003) and *Oreochromis* (El Sayed & Kawanna, 2004).

Lengthening photoperiod may stimulate growth in juvenile snapper for a variety of reasons. Entraining fish to regular light–dark transitions is important in synchronizing locomotor activity rhythms (Boeuf & Le Bail, 1999), therefore it is plausible that regular feed delivery under longer photoperiod stimulated an endogenous rhythm that allowed snapper to synchronize with metabolic demands and utilize nutrients more efficiently. This is supported by lower AFCR and higher TGC under 18L:6D for snapper. Lengthened photoperiod may indirectly modify growth by creating a longer foraging time for visual feeders and increasing food intake or muscle mass by exercise (Boeuf & Le Bail, 1999).

Shorter photoperiod has been shown to retard growth by demanding higher energy expenditure for synchronization into an endogenous rhythm, leading to a reduction of somatic growth (El Sayed and Kawanna, 2004). In contrast, without a period of total darkness, fish condition, growth and feed conversion rates are compromised (Thorarensen, Clarke & Farrell, 1989; Boeuf & Le Bail, 1999; Gines, Afonso, Argello, Zamorano & Lopez, 2004).

Based on determination of a digesto-somatic index (i.e. 100 * digestive system weight / carcass weight), juvenile snapper (0+ year class) sampled from the wild were shown to exhibit a diel feeding rhythm (sinusoidal), with the digesto-somatic index increasing throughout the daylight hours before peaking at sunset (Fancis, 1997). This suggests that, in the wild, juvenile snapper are predominantly daylight visual feeders, and that increasing day length under artificial conditions will likely have a positive outcome on growth. This hypothesis is supported by the results recorded for newly weaned snapper reared under the 18L:6D in our study, but the benefits of photoperiods longer than 18L:6D remain to be tested.

In general terms, all the performance indices presented in this study improved as a consequence of more frequent feeding. Obviously, the greater weight gain and TGC's of snapper fed 4 and 8 times per day compared with snapper fed twice per day is related directly to improved feed conversion efficiency (Table 2). It is likely that more frequent feeding also resulted in less competition between individuals through better or fairer distribution of feed, an hypothesis supported by the smaller CV_{hw} for fish fed 4 and 8 times per day. One possible reason for the improved AFCR in more frequently fed snapper is that fish consuming at or close to apparent satiation many times per day were more capable of satisfying their maintenance requirements and so had collateral nutrients and energy available for somatic growth. Similar results were found for gilthead sea bream (Andrew, Holm, Kadri & Huntingford, 2004). Murai & Andrews (1976) also found that small channel catfish (1.5 g) grew best when fed 8 times per day and concluded that the effects of long intervals between feeding are greater for smaller fish with higher metabolic demands. Some caution must be exercised when interpreting the results for AFCR in our study, because uneaten feed was not recorded.

Increased survival of newly weaned snapper appears to be related to a concomitant reduction in the size heterogeneity of fish which are fed more frequently or at different times of the day (Table 2). Although not significant, the higher CV_{hw} for snapper fed twice daily compared to those fed more frequently suggests that competition from larger dominant juveniles may have prevented smaller fish from securing enough feed to promote optimal growth. Our results are consistent with those of Battaglene & Talbot (1992), who showed that size heterogeneity increased the likelihood of cannibalism in snapper larvae, and in experiments on other species such as juvenile gilthead sea bream (Goldan, Popper * Karplus, 1997) and Atlantic cod (Folkvord & Ottera, 1993). Successful cannibalistic attacks among fish of a

similar size may impart a growth spurt to the attacker that can exacerbate further predation on smaller fish (Paller & Lewis, 1987), hereby presenting problems of increased cannibalism. A study by Wang, Hayward & Noltie (1998) showed size heterogeneity was reduced in green sunfish by increasing the number of daily feeds.

Feed conversion and nutrient utilization are complex processes that are affected by a number of factors including entrainment, maintenance energy requirements (Sanchez-Muros, Corchete, Suarez, Cardenete, Gomez-Milan & de la Higuera, 2003) and other endogenous mechanisms (Bolliet, Azzaydi & Boujard, 2001). For the snapper in this study, nutrient utilization appears to be influenced by time of feeding, since AFCR and TGC were improved when fish were fed the same amount but later in the same photoperiod. Reasons for this improvement are unclear, but numerous other studies with fish have documented distinct preferences for feeding in the morning or afternoon (Boujard & Leatherland, 1992; Jørgensen & Jobling, 1992; Dwyer *et al*., 2002; Sanchez-Muros *et al*., 2003). Feeding time may influence the phase or amplitude of some of the endocrine cycles involved in the physiological regulation of feeding, perhaps coinciding with natural rhythms of secretion, activation or synthesis of digestive or metabolic enzymes (Sanchez-Muros *et al*., 2003).

5. CONCLUSIONS

This study has demonstrated that weight gain, AFCR and size heterogeneity in newly weaned snapper can be dramatically improved if they are reared under an 18L:6D as opposed to a 12L:12D photoperiod and, when fed 10% BW d^{-1}, fed at least 8 times per day. The interaction between photoperiod and feeding frequency acted synergistically on the weight gain and TGC of snapper, but, the optimal feeding frequency was not identified as further increases in weight gain and performance may have been recorded had snapper been offered a greater number of feeds. Interestingly, this study has shown that newly weaned snapper are sensitive to the timing of feeds, with significant differences in weight gain and performance recorded between fish fed early (2FE) or later (2FL) in the same photoperiod. Further research is needed to clarify this observation.

Adoption of this rearing strategy should improve hatchery efficiency by increasing survival and reducing size variation and AFCR among new cohorts. Reduction in size variation alone will reduce the potential for competition and cannibalistic behaviour and improvements in AFCR will reduce feed costs and increase water quality. This strategy will indirectly benefit the snapper grow-out industry by ensuring farmers have access to large numbers of vigorous and similar sized fingerlings.

ACKNOWLEDGEMENTS

We would like to thank the staff of the Marine Fish Breeding Facility (MFB) at NSW DPI Fisheries for supplying the snapper used in this study and Ian Russell and Peter Dickson for their technical assistance running the experiment. Special thanks to Dr Trevor Gibson, Dr Wayne O'Connor and Dr Mike Dove for internal review of this manuscript. The research presented here forms part of a project of the Aquafin CRC, and received funds from the Australian Governments CRC Program, the Fisheries R&D Corporation and other CRC Participants.

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TABLE 1

Feeding protocols for newly weaned snapper reared under a 12L:12D or 18L:6D photoperiod[1].

	Feeding time[2] (h)							
12L:12D photoperiod (0700-1900 h)								
2FE	0800	1200	-	-	-	-	-	-
2FL	1100	1500	-	-	-	-	-	-
4F	0800	1120	1440	1800	-	-	-	-
8F	0800	0925	1050	1215	1340	1500	1630	1800
18L:6D photoperiod (0700-0100 h)								
2FE	0800	1600	-	-	-	-	-	-
2FL	1200	2000	-	-	-	-	-	-
4F	0800	1320	1845	1145	-	-	-	-
8F	0800	1015	1230	1445	1700	1915	2130	2345

[1] Photoperiod regimes = 12 h light: 12 h dark (12L:12D) or 18 h light: 6 h dark (18L:6D)
[2] Feeding frequencies = 2 feeds early (2FE), 2 feeds late (2FL), 4 feeds (4F) or 8 feeds (8F) delivered during the daylight phase of each photoperiod.

TABLE 2

Performance of newly weaned snapper reared under different photoperiod and feeding regimes after 32 days.

	Photoperiod regime							
	12L:12D				18L:6D			
Feeding frequency	2FE	2FL	4F	8F	2FE	2FL	4F	8F
Performance characteristic								
Individual stock weight (g)	0.15	0.15	0.14	0.14	0.15	0.15	0.14	0.15
Individual harvest weight (g)[1]	0.70	0.79	1.02	1.47	0.70	0.89	1.14	1.80
Harvest biomass (g tank^{-1})	59.38	74.26	100.54	149.13	62.22	77.91	112.34	172.17
Survival (%)[2]	81.56	91.25	91.80	95.70	84.90	87.86	94.62	96.78
Feed input (g tank^{-1})	87.68	97.44	117.82	134.80	95.45	102.19	130.69	155.26
Apparent FCR	2.02	1.66	1.40	1.01	2.06	1.64	1.35	0.99
TGC[3]	0.44	0.49	0.59	0.75	0.44	0.52	0.63	0.83
CV individual harvest weight (%)[4]	52.11	51.24	46.62	38.32	46.97	42.63	40.64	34.98

See Table 1. for abbreviations describing photoperiod and feeding frequency.
Except where indicated, data represent untransformed treatment means of n= 6 replicate tanks.
[1] Individual harvest weight = harvest biomass / number of fish at harvest
[2] Survival (%) = number fish at harvest / (number fish at harvest + sum of all recovered mortalities) x 100.
[3] Thermal growth coefficient calculated according to equation presented in Alanara, Kadri & Paspatis (2001).
[4] Coefficient of variation (CV) on individual harvest weight based on n=3 replicate tanks.

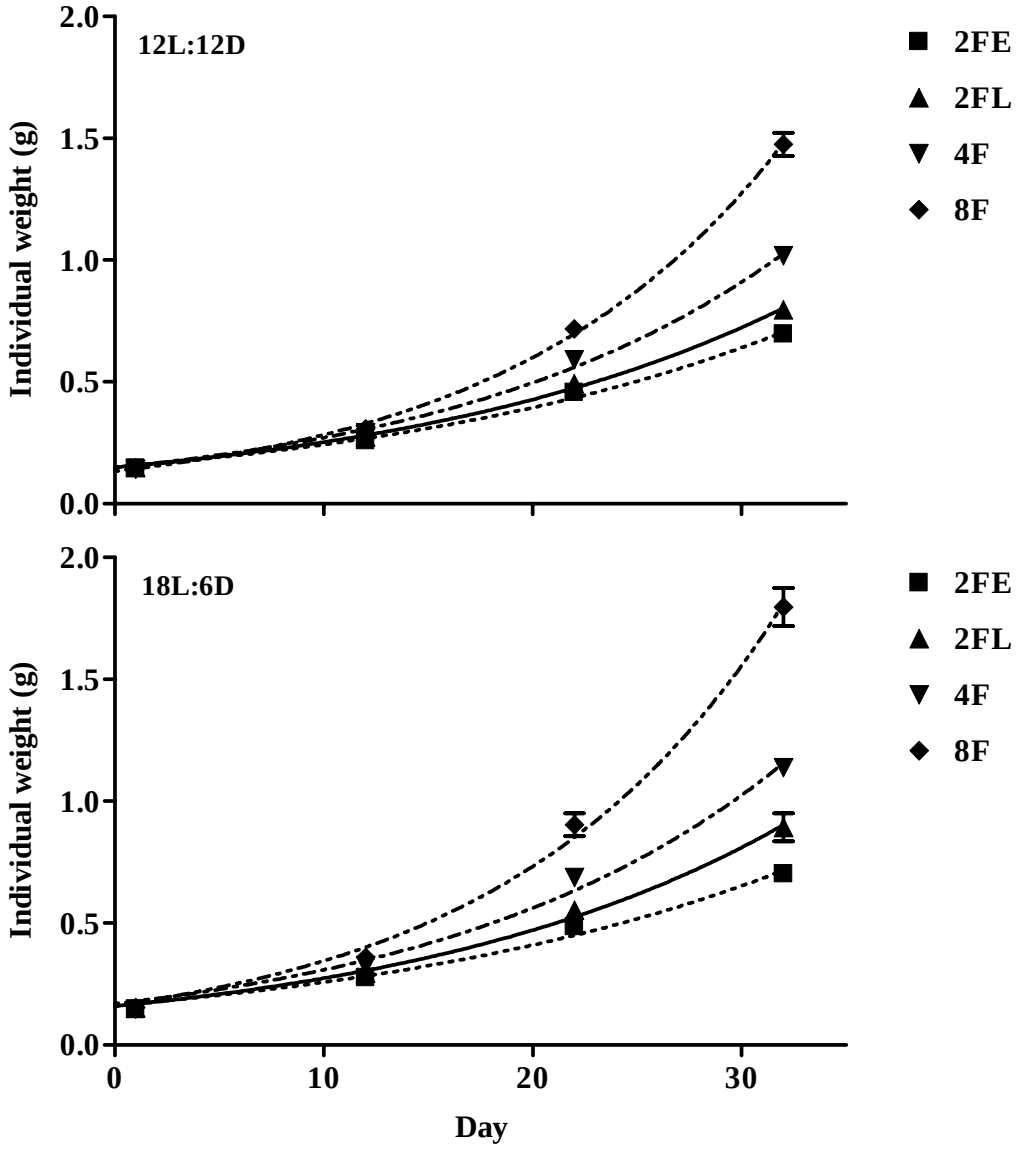

FIGURE 1

Effect of feeding frequency on the individual weight of newly weaned snapper reared under a 12L:12D or 18L:6D photoperiod for 32 days. Data are means ± SEM for *n*=6 replicate tanks.

4.7 Effect of feeding regime and fish size on weight gain, feed intake and gastric evacuation rates in juvenile snapper *Pagrus auratus*

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ABSTRACT

We investigated the interactive effects of nine feeding regimes (1 feeding early, 1 feeding late, 2 feedings early, 2 feedings late, 4 feedings, 4 feedings early, 4 feedings late, 6 feedings or 8 feedings per day; hereafter IFE, 1FL, 2FE, 2FL, 4F, 4FE, 4FL, 6F or 8F, respectively) and two fish sizes (small ≈ 5 g or large ≈ 20 g) on the harvest weight, feed intake and feed conversion ratio (FCR) of juvenile Australian snapper reared under an 18L:6D photoperiod at a temperature of 23˚C for 42 days. At the completion of the feeding study, small or large fish which had been accustomed to 1FE or 1FL were sacrificed to model gastric evacuation of a single meal. The feeding study indicated that optimum to maximum weight gain and FCR in juvenile snapper can be achieved by feeding fish to apparent satiation twice per day. This regime equated to relative feed intakes of 36 or 47 g $kgBW^{-1}$ day^{-1} for large or small snapper, respectively. In addition, there were no improvements in weight gain as a consequence of feeding a similar number of feeds (i.e. once, twice or four times) earlier in the day as opposed to later in the day. Size heterogeneity in both groups of snapper measured as the coefficient of variation in harvest weight (CV_{hw}), was not affected by feeding regime, but smaller snapper recorded slightly higher CV_{hw} (0.14) than larger juveniles (0.11). The gastric evacuation rates (GER) of small or large snapper fed a single meal proved to be similar (relative feed $content_{Combined} = 2.733 \pm 0.195 \times \exp^{(-0.139 \pm 0.013)}$; $R^2=0.72$), with approximately half the meal passed within 5 h and the whole meal cleared from the stomach within 16-20 h. This information will help operators of snapper hatcheries/nurseries plan their feeding regimes and assist with benchmarking performance.

1. INTRODUCTION

Feeds and feed delivery represent major economic costs in intensive marine fish hatcheries. Juvenile fish grow rapidly during this period and their feeding preferences can often be affected by ontogeny (Bolliet, Azzaydi & Boujard, 2001; Fielder, Bardsley, Allan & Pankhurst, 2002). Therefore it is critical to match an appropriate feeding strategy to each stanza of growth. In addition, it has been shown that feeding intervals or feeding frequency are strongly correlated with gastric evacuation time (Lee, Hwang & Cho, 2000; Riche, Haley, Oetker, Garbrecht & Garling, 2004) and that return of appetite is linked to gastric emptying rate (Riche et al., 2004). Thus matching the timing or frequency of feeding with peak appetite may improve the efficiency of production by enhancing growth and reducing feed conversion ratio (Boujard et al., 2001; Dwyer, Brown, Parrish & Lall, 2002). Optimising feeding regimes may also minimize feed wastage, leading to improvements in water quality and or reductions in size heterogeneity (Dwyer *et al*., 2002; Tucker, Booth, Allan, Booth & Fielder, 2006) while poorly timed or sporadic feeding regimes may lead to increased hunger, intra-specific aggression and increased rates of cannibalism (Folkvord & Oterra, 1993), all problems that decrease the efficiency of production and ultimately increase labour input costs.

We recently demonstrated that different feeding regimes and photoperiods can significantly affect the growth of newly weaned snapper (Tucker *et al*., 2006). The results of that study indicated that weight gain of snapper weighing between 0.14-1.80 g could be maximised by rearing them under an 18L:6D photoperiod and feeding them at least 8 times per day. The study also showed that newly weaned snapper generally performed better when fed later rather than earlier under the same photoperiod regime. While the aforementioned study was successful in recommending a feeding strategy for newly weaned

fish, there remained a need to investigate feeding strategies for larger juvenile snapper reared under similar intensive nursery conditions. Therefore, the aims of the present study were 1) determine feeding strategies that maximised feed intake, weight gain and feed conversion ratio in juvenile snapper weighing 5-20 g and 2) investigate the passage of a single meal through the stomach and intestinal organs of juvenile snapper in order to model gastric evacuation.

2. MATERIALS AND METHODS

2.1 Design of feeding experiment

A factorial experiment was designed to investigate the effects of 9 feeding regimes (1 feeding early, 1 feeding late, 2 feedings early, 2 feedings late, 4 feedings, 4 feedings early, 4 feedings late, 6 feedings or 8 feedings per day; hereafter IFE, 1FL, 2FE, 2FL, 4F, 4FE, 4FL, 6F or 8F, respectively) and two fish sizes (small ≈ 5 g or large ≈ 20 g) on weight gain and performance of juvenile Australian snapper. The combined effect of both factors on different performance indices was assessed under an 18L:6D photoperiod following the recommendations of previous research on this species (Fielder et al., 2002; Tucker *et al*., 2006). The feeding regimes chosen for this study were based on outcomes of earlier work (Tucker *et al*., 2006) as well as feeding regimes of interest to operators of commercial finfish hatcheries and nursery systems in Australia. All feedings were offered to fish within the daylight hours of the 18L:6D photoperiod. The nine feeding regimes are described in Table 1. Each of the feeding regimes (i.e. treatments) was randomly assigned to 4 replicate tanks within a temperature controlled laboratory and the experiment was run for 42 days.

2.2 Stocking, weight check and harvest procedures

The Australian snapper (*Pagrus auratus*) used in this study were progeny of first generation brood-stock held at the NSW DPI Port Stephens Fisheries Centre (PSFC) Marine Fish Hatchery. Two juvenile size classes, each held in separate tank systems were used; one of approximately 5 g and the other of approximately 20 g. Prior to their use in the experiment both groups of fish were held under a natural photoperiod (late spring - 13L:11D; 32°30'S, 152° 0'E) and at a relatively constant temperature (24.0 ± 2.0 ºC). Each group of fish was also fed the same commercial diet formulation, however, the size of the pellets varied according to fish size.

Each size class was moved to the laboratory separately after which a sample of individuals from each population was anaesthetised (15-20 mg L^{-1} ethyl-ρ-aminobezoate) and weighed (Precisa 3kg ± 0.01g top-loading balance) in order to determine a suitable weight range for stocking. Following this, snapper from within each size class were anaesthetised, weighed individually to ensure they fell within the desired range and then systematically stocked into experiment tanks. Eight larger (mean weight ± sd = 18.69 ± 1.79 g; n=288) or 20 smaller (mean weight ± sd = 4.69 ± 0.44 g; n=720) snapper were stocked into one of 72 experiment tanks giving 36 replicate tanks per size class.

All fish were reweighed after 14 and 28 days in order to monitor weight gain. At these times snapper were lightly anaesthetised in-tank, gently captured in a small dip net or by hand and bulk weighed before being returned to clean aquaria. At the completion of the experiment all fish were individually weighed and counted. Only three fish died during the experiment. The dead fish were weighed and then replaced with live fish of similar weight in order to maintain the original stocking densities.

2.3 Diet and feeding protocols

Snapper were fed a commercial Atlantic salmon starter diet prior to and during the experiments (Skrettings Pty. Ltd., Cambridge, Tasmania, Australia; typical analysis 52% crude protein, 18 MJ kg^{-1}gross energy). Pellets used in experiments were made from a single batch of the commercial feed which was finely ground (Raymond Laboratory Mill, Transfield Technologies, Rydalmere, NSW, Australia), fortified with 1g kg^{-1} vitamin-C (Rovimix® Stay-C® 35; F. Hoffman-La Roche, Basel, Switzerland) and then thoroughly dry-mixed before being moistened with distilled water and cold pressed into 1.5, 2.5 or

3.0 mm diameter pellets (Barnco Mincer, Australia Pty Ltd, Leichhardt, NSW, Australia). Moist pellets were dried in a convection drier at < 40˚C until moisture contents were < 50 g kg^{-1}. Pellet strands were broken to < 5 mm in length, sieved of dust and then stored frozen at < -15˚C.

Fish were fed to apparent satiation by adopting the following procedures. All tank rations were gradually increased from 1.5 to 5.0% of tank biomass in the three days following stocking (i.e. acclimation). During this period the allocated ration for each tank was split equally between a morning and afternoon feeding. During the experimental phase the daily quantum of feed for each tank was based on the previous days feed intake. In this way, feed rations were adjusted retrospectively, however, it ensured that all feeding was completed in a timely manner. Daily rations were adjusted according to the following protocol: if more than 50% of the daily quantum of feed was collected from a tank, the next days ration remained unchanged; if less than 50% of the daily quantum of feed was collected, the next days ration was increased by 10%. The calculated daily ration for each tank was split into equal portions according to the feeding regimes outlined in Table 1 and fish were offered their periodic ration/s gradually to ensure most feed was consumed from the water column before pellets settled on the floor of the tank. Nonetheless, any uneaten pellets were collected approximately 10 min after feeding, dried to a constant weight and subtracted from the total allocated ration to ensure accurate determination of feed intake. Fish were not fed in the 24 h prior to any weighing procedures.

2.4 Laboratory facilities

Individual experiment tanks (60 L rectangular clear acrylic) were supplied with continuously flowing (0.9 – 1.0 L min^{-1}), temperature controlled saltwater. Saltwater was obtained from an estuary adjacent to PSFC. This water was initially pre-filtered by sand-filters, disinfected with liquid chlorine then de-chlorinated with sodium thiosulphate solution and stored in a 50 kL reservoir. Recirculated water from the laboratory was pumped through a twin-cartridge pool filter (nominal pore size 20 μm) and over a 2 m^3 trickling bio-filter before reaching the experiment tanks. Between 50 to 200% of effluent stream water was exchanged daily and replaced with clean water depending on the rise and fall of total ammonia nitrogen (TAN) in the system.

Each tank was covered with a clear perspex lid and aerated with two air-stone diffusers. Individual fluorescent lighting was provided over each tank and was automatically controlled by an electric time-clock to provide the 18L:6D (0700 to 0100 h) photoperiod. Visible light intensity in experiment tanks was measured as photo synthetically active radiation (≈ PAR 26 μmol s^{-1} m^{-2} ; 1580 lux) using a LI-188B Quantum/Radiometer/Photometer and a LI-192SB Underwater Quantum Sensor (LI-COR, Lincoln, Nebraska, USA).

Water quality parameters were monitored daily with a Model 611-Intelligent Water Quality Analyser (Yeo-Kal Electronics Pty., Ltd., Brookvale, NSW, Australia). The mean ± sd values recorded were; water temperature (23.50 ± 0.86˚C), dissolved oxygen (5.40 ± 0.41 mg L^{-1}), pH (7.80 ± 0.17), salinity (35.50 ± 1.13 ‰). TAN levels were monitored regularly using a rapid test kit procedure (E. Merck, Model 1.08024, Germany) and always remained < 0.8 mg L^{-1}.

2.5 Gastric evacuation in small or large juvenile snapper

At the completion of the feeding experiment, snapper entrained to a single meal per 18L:6D photoperiod (1FE or 1FL) were remixed, individually selected, weighed and then randomly allocated to clean experiment tanks. The physical set-up of the laboratory and the photoperiod remained unchanged. Sixteen experiment tanks were each stocked with 5 small fish (17.8 ± 3.1 g; n=80) and 16 tanks were each stocked with 4 larger fish (46.7 ± 6.9 g; n=64). Fish were then acclimated to a single satiation feeding each day at 0800 h for the next 3 days and fed the 3 mm pellets described above. Prior to feeding on day 4, one tank of small and one tank of large snapper was randomly selected and euthanased with a lethal dose of ethyl-ρ-aminobenzoate to provide a starting point in the 36 h serial slaughter study. All remaining fish were then fed as usual, after which one tank containing large and one tank containing

small snapper was randomly selected at 2 or 4 h intervals and euthanased. Euthanased fish were dried and placed in labelled bags then frozen (-15°C).

Fish were thawed at room temperature, blotted dry and weighed before the stomach and intestine from each fish were carefully excised. Surgical clamps were used to retain the contents of the stomach or intestine during dissection. Stomach contents included all material contained between the posterior end of the oesophagus and the anterior end of the small intestine. Intestinal contents included all material between the top of the small intestine and the anal opening. The contents of both organs were removed by gentle pressure into separate pre-weighed oven-proof containers for determination of dry matter (105°C for 16 h).

2.6 Performance calculations

The following performance indices were calculated to interpret the results. Data for use in statistical tests were based on indices derived from tank averages. Interpretation of the study on feed passage relied on the spread of data from individual fish within each randomly sampled tank.

- Harvest weight (g) = wet weight of fish after 42 days
- Feed intake (g $kgBW^{-1}$ day^{-1}) = total individual feed intake / (GMBW/1000) / 42 days; where GMBW = geometric mean body weight of fish i.e. $(FBW \times IBW)^{0.5}$
- Feed conversion ratio (FCR) = total feed intake per tank / total wet weight gain of tank
- Thermal growth coefficient (TGC) = $[(FBW^{0.3333} – IBW^{0.3333}) / (T \times D)] \times 1000$; where FBW = final body weight, IBW = initial body weight, T = temperature (i.e. 23.5°C), D = days (i.e. 42) (Alanara et al., 2001).
- Coefficient of $variation_{harvest\ weight}$ = mean standard deviation of harvest weight per tank / mean harvest weight of fish per tank
- Relative stomach or intestinal content (g dry matter 100g BW^{-1}) = (grams of dry matter removed from either the stomach or the intestine / g wet body weight of fish) x 100

2.7 Statistical analyses

Two-way analysis of variance (ANOVA) was used to investigate the interactive effects of feeding regime and fish size (fixed factors) on individual harvest weight, relative feed intake, feed conversion ratio (FCR) or thermal growth coefficient (TGC) of juvenile snapper. Two-way ANOVA was also used to assess the interactive effects of the fixed factors on size heterogeneity in snapper by employing the coefficient of variation of harvest weight from each replicate tank (CV_{hw}) as the response variable.

Three-way ANOVA was used to evaluate the effect of early and late feeding regimes on harvest weight and TGC of juvenile snapper fed once, twice or four times per daylight phase. Fixed factors were: fish size (big or small), feeding time (early or late) and frequency of feeding (once, twice or four feeds per photoperiod). Prior to ANOVA, data were tested for homogeneity of variance using Cochran's C-test. All data satisfied this assumption. If ANOVA tests proved significant, a Tukey HSD multiple comparisons procedure was used to discriminate among means. All statistical tests were performed at the 95% confidence interval using Statgraphics Plus for Windows 4.1 (Manugistics Inc. Rockville, Maryland, U.S.A.). The relative amount of feed recovered from the stomach and intestinal contents of juvenile snapper and data concerning feed intake, FCR and growth were modelled using GraphPad Prism V4.01 for Windows (GraphPad Software Inc).

3. RESULTS

Fingerlings remained healthy and vigorous throughout the trial. Mortality was extremely low and only 3 fish died during the early part of the experiment. The effect of feeding regime and fish size on survival is not considered further here. Performance indices (i.e. tank means ± pooled sem) for large and small juvenile fish are presented in Tables 2 and 3, respectively.

3.1 Effect of feeding regime and fish size on harvest weight or TGC in juvenile snapper

Feeding regime (P<0.0001) and fish size (P<0.0001) but not their interaction (P=0.3875) affected the individual harvest weight of juvenile snapper. The multiple comparisons procedure indicated that juvenile snapper fed once daily, regardless of the time they were fed, gained significantly less weight than all other feeding regimes which were statistically similar (ranked order of marginal means; $1FE^{a}$ $=1FL^{a} < 2FL^{b} = 4FL^{b} = 4F^{b} = 4FE^{b} = 2FE^{b} = 8F^{b} = 6F^{b}$; superscript letters indicate homogenous groups; n=8).

Similarly, only feeding regime (P<0.0001) and fish size (P<0.0001), significantly affected the TGC of juvenile snapper. The TGC (marginal mean) of larger snapper was significantly higher at 1.0556 (n=36) than that of smaller snapper which recorded a TGC of 0.9367 (n=36). In terms of feeding regime the TGC's of juvenile snapper fed once daily were statistically similar but significantly lower than all other feeding regimes (ranked order of marginal means; $1FE^{a} =1FL^{a} < 2FL^{b} = 4FL^{b} \leq 4FE^{bc} = 4F^{bc} = 2FE^{bc} = 8F^{bc} \leq 6F^{c}$; superscript letters indicate homogenous groups; n=8).

Three-way ANOVA indicated there were no significant interactions among any of the fixed factors when testing their effects on harvest weight or TGC of juvenile snapper. For harvest weight, only the effects of fish size and frequency of feeding were significant (P<0.0001 for both main effects). Time of feeding (early or late) did not affect harvest weight (P=0.1199). A comparison of marginal means grouped the frequency of feeding with respect to harvest weight as; 1 feeding^{a} < 2 feedings^{b} = 4 feedings^{b} (n=16). Similarly, both fish size and frequency of feeding (P<0.0001 for both main effects) but not time of feeding (P=0.1175), significantly affected TGC. The TGC of larger snapper was greater than smaller snapper (i.e. large = 1.0337 vs small = 0.8907). A comparison of marginal means grouped the frequency of feeding with respect to TGC as; 1 feeding^{a} < 4 feedings^{b} = 2 feedings^{b} (n=16).

3.2 Effect of feeding regime and fish size on relative feed intake or FCR in juvenile snapper

Both relative feed intake (g kgBW^{-1} day^{-1}) and FCR were significantly affected by feeding regime, fish size and the interaction of the main factors (P<0.05). The interaction mean square and F-ratio in the test of feed intake was relatively weak ($F_{8,54}$ = 3.13, P=0.0056) compared to the variance explained by the main effects (feeding regime $F_{8,54}$ = 36.41, P<0.0001; fish size $F_{1,54}$ = 282.28, P<0.0001). The interaction term with respect to FCR was much stronger (P<0.0001) and accounted for as much variance as the main effect of feeding regime. The strong interaction term occurred because of the poorer (i.e. higher) FCR recorded for larger snapper fed 4F, 6F or 8F compared to the FCR of smaller snapper fed similar regimes. Consequently, each analysis was reduced to a comparison of simple main effects concentrating on the effect of feeding regime within size class (one-way ANOVA; Tukey HSD). Results of multiple comparison procedures for large and small fish are detailed in Tables 2 and 3, respectively. Larger snapper fed the 4F, 6F and 8F treatments recorded significantly higher feed intake than most other treatments but the increase in consumption was offset by poorer feed conversion ratio (Table 2). This was not as evident in smaller snapper with the poorest feed conversion ratio recorded in fish fed only once a day (Table 3).

3.3. Non-linear relationships between feed intake, TGC and FCR

In order to explore the theoretical relationships between feed intake and growth or feed intake and FCR (Hepher, 1989; Lovell, 1989) the average value of feed intake per single, duplicate or quadruplicate feeding regimes was plotted against treatment means for TGC or FCR. Data for 6F and 8F were also

included. The relationship between feed intake and TGC was modelled using a nonlinear equation that approaches an asymptotic value (i.e. $y = a \times [1 - \exp^{(-b(x-c))}]$) while the relationship between feed intake and FCR was modelled using 2nd or 3rd order polynomial functions. All independent data points (treatment means) were used to derive each model relying on the assumption that the independent variable was measured without error. Parameter estimates for the relationship between feed intake and TGC for larger and smaller fish are presented in figure headings accompanying Figures 4 and 5, respectively. Optimal growth rates were estimated by multiplying the derived asymptotic values of TGC by a conservative value of 0.95. As such, optimal TGC coincided with feed intakes of approximately 36.2 and 46.9 g kgBW^{-1} day^{-1} for larger and smaller snapper, respectively. Optimal FCRs were based on the 1st inflection point in each of the polynomial equations and corresponded to feed intakes of 36.0 g kgBW^{-1} day^{-1} for large snapper and 47.8 g kgBW^{-1} day^{-1} for small snapper.

3.4 Size heterogeneity

Two-way ANOVA revealed that fish size (P<0.001), but not feeding regime (P=0.2620) nor the interaction of the main factors (P=0.6543), significantly affected CV_{hw} in juvenile snapper. The marginal mean of the CV_{hw} of small snapper was higher (CV_{hw} = 0.14) than that of larger snapper (CV_{hw} = 0.11).

3.5 Gastric evacuation in small or large juvenile snapper

The passage of feed (dry matter) through the stomach or intestinal organs of larger and smaller juvenile snapper fed a single meal at 0800 h is presented in Fig. 1 and 2, respectively. The overall pattern of response was very similar with relative stomach contents peaking around 2g 100g BW^{-1} and falling within 2 - 4 h of feeding. Clearance of the single meal was reasonably rapid and in both cases there was no feed recorded from the stomach after 20 h. The passage of intestinal contents from the same meal closely mirrored that of the stomach, however, the quantum of dry matter in the intestinal tract appeared to peak approximately 2 h later. The quantum of feed moving through the intestinal tract was also well regulated and was generally less than 0.5 g 100g BW^{-1}. The amount of dry matter removed from the stomach or intestine of snapper that were re-fed 24 h later was somewhat reduced, however, the fluctuation in feed collected from either organ was similar to that recorded over the previous 24 h cycle.

Gastric (stomach) evacuation rate (GER) was estimated by fitting data recorded between 2 and 20 h after feeding to a model of exponential decay, $y = a \times \exp^{(-k \times x)}$. Data points for 0 and 24 h were excluded to reduce bias in the estimates of the exponential model because no feed was recorded in the stomach of either size fish at these times. The parameter estimates ± sem for each of the fitted equations are presented below and a plot of the models is presented in Fig. 3.

- Relative feed content$_{Large}$ = 2.624±0.233 x exp$^{(-0.138±0.016)}$; R^2=0.80, n=36 fish
- Relative feed content$_{Small}$ = 2.822±0.303 x exp$^{(-0.140±0.019)}$; R^2=0.68, n=45 fish

Subsequently, the null hypothesis that one equation could describe both data sets was tested using a global modelling approach that compared the difference between similar parameter estimates from each model (F-test; α = 0.05; GraphPad Prism). The statistical analysis supported the null hypothesis indicating there was no significant difference between the specific parameters describing the relationship between stomach content and time in small or larger snapper (F=0.2995; df=2,77; P=0.7421). The combined model is;

- Relative feed content$_{Combined}$ = 2.733±0.195 x exp$^{(-0.139±0.013)}$; R^2=0.72

GER as predicted by the rate constant (*k)* in the global model is approximately 0.139 g 100g BW^{-1} h^{-1} (i.e. 0.139 % h^{-1}). Juvenile snapper required approximately 4.98 h to pass 50% of their ingested meal through the gastric chamber (i.e. T½ = 0.693 / 0.139).

4. DISCUSSION

Short term feeding studies such as the one presented here aim to match the feeding preference of the animal to a limited number of predetermined feeding regimes. If the range of feeding regimes is great enough then the chance of matching the test animal to their preferred feeding option is increased. As such, if increases in harvest weight and TGC are considered the principal indicators of performance then this study has shown that juvenile snapper weighing 5-50 g need only be fed to apparent satiation twice per day to reach their growth potential. This study has also shown there is no significant improvement in growth as a result of offering a similar number of feeds earlier in the photoperiod as opposed to later in the photoperiod and that increasing the number of daily feedings does not lead to major changes in size heterogeneity among fish of this size.

The results of the present work differ from those we reported for newly weaned snapper weighing between 0.14 – 1.80 g reared under a 18L:6D photoperiod (Tucker et al., 2006). For example, we found significant increases in the harvest weight, TGC, survival, CV_{hw} and apparent FCR of newly weaned snapper fed 2FE compared to 2FL. Other differences include the fact that newly weaned snapper were fed a fixed ration of 10% BW^{-1} day^{-1} delivered as 2FE, 2FL, 4F or 8F on the premise that this amount of feed was likely to be in excess of their requirements and would not restrict weight gain. However, unlike the present study our previous results demonstrated that growth did not plateau and we were unable to identify an optimal feeding frequency using the rations that we selected; weight gain continued to increase in response to more frequent feeding as well as concomitant increases in the absolute amount of the fixed ration (Tucker *et al*., 2006). These differences may relate to the greater impact of the prevailing biotic and abiotic experimental conditions on endogenous mechanisms in very small, newly weaned fish compared to the impact similar factors have on larger fingerlings (Madrid, Boujard & Sanchez-Vazquez, 2001).

From a production point of view an optimum ration is one which simultaneously promotes best growth and FCR (Hepher, 1989; Lovell, 1989). In this study the amount of feed necessary to optimise growth (i.e. 95% of maximum TGC) and FCR were in close agreement. However, as with many studies maximum growth and feed utilisation did not coincide and the choice is one between feeding for maximum growth as opposed to feeding for economic return (Hepher, 1989; Lovell, 1989). The intake values determined in this study compare favourably to those given in daily ration x water temperature tables for juvenile red sea bream (Koshio, 2002). According to these tables juvenile red sea bream weighing between 5-20 g and reared at 22-23˚C require between 48-60 g dry feed kgBW day^{-1} and those weighing between 20-50 g require as much as 39-53 g dry feed $kgBW^{-1}$ day^{-1} (note: no data were included on the nutrient or energy density of feeds by the author).

It is clear from these results that weight gain of juvenile snapper in the present study was affected more by the total amount of feed that they were able to consume *per sė* as opposed to any direct benefit associated with the particular feeding regimes selected. This is especially true for treatments where more than one feeding was offered per day. Thus it appears that peak appetite of the juvenile snapper within the weight range we studied was satisfied by the amount of feed they could physically consume in 2 to 8 feedings per day (i.e. this particular feed, its ingredient composition, nutrient and energy density). Nile tilapia have also been shown to consume similar amounts of feed whether offered 3 or 5 meals per day when fed to apparent satiation, however, growth and feed efficiency were better in fish fed 3 times a day (Riche, 2000, cited in Riche, Haley, Oetker, Garbrecht & Garling, 2004).

A comparison of non-linear model parameters found no difference between the GER of smaller or larger juvenile snapper fed one meal to apparent satiation at 0800 h. In contrast, gastric emptying time (GET) measured by x-radiography has been shown to vary with body weight, temperature and relative meal size in flatfish (Gwyther & Grove, 1981). In addition, while the effect of feeding frequency on GER was not investigated here, Riche *et al*. (2004) found it had no affect on GERs in Nile tilapia fed either 3 or 5 meals per day. This finding has implications for the present study as it suggests GER is independent of feeding frequency and there is a regulated flow of energy from the gastric chamber (Riche *et al*., 2004) which may partly explain why feeding frequencies greater than one feeding per day had little impact on

the performance of snapper. By way of comparison the passage of feed through the stomach of juvenile snapper was remarkably similar to that reported for juvenile Korean rockfish (Lee et al., 2000).

The GER of snapper fed once daily to apparent satiation was estimated to be k=0.139 while half a single meal of about 2.7% BW could be evacuated in around 5 h. Complete evacuation of the gastric chamber occurred within 16-20 h of feeding. The constant in all the aforementioned models represents an estimate of the relative quantity of feed recorded in the stomach of fish at the beginning of the time course study. It was slightly lower than the average feed intake calculated in snapper fed once daily over the life of the whole experiment (i.e. between 3-5%), however, allowances must be made for daily fluctuations in feed intake as well as the fact that fish in the time course study were first sampled 2 h after feeding. Interestingly, while the peak in feed recovered from the intestinal tract occurred somewhere between 2-4 h after feeding the fact that feed was found in the upper intestinal tract at first sampling suggests that the gastric phase of digestion in juvenile snapper is fairly rapid.

It is commonly acknowledged that GER along with other exogenous and endogenous drivers act on the return of appetite and the desire to feed (Gwyther & Grove, 1981; Riche *et. al*., 2004). But feeding too frequently can result in poorer feed conversion ratio due to increased GERs or "gastrointestinal overload" where intake of the next meal occurs before the previous bolus has been subjected to adequate gastric attack. When this occurs the existing chyme enters the anterior intestine only partially digested (Riche *et al*., 2004). Excluding single daily feedings, snapper were re-fed to apparent satiation 8 h (2FE, 2FL), 5¼ h (4F), 2¼ h (4FE, 4FL), 3 h (6F) or 2 ¼ h (8F) after the first allocation of feed. The similarity in FCRs recorded in smaller snapper fed more than once a day indicate there is little evidence to suggest they were suffering from gastrointestinal overload. Likewise the evidence for this effect is lacking when reviewing the FCRs of larger fish as FCRs were better in fish fed at closer intervals (e.g. 4FE or 4FL vs 4F; 8F vs 6F).

Selection of GET is somewhat arbitrary and its relationship to return of appetite is at best unclear. In gilthead sea bream GET was based on the time taken to empty half the stomach contents and varied between 3.8 to 8.6 h according to whether test feeds contained raw or extruded forms of wheat or corn. The concomitant GER (exponential model) for fish fed these diets ranged between 1.46 to 4.17 x 10^{-3} % min^{-1} which translates to about 0.087 to 0.252 % h^{-1} (Venou, Alexis, Fountoulaki, Negas, Apostolopoulou & Castritsi-Carthariou, 2003). These values bracket the ones we determined for snapper (see above). Another study on gilthead sea bream compared the GER of fish fed a natural feed (*Nereis diversicolor*) to fish fed commercial pellets. The GER of pelleted feed was 7.97% h^{-1} while that of natural feed was 6.24% h^{-1} (Andrade et al., 1996). Our data return values of 4.7 and 5.9 % h^{-1} for large and smaller snapper, respectively when converted to similar units and modelled using linear regression analysis.

CONCLUSION

Juvenile Australian snapper weighing between 5 to 60 g and reared at 23˚C can achieve optimum to maximum weight gain and feed conversion ratio if fed a commercial diet containing approximately 52% crude protein and 18 MJ kg^{-1} gross energy to apparent satiation at least twice a day. This amount corresponds to approximately 36.2 and 46.9 g $kgBW^{-1}$ day^{-1} for larger and smaller snapper, respectively. In addition we found no evidence of a difference in the performance of fish fed earlier in the photoperiod compared with those fed later in the photoperiod. A simple study investigating the passage of ingested feed found that there was no difference in the GERs of smaller and larger juveniles. Approximately 50% of a single meal was passed within 5 h and the stomach was fully evacuated within 16-20 h. This information will help operators of snapper hatcheries / nurseries better plan their feeding regimes and assist with benchmarking performance.

ACKNOWLEDGMENTS

We would like to thank Ian Russell and Peter Dickson for their technical assistance in running experiments and handling fish. We would also like to thank Wayne O'Connor and Mike Dove for

critical internal review of this manuscript. We gratefully acknowledge the financial support of this work by the Australian Aquafin Cooperative Research Centre (Aquafin CRC) for the Sustainable Aquaculture of Finfish and the Fisheries Research & Development Corporation (FRDC).

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TABLE 1

Feeding protocols for juvenile snapper reared under a 18L:6D photoperiod for 42 days.

Feeding regime	Feeding time (24 h)							
1 feeding early	1000	-	-	-	-	-	-	-
1 feeding late	2000	-	-	-	-	-	-	-
2 feedings early	0800	1600	-	-	-	-	-	-
2 feedings late	1200	2000	-	-	-	-	-	-
4 feedings	0800	1320	1845	2345	-	-	-	-
4 feedings early	0800	1015	1230	1445	-	-	-	-
4 feedings late	1445	1700	1915	2130	-	-	-	-
6 feedings	0800	1100	1400	1700	2000	2300	-	-
8 feedings	0800	1015	1230	1445	1700	1915	2130	2345

N.B. The light period commenced at 0700 h and ceased at 0100 h the following day. All feedings were delivered during the daylight phase.

TABLE 2

Performance characteristics of large snapper subjected to different feeding regimes and reared under a 18L:6D photoperiod for 42 days.

Feeding regime	Initial weight (g)	Harvest weight (g)	Feed intake (g $kgBW^{-1.0}day^{-1}$)	FCR	TGC	CV_{hw}
1 feeding early	18.79	46.00	33.09^{a}	1.51^{ab}	0.92	0.15
1 feeding late	18.74	47.45	33.84^{ab}	1.48^{a}	0.96	0.14
2 feedings early	18.79	54.18	37.19^{ab}	1.41^{a}	1.12	0.11
2 feedings late	18.60	51.40	34.91^{ab}	1.39^{a}	1.06	0.08
4 feedings	18.66	52.55	45.38^{d}	1.76^{bc}	1.08	0.10
4 feedings early	18.80	53.75	38.73^{bc}	1.48^{a}	1.11	0.11
4 feedings late	18.63	51.08	37.27^{ab}	1.49^{a}	1.05	0.11
6 feedings	18.50	53.25	47.26^{d}	1.80^{c}	1.11	0.10
8 feedings	18.75	53.67	43.07^{cd}	1.65^{abc}	1.11	0.12
Pooled SEM	0.14	1.20	1.14	0.06	0.03	0.02

Table values are group means and based on average of 8 fish in n=4 replicate tanks.
Thermal growth coefficient based on temperature of 24˚C.
Results of one-way ANOVA on feed intake or FCR: similar superscript letters in table columns indicate homogenous groups (one-way ANOVA; Tukeys HSD*; n*=4).
Results of two-way ANOVA on interactive effects of fish size and feeding regime are detailed in the results section.

TABLE 3

Performance characteristics of small snapper subjected to different feeding regimes and reared under a 18L:6D photoperiod for 42 days.

Feeding regime	Initial weight (g)	Harvest weight (g)	Feed intake (g $kgBW^{-1}day^{-1}$)	FCR	TGC	CV_{hw}
1 feeding early	4.64	14.63	42.32^{a}	1.48^{cd}	0.77	0.15
1 feeding late	4.69	14.65	44.15^{ab}	1.55^{d}	0.77	0.14
2 feedings early	4.71	18.78	46.21^{bc}	1.29^{ab}	0.97	0.14
2 feedings late	4.72	17.80	42.32^{a}	1.25^{a}	0.93	0.13
4 feedings	4.70	19.4	50.08^{cd}	1.37^{abc}	1.00	0.15
4 feedings early	4.70	18.30	49.65^{cd}	1.42^{bcd}	0.96	0.15
4 feedings late	4.70	18.25	45.13^{ab}	1.29^{ab}	0.95	0.13
6 feedings	4.71	20.68	50.61^{d}	1.32^{abc}	1.06	0.13
8 feedings	4.71	19.85	50.83^{d}	1.37^{abc}	1.02	0.14
Pooled SEM	0.04	0.36	0.81	0.04	0.02	0.01

Table values are group means and based on average of 20 fish in n=4 replicate tanks.
Thermal growth coefficient based on temperature of 24˚C.
Results of one-way ANOVA on feed intake or FCR: similar superscript letters in table columns indicate homogenous groups (one-way ANOVA; Tukeys HSD*; n*=4).
Results of two-way ANOVA on interactive effects of fish size and feeding regime are detailed in the results section.

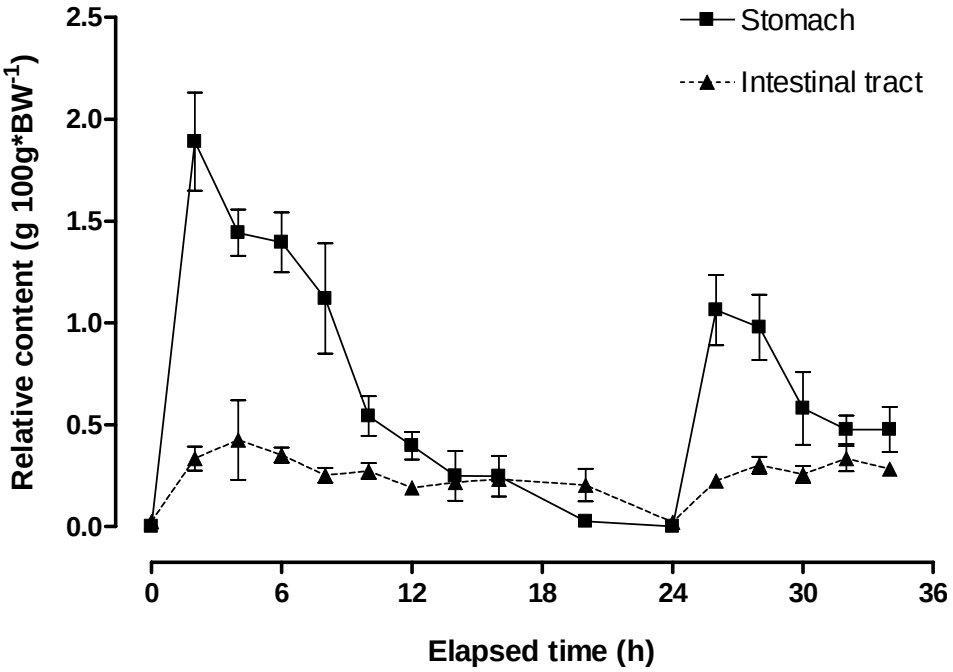

FIGURE 1

Temporal changes in the contents (g dry matter per 100g wet body weight) of stomach and intestinal organs in larger juvenile snapper (mean weight = 54.8 g). Points and error bars represent mean ± SEM of 4 fish fed a commercial diet to apparent satiation at 0800 h.

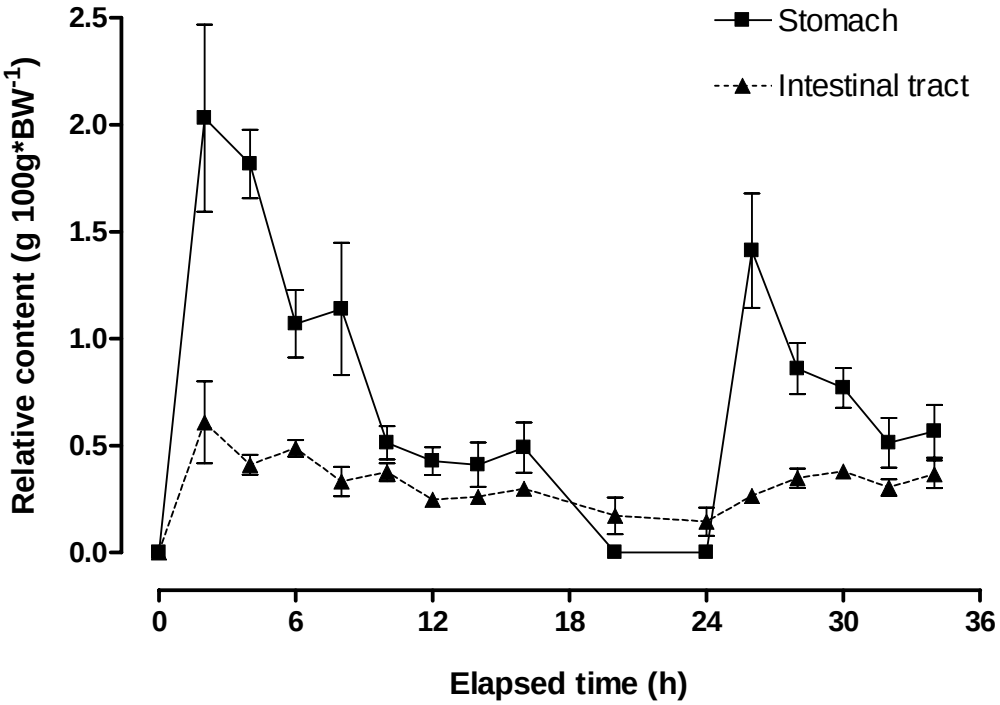

FIGURE 2

Temporal changes in the contents (g dry matter per 100g wet body weight) of stomach and intestinal organs in smaller juvenile snapper (mean weight = 17.8 g). Points and error bars represent mean ± SEM of 5 fish fed a commercial diet to apparent satiation at 0800 h.

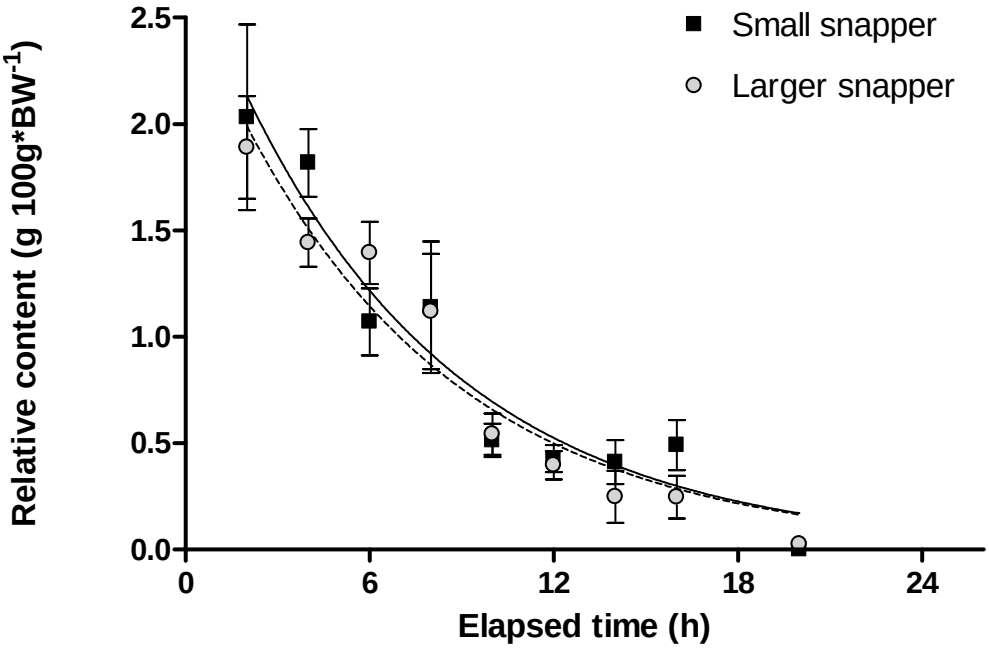

FIGURE 3

Gastric evacuation rates in small (14.6 g) and larger (46.7 g) snapper fed a single meal at 0800 h. Parameters for each of the fitted exponential curves and the global model are described in the text.

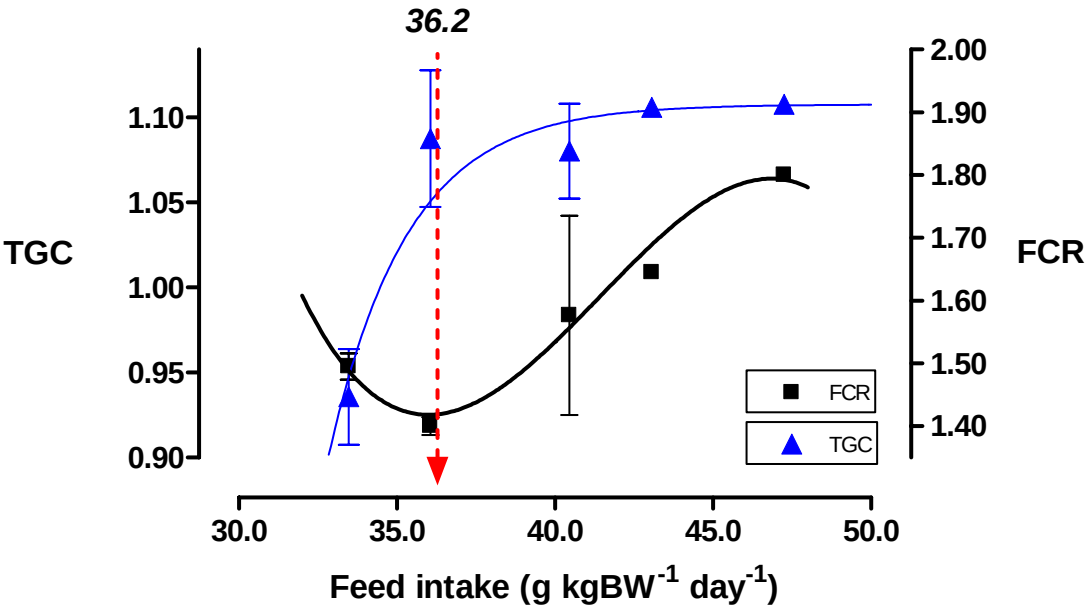

FIGURE 4

Effect of relative feed intake on TGC or FCR in larger snapper. Parameter estimates for TGC are; a = 1.108±0.015, b = 0.400 (constrained), c = 28.640±0.436; R^2 = 0.81. Dotted line indicates 95% asymptotic value of TGC.

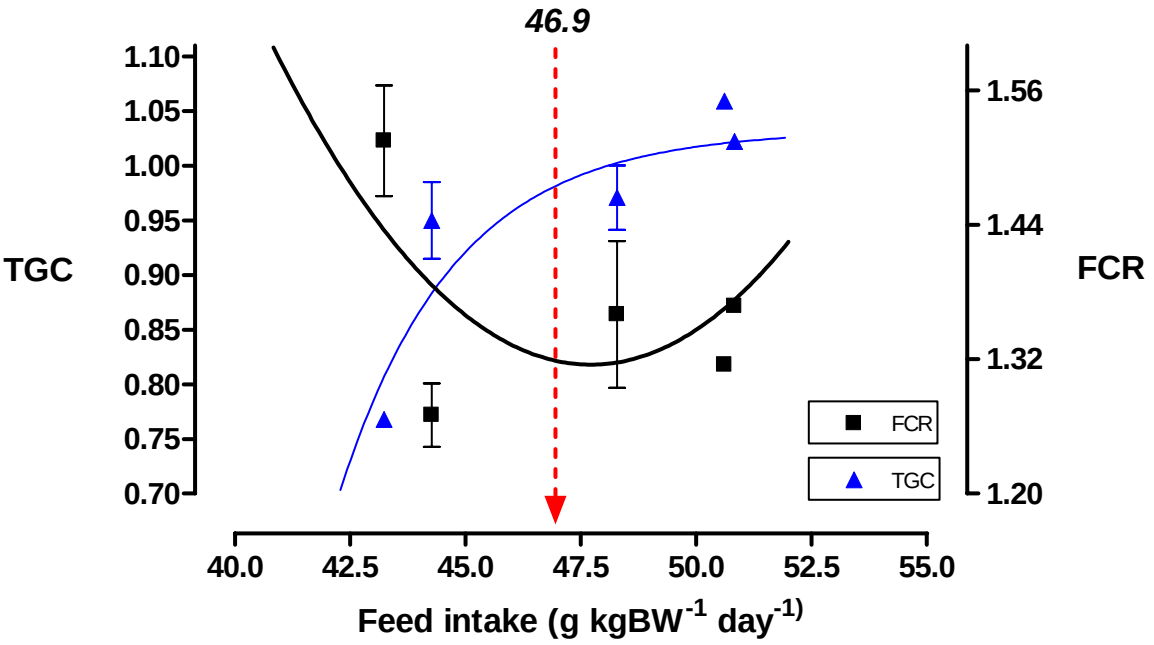

FIGURE 5

Effect of relative feed intake on TGC or FCR in smaller snapper. Parameter estimates for TGC are; a = 1.032±0.026, b = 0.400 (constrained), c = 39.430±0.465; R^2 = 0.77. Dotted line indicates 95% asymptotic value of TGC.

4.8 *Amyloodinium ocellatum*: Introduction and literature review[1]

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1. INTRODUCTION

Few scientists will question that aquaculture production losses due to parasitic infections are significant on a global scale. With diminishing wild populations of many commercial fish species, aquaculture production is becoming a more important source of protein for human consumption. With progressive advances in technology of aquaculture systems, productivity is assessed and managed at an increasingly finer scale, thereby exposing previously hidden limitations in an attempt to fully maximise harvest. Nonetheless, significant diseases that have been present for many years can often receive relatively little research effort even though their level of their impact may be high.

Since its description by Brown (1931), *Amyloodinium ocellatum*, has been identified and implicated in mortality events in aquaria and aquaculture facilities spanning tropical and sub-tropical regions. A range of tropical aquaculture species are affected by the parasite. Despite its common occurrence, management of *A. ocellatum* has not changed in over two decades, in large part due to deficiencies in knowledge of the basic biology of the parasite.

This review focuses on existing knowledge of *A. ocellatum* and how it affects aquaculture production, with the intention of identifying those information gaps that this study addresses and to explore their potential to better manage this parasite.

2. THE DINOFLAGELLATA

The phylum Dinoflagellata is a diverse group of free living and parasitic protozoans distinguished by the motile life stage possessing two flagella; one a regular, posteriorly beating flagella, the other a ribbon-like flagella that beats to the cell's left. The importance and diversity of the Dinoflagellata cannot be overestimated. These organisms are found in freshwater and marine environments, ranging from planktonic to benthic habitats. Many species of dinoflagellate are implicated in cases of human illness. These are toxin producers and can bring about diarrheic shellfish poisoning, neurotoxic shellfish poisoning, paralytic shellfish poisoning and ciguatera poisoning. These are of enormous importance to global shellfish and finfish aquaculture because the presence of these toxins in aquaculture products affects immediate saleability, and consequently impinges on marketability and consumer trust in the products.

In addition to free-living forms, many dinoflaglellate lineages have adapted to symbiotic or parasitic life styles. Coral reefs are built with the help of symbiotic zooxanthellae, while parasitic forms can be either extracellular (tribe Blastodinida) or intracellular (tribe Syndinia). However, for parasitic forms the boundary becomes less clear with some species exhibiting characteristics of both (tribe Duboscquodinida).

Within the parasitic assemblages, a number of species are important to aquaculture. Of the intracellular species, species of the genus *Hematodinium* Chatton and Poisson, 1931, (Family Syndinidae Chatton, 1910) are of the most concern (Gruebl, Frischer, Sheppart & Neumann, 2002). The potential of these

[1] Sections 4.8 to 4.13 of this report are taken from a thesis entitled *Managing velvet disease in marine fish hatcheries* submitted for the degree of Doctor of Philosophy at the University of Queensland, School of Molecular and Microbial Sciences, in October 2007 by Ashley Roberts-Thomson.

parasites to affect culture of crabs and lobsters will become more apparent as the production of these emerging aquaculture targets increases. The possibility that these species could spread into aquaculture facilities is more likely with the recent discovery of a free-living life stage of *Hematodinium* (see Frischer, Lee, Sheppard, Mauer, Rambow, Neumann, Brofft, Wizenmann & Danforth, 2006). Another of the genera within the Family Syndinidae, *Ichthyodinium* Hollande & Cachon, 1953, should be of potential concern to aquaculture. This is currently a single species genus, infecting the eggs of *Sardinia pilchardus* and *Maurolicus pennanti* (see Lom & Dykova, 1992). The potential exists for devastating effects on egg viability in hatcheries if it is able to exploit aquaculture systems.

The Tribe Blastodinida contains the extracellular parasites, primarily of fish and annelids. Many of their species are of particular importance to aquaculture. *Crepidoodinium* spp. Lom & Lawler (1981) species are gill parasites on fish, and are considered non-pathogenic (Lom & Dykova, 1992). Yet, high intensity infections in closed aquaculture systems have not been observed. It may be that pathology is relative to intensity of infection. *Piscinoodinim pillulare* (Schaperclaus, 1954) Lom, 1981 is a parasite of fish and is described as 'indiscriminate' when seeking hosts (Lom & Dykova, 1992). It is implicated in freshwater fish disease outbreaks (Martins, Moraes, Andrade, Schalch & Moraes, 2001). *Pfiesteria piscicida* Steidinger et Burkholder, 1996, *Pseudopfiesteria shumwayae* (Glasgow et Burkholder, 2001) Litaker, Steidinger, Mason, Landsberg, Shields, Reece, Haas, Vogelbein, Vandersea, Kibler & Tester, 2005, and *Pfiesteria*–like organisms have created much interest in scientific literature and also general media, due to their implication in fish kills and their human health issues. Controversy exists in the literature regarding the parasitic nature of these species and their life cycles (Burkholder, Noga, Hobbs & Glasgow, 1992). A 24 stage life cycle for *P. piscicida*, including amoeboid forms proposed by Burkholder & Glasgow (1997), is certainly unique among the dinoflagellates. This was disputed, as was the toxicity of the species, and a more conventional dinoflagellate life cycle suggested (Litaker, Vandersea, Kibbler, Madden, Noga & Tester, 2002). The work by Litaker (2002) rigorously examined the evidence and was based on excellent experimental design.

The most commonly encountered, and earliest described species of blastodinid is *Amyloodinium ocellatum* (Brown, 1931) Brown & Hovasse, 1946. Its frequent implication in disease outbreaks make it the possibly the most important dinoflagellate species that currently impacts on aquaculture production (Noga, Smith & Landsberg, 1991, Ellis & Watanabe, 1993, Colorni, 1994, Jenkins, Heyward & Smith, 1998, Rigos, Christofilogiannis, Giahnishi, Andriopoulou, Koutsodimoy, Negas & Alexis, 1998, Fielder & Bardsley, 1999, Liao, Huang, Tsai, Hseueh, Chang & Leoano, 2004, Abreu, Robaldo, Sampaio, Bianchini & Odebrecht, 2005). From its initial description, debate existed on the exact morphology of *A. ocellatum* (Brown, 1934, Nigrelli, 1936, Brown & Hovasse, 1946). It is entirely possible, that the description by Nigrelli, 1936 is in fact a different species of parasitic dinoflagellate, and this is alluded to in Brown &Hovasse's, 1946 revisitation of the original description. What is important to note is that both studies comprise the first records of a dinoflagellate infection in fish species. Both initial records originated from public aquaria, but were later shown to have primary influences on finfish species in aquaculture. Since then, the list of hosts infected by *A. ocellatum* has grown to include teleosts, elasmobranchs and even 'hyperinfecting' monogenean helminths (Lawler, 1980; Colorni, 1994).

3. TAXONOMY

Precise characterization of any organism that causes disease outbreaks in aquaculture is a fundamental tenet in effectively managing the infection. The *Amyloodinium* genus is currently represented by a single species, a concept supported by equivocal morphological and genetic evidence (see Levy, Poore, Colorni, Noga, Vandersea & Litaker, 2007). Interestingly, throughout its recorded history, variation in *A. ocellatum* behavior, morphology and environmental tolerance has been recorded by numerous authors which leads to the suggestion that *A. ocellatum* is a species complex (e.g. Lawler, 1980, Landsberg, Steidinger, Blakesley & Zondervan, 1994, Noga & Levy, 1995, Abreu *et al.,* 2005). The most recent taxonomic investigation by Levy *et al.*, 2007, describes two consistently distinct behavioural forms of the parasite as well as consistent morphological variation.

Informative morphological characters used for dinoflagellate taxonomy primarily focus on thecal plate tabulation in the dinospore stage, using scanning electron microscopy and preparation of samples using a technique known as suture swelling. Differences of only a single thecal plate are considered enough to merit placement of dinoflagellates in new species (Litaker *et al*., 2005). Presently, a single study has examined thecal plate tablulation of *A. ocellatum* (Landsberg *et al,*. 1994), looking at dinospores from a relatively small geographic area. While the results showed identical plate tabulation from that locality, a lack of thecal plate data exists for geographically distinct isolates of *A. ocellatum* and making comparative morphological studies using this technique difficult.

Genetic studies of *A. ocellatum* isolates have been limited to a single study of intra-specific rDNA variation (Levy *et al.,* 2007), followed by a phylogenetic analysis to place it within the Dinoflagellata (Litaker, Tester, Colorni, Levy & Noga, 1999). The study of intra-specific diversity did not discern cryptic species in the genetic analysis, but did identify two novel, isolate specific, behavioural differences. Considering the value of molecular techniques to modern science, the relative paucity of genetic information is surprising for such an economically important parasite.

Detailed characterisation of pathogens in aquaculture underpins further biological investigation targeting their control. Even, slight differences found consistently in different populations of a disease agent, may be exploitable in management of the infection. With this in mind, extensive morphological and genetic study of isolates from around the globe would be required to properly assess opportunities to control *A. ocellatum*.

4. LIFE CYCLE AND MORPHOLOGY

Three stages are characterised in the life cycle of *Amyloodinium ocellatum*: A trophont, which attaches to and feeds primarily on fish gill cells, but also epithelial tissue; a tomont that undergoes division on the substrate; and a free-swimming, infective dinospore (Brown & Hovasse, 1946). The parasite apparently tolerates broad environmental variation. It remains viable in salinities ranging from 1ppt to 78 ppt (Paperna, 1984b). Temperature changes may slow the life-cycle below 23° C (Paperna, 1984b), and suspend it temporarily below 14°C (Noga, 1995a). This allows the parasite to tolerate broader environmental changes than many of its hosts.

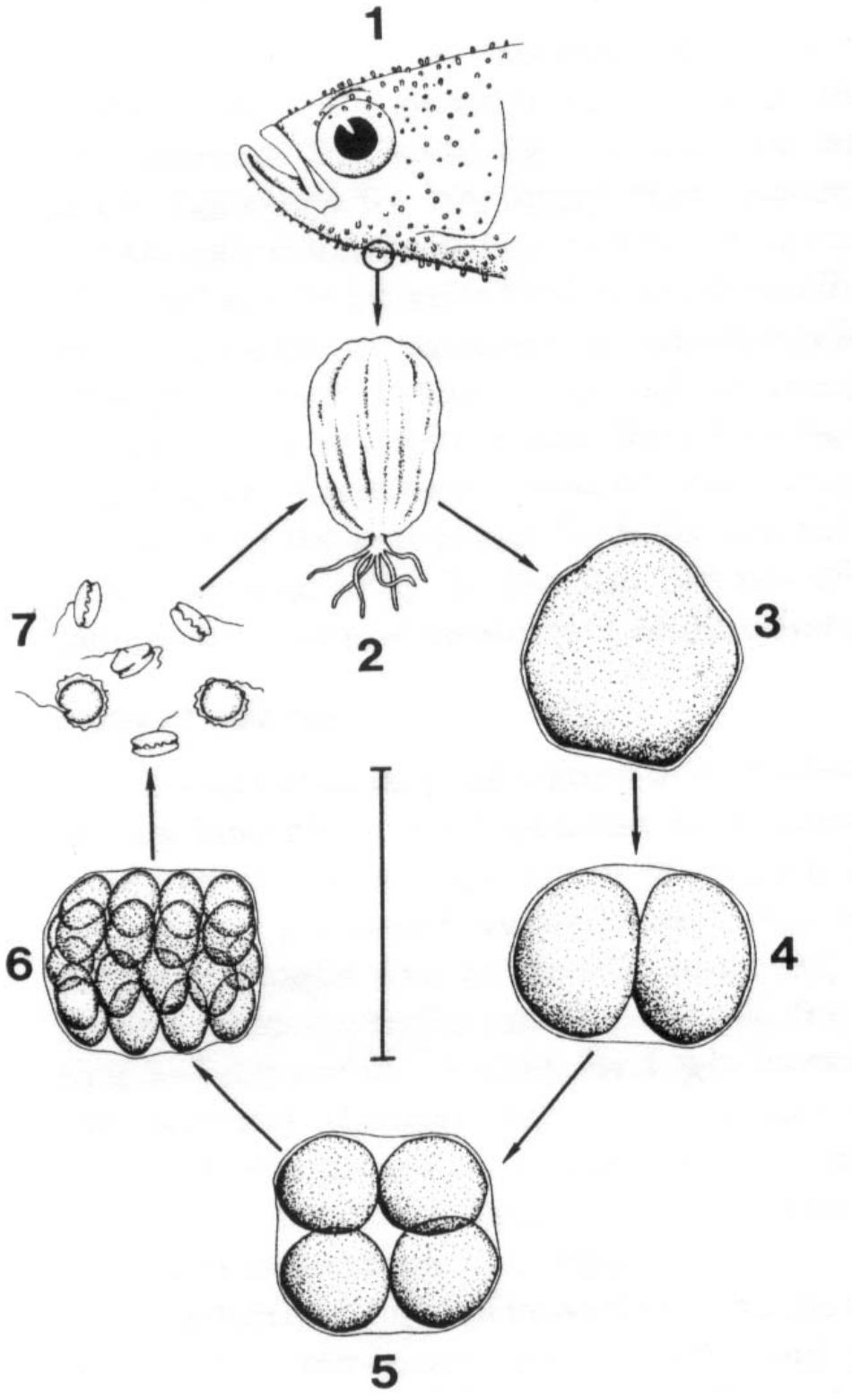

FIGURE 1

Life cycle of *Amyloodinium ocellatum* from Lom, 1992.

Attachment of the trophont to the host is facilitated by root-like structures called rhizoids (Brown & Hovasse, 1946). These penetrate the epithelial cells of the host and serve as an anchor leading to the suggestion that the parasite uses its host only as a substrate (Roberts, 1989). However, ends of the rhizoids have been observed to contain vesicles, supporting the original proposition, that these structures have some absorptive or digestive function (Nigrelli, 1936; Brown & Hovasse, 1946, Cachon & Cachon, 1987). Additionally, a tentacular process, the stomopode, is present in this life-stage, in which small vesicles and organelles, known as clove-like and dense bodies, are thought to hold lytic substances that are injected into host epidermal cells (Cachon & Cachon, 1987; Noga & Levy, 1995). Noga (1995) proposed a feeding mechanism comprising cellular debris collected by the stomopode being delivered to and taken up by a cytopharangeal feeding apparatus, associated with numerous food vacuoles within the main body of the trophont. Observation of constant twisting motion of the trophont while attached to the host has also been suggested to assist in the feeding process (Lom & Dykova, 1992). However, precise details of feeding mechanisms, function of rhizoids and the stomopode are yet to be determined and represent a significant gap in the knowledge of the biology of *A. ocellatum*.

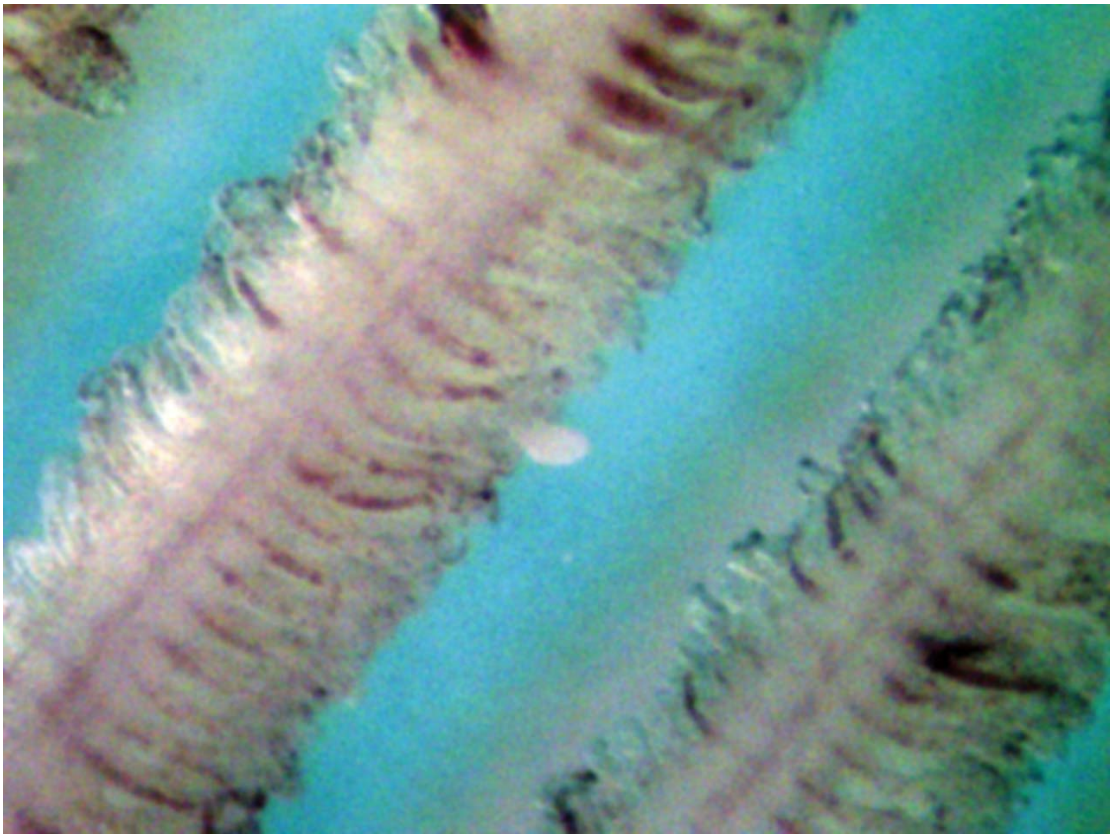

FIGURE 2

Trophont of *Amyloodinium ocellatum* on the gills of barramundi (*Lates calcarifer*)

Duration of infection in the trophont varies between 24 hours up to four or five days with time increasing as temperature decreases (Paperna, 1984b). Optimum temperatures, allowing the life cycle to complete in the shortest time are between 23°C and 27°C.At the end of this infection stage, the trophont measures an average maximum length of 100-150µm, although, some individuals may be up to 350µm long (Brown & Hovasse, 1946; Lawler, 1980; Noga *et al*., 1991; Colorni, 1994; Noga, 1995; Noga & Levy, 1995; Rigos *et al*., 1998; Coats, 1999). It is at this point that the trophont retracts its rhizoids and drops from the host to the substrate, changing in shape from pyriform to spherical (Brown & Hovasse, 1946).

The life stage present on the substrate is known as the tomont. When the tomont has been detached from the host for approximately 12 hours, mitotic division begins and continues until up to eight divisions have occurred. During this division process, the tomont begins to resemble a bunch of grapes (see Figure 3).

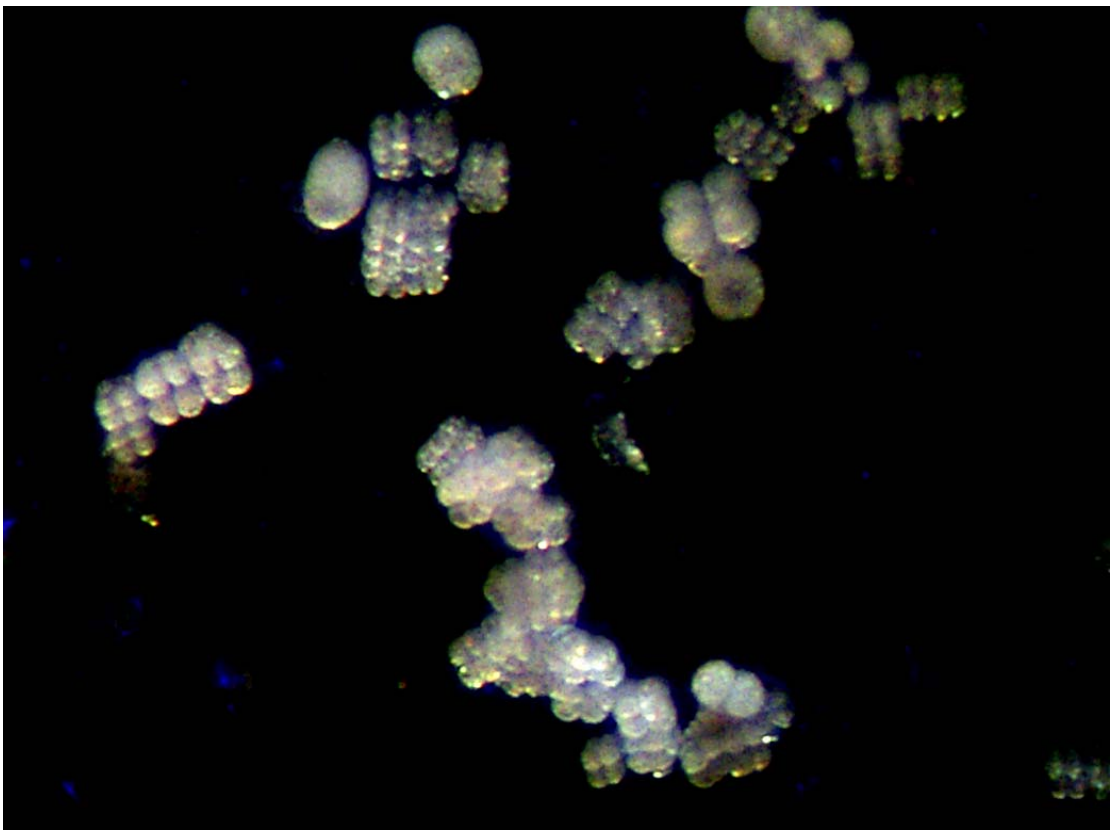

FIGURE 3

Tomont stages of *Amyloodinium ocellatum* in various stages of division

After all divisions are finished, sporulation of the tomont occurs and up to 256 infective dinospores emerge. There is some suggestion that phototaxis may play a role in the host seeking strategies (Montgomery-Brock & Brock, 2001). However, host-seeking mechanisms of *A. ocellatum* remain untested and represent a significant area of future research.

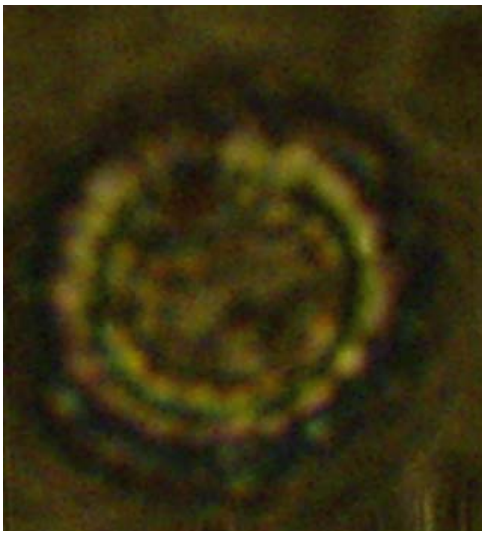

FIGURE 4

Dinospore stage of *Amyloodinium ocellatum* as observed through a compound microscope

5. PATHOLOGY & HOST IMMUNE RESPONSE

The life cycle of *A. ocellatum* can be completed in as little as five days at optimum temperatures. In aquaculture systems, where stocking density is high and susceptibility of fish may be accordingly high. As such, entry of the pathogen into a facility can lead rapidly to high intensity infections in short order. Indeed, entry pathways of the pathogen into the facility may be on introduced stock, contaminated equipment, influent water or even aerosols (e.g. *Ichthiopthirius multifiliis*). Once inside a facility, clinical signs of infection usually manifest as parasite intensity increases rapidly. Loss of appetite is a key indicator of chronic *Amyloodinium ocellatum* infection at low intensity levels (Bill Bardsley, pers. comm.). Other signs of infection include flashing, anorexia, irregular or rapid opercular beat and uncoordinated movement (Lawler, 1977; Noga & Levy, 1995). Unfortunately, these clinical signs are often encountered in many other parasitic infections and are therefore not diagnostic for *A. ocellatum*.

The gills are considered the primary attachment site for *A. ocellatum* infections (Noga & Levy, 1995). However, infections can also occur on skin and eyes. Attachment to the host epithelia with rhizoids can cause physical damage to several cells surrounding each trophont (Noga, 1995; Noga & Levy, 1995). Damage is thought to be exacerbated by the constant twisting motion of the trophont (Noga & Bower, 1987; Lom & Dykova, 1992). In, heavy infestations this damage from each individual trophont, can lead to gill hyperplasia, inflammation, haemorrhage and necrosis (Roberts, 1989; Noga *et al*., 1991; Noga, 1995). Anoxia and impaired osmoregulation are considered the cause of mortality, within as little as 12 hours for high-intensity infections (Noga, 1995; Noga & Levy, 1995).

Host immune response to *Amyloodinium ocellatum* was first identified in Tilapia. *Oreochromis aureus* (Blue Tilapia) serum was found to show potent inhibition of parasite growth *in vitro* (Landsberg, Smith, Noga & Richards, 1992). This study also identified the potential of mucus to have anti-*Amyloodinium* activity. Definitive characterisation of the response was confounded by microbial contamination, which is known to independently inhibit parasite development (Noga & Bower, 1987; Noga 1989, Oestmann & Lewis, 1995). An assay was developed for *in vitro* assessment of *A. ocellatum* infectivity after exposure to various immune factors (Noga, 1992).

An enzyme-linked immunosorbent assay (ELISA) was developed to detect antibody-mediated responses to the parasite (Smith, Levy & Noga, 1992). This ELISA protocol was subsequently used to quantify the serum response of *O. aureus* to intraperitoneal (IP) immunisation with dinospore antigens (Smith, Noga,

Levy & Gerig, 1993). Stronger response to IP immunisation occurred with live dinospores rather than sonicated dinospores (Smith *et al.*, 1993).

Fish develop strong resistance to infection by *A. ocellatum* after repeated non-lethal challenge (Cobb, Levy & Noga, 1998b). Dinospore attachment is apparently not affected, however, an anti-trophont mechanism has been proposed as the means of resisting infection. Immune fish are able to reject trophonts, or at least severely retard trophont development. This is thought to be mediated by mucosal antibody secretions (Cobb, Levey & Noga, 1998a; Cobb *et al.*, 1998b). Protective response to *A. ocellatum* can last for up to 6 months in *Amphiprion frenatus* (Tomato clownfish) (Cobb *et al*., 1998b).

Certain piscean innate immune factors have also shown anti-trophont activity. Histone-like proteins (HLPs) in skin mucus may inhibit *A. ocellatum* growth (Noga *et al*., 2002a). HLP-1 and HLP-2 are known to possess potent anti-parasitic activity (Noga *et al*., 2001). HLP1 levels can become depressed in the skin of chronically stressed fish, increasing the risk of fish developing Velvet disease (Noga, Fan & Sillphaduan, 2002b). Chronic stressors are often present in aquaculture systems and as such may play a role in making fish susceptible to *A. ocellatum* infections.

Noga & Wooster (2005) suggest that vaccination is the way forward for management of *A. ocellatum* in aquaculture. However, development of a successful vaccine may be some way off. The most pertinent consideration with respect to this pathway is that, currently, no vaccine exists for any fish ecto-parasitic protozoan. This is despite much focus on this particular field of aquaculture health in recent years. While a vaccine remains a beacon to aim for in the control of this disease, there are certainly further investigations necessary to fully understand the host parasite interaction of *A. ocellatum*. As such, more conventional control strategies still offer the best strategy to combat *A. ocellatum* infections.

6. DIAGNOSIS

Rapid and accurate identification of the organisms causing disease in aquaculture is essential to the timely management of infections. Until very recently, definitive diagnosis could only be confirmed through pathological examination of gill or epithelial tissue (Noga, 1995). Certainly, other techniques could be used for diagnosis, for example, kofoidian thecal plate tabulation of the dinospore is a very accurate method of diagnosing dinoflagellates (Litaker *et al*., 2005). However, the methods used to visualise the tabulation pattern are delicate and time intensive, making the technique far from rapid and not cost effective. Detection via the host immune response is also possible with ELISA (Noga, 1992). However, a lag between infection and immune response is not conducive to rapid diagnosis of the parasite.

The advent of PCR detection methods for rapid and accurate diagnosis of *A. ocellatum* (Levy *et al*., 2007) is a crucial step in improving management of the parasite in aquaculture. However, this diagnostic is based in the small sub-unit region of rRNA, where there is a high level of sequence homology for all isolates of *A. ocellatum*. The rate of mutation in this region is relatively slow compared to other regions of the gene (e.g. ITS) and therefore may lack sufficient resolution to identify genetic differences in isolates that correlate to biological characters. From an aquaculture point of view, isolate differentiation only becomes relevant if consistent differences in the morphology, physiology or behaviour (such as those described by Levy, 2007), are able to be exploited in varied management strategies for the parasite.

7. PARASITE CULTURE FOR IN VITRO STUDIES

Successful control of *Amyloodinium ocellatum* in aquaculture necessitates a ready supply of the parasite for use in laboratory experimentation. Consequently, numerous culture protocols have been developed to produce the parasites required for manipulative experiments although culture methods have been shown to be problematic. The first developed was *in vivo* culture which required at least one life stage of the parasite being present in conjunction with a live teleost to propagate the parasite (Bower, Turner & Beiver, 1987). Such attempts were complicated by the presence of bacterial, protozoan and mycotic

contaminants (Noga & Bower, 1987; Noga, 1989; Oestmann & Lewis, 1995). To combat these contaminants and maximise parasite yields in culture, tomonts were treated with solutions of penicillin and streptomycin-sulphate in artificial seawater (Noga, 1987).

After the protocol for culture was established, propagation of the parasite was first developed using neonate, guppy larvae as an assumed gnotobiotic host to minimise further contamination (Noga & Bower, 1987). Larvae were removed from an alcohol swabbed gravid female guppy using sterilised forceps then placed in a solution of seawater and gentamicin sulphate. This method provided a measure of success. Further studies revealed that the tomont phase of the parasite was most affected by microbial contaminants in culture. As such, culture methods were further refined through the use of a silica based density gradient to remove contaminants using centrifugation to separate contaminated tomonts from non-contaminated tomonts (Oestmann & Lewis, 1995).

Cultivation of the parasite *in vitro* eliminated the need to maintain fish in a laboratory, which provided a significant step forward in propagation of the parasite. However, maintenance of any pathogen through cell culture is expensive in time and resources. Additionally, passage through successive generations of cell culture can place selection pressure on the parasite and lead to genetic drift from the original wild strains. Some cultures of *A. ocellatum* have now been maintained for nearly 20 years (Noga, 1987; Levy *et al*., 2007) and may not represent the genotype of the original strain. Nonetheless, the use of cell culture techniques provides an opportunity to provide sufficient parasite numbers for experimental purposes.

Given the limitations described above, it would be valuable if the parasite could be stored in stasis for long periods, and remain viable and infective. Noga (1995) found that tomonts were able to undergo stasis for up to 17 weeks at temperatures below 14º C, and remain viable when temperature was increased. This observation prompted the possibility that *A. ocellatum* could be stored through the process of cryopreservation, which has been widely used and originated in the late 1940's and 50's (Hubalek, 2003).

8. CURRENT MANAGEMENT PRACTICES

Management of *A. ocellatum* in aquaculture facilities is a difficult task. The parasite itself is able to reproduce in salinities ranging from 3ppt up to 45ppt and temperatures from 17°C up to 40°C, making it more resistant to environmental change than many of its hosts (Paperna 1984b; Noga 1995; Kuperman & Matey, 1999). Treatment also becomes problematic when targeting the tomont stage. This is isolated from the environmental conditions by its cyst wall, making it near impervious to chemicals (Noga, 1995). Despite these hurdles, a measure of success has been reached in the implementation of chemotherapeutic treatments. These are currently the best method proposed to combat the parasite on the ground.

Substances affecting the trophont stage are generally non-specific chemicals. These include formalin, copper compounds, hydrogen peroxide, and fresh water baths (Paperna 1984a; Noga, 1995; Stopford, 2000, Montgomery-Brock, Sato, Brock & Tamaru, 2001). While these compounds are effective in shocking the parasite from its host they do not generally arrest development and trophonts are able to quickly form a tomont. Many of these substances are also toxic to the fish, making them generally ineffective as control measures. Copper compounds can be useful in dinospore elimination (Oestmann & Lewis, 1996a). Chloroquine, a common human anti-malarial drug, has also been found effective against *A. ocellatum* (Lewis, Wang, Ayers & Arnold, 1988). However, its expense and long half-life in flesh make it unsuitable for large-scale, food-fish production.

While malachite green, acriflavine, furnance and nitrofurazone have been found to act on the tomont stage, none are acceptable treatments in food fish production due to their persistence in the fish or adverse affects to human health (Paperna, 1984a; Oestmann & Lewis, 1996a). However, 3,N-methylglucamine has been found to be effective against trophont and tomont life stages in in vitro

studies (Oestmann & Lewis, 1996a). This ionophorous antibiotic lasolocid is approved for treatment of coccidian protozoa of poultry, potentially paving the way for its use in aquaculture.

Dinospores have been shown to be susceptible to UV exposure (Lawler, 1977). Many advances in UV and ozone sterilisation have been made since this discovery and they may prove useful as control measures for *A. ocellatum*.

9. CONCLUSIONS

Despite a long although sporadic history of study, *A. ocellatum* remains a significant threat to global tropical aquaculture production. It can lead to mass mortalities in production systems, yet the precise mechanisms of how it finds its hosts and how it feeds on the host remain unknown. A number of characteristics that, in other species, have indicated cryptic speciation are well documented in *A. ocellatum*. A thorough investigation of the intra-specific variation is essential so that management of the parasite can be targeted at more specific aspects of the parasite's biology. Current PCR diagnostic methods are not able to differentiate strains of geographically distinct isolates. This may be crucial if taxonomic studies reveal cryptic species that necessitate different management strategies. Numerous chemotherapeutics in use against *A. ocellatum* have not been thoroughly tested against all life stages of the parasite, and new potential chemotherapeutics are available that remain untested. Additionally, having accurate laboratory models that reflect subtle differences in the wild strains is essential for this testing. While immunisation remains an important milestone in combating this disease in aquaculture, conventional management strategies should not be neglected. Biosecurity is a fundamental tenant of efficient aquaculture production. Securing facilities against the intrusion of *A. ocellatum* will always remain the best method of control once all pathways into aquaculture systems have been identified.

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4.9 Investigating cryopreservation of *Amyloodinium ocellatum*

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1. INTRODUCTION

Amyloodinium ocellatum is an ectoparasitic dinoflagellate of the order Peridinales. Its life cycle comprises three stages, incorporating a trophont, which attaches to and feeds on fish gill and epithelial tissue; a tomont undergoing division on the substrate; and a free-swimming, infective dinospore (Brown & Hovasse, 1946). Infections are able to proliferate dramatically in closed aquaculture systems, causing significant mortality in hatcheries and grow out facilities globally.

Successful control of *Amyloodinium ocellatum* necessitates a ready supply of the parasite for use in laboratory experimentation. Consequently, numerous culture protocols have been developed to produce the parasites required for manipulative experiments although culture methods have been shown to be problematic. The first developed was *in vivo* culture which required at least one life stage of the parasite being present in conjunction with a live teleost to propagate the parasite (Bower, Turner & Biever, 1987). Such attempts were complicated by the presence of bacterial, protozoan and mycotic contaminants (Noga & Bower, 1987; Noga, 1989; Oestmann & Lewis, 1995). To combat these contaminants and maximise parasite yields in culture, tomonts were treated with solutions of penicillin and streptomycin-sulphate in artificial seawater (Noga, 1987).

After the protocol for culture was established, propagation of the parasite was first developed using neonate, guppy larvae as an assumed gnotobiotic host to minimise further contamination (Noga & Bower, 1987). Larvae were removed from an alcohol swabbed gravid female guppy using sterilised forceps then placed in a solution of seawater and gentamicin sulphate. This method provided a measure of success. Further studies revealed the tomont phase of the parasite was most affected by microbial contaminants in culture. As such, culture methods were further refined through the use of a silica based density gradient to remove contaminants using centrifugation to separate contaminated tomonts from non-contaminated tomonts (Oestmann & Lewis, 1995).

Cultivation of the parasite *in vitro* eliminated the need to maintain fish in a laboratory, which provided a significant step forward in propagation of the parasite. However, maintenance of any pathogen through cell culture is expensive in time and resources. Additionally, passage through successive generations of cell culture can place selection pressure on the parasite and lead to genetic drift from the original wild strains. Some cultures of *A. ocellatum* have now been maintained for nearly 20 years (Noga, 1987; Levy, Poore, Colorni, Noga, Vandersea & Litaker, 2007) and may not represent the genotype of the original strain. Nonetheless, the use of cell culture techniques provides an opportunity to provide sufficient parasite numbers for experimental purposes.

Given the limitations described above, it would be valuable if the parasite could be stored in stasis for long periods, and remain viable and infective. Noga (1995) found that tomonts were able to undergo stasis for up to 17 weeks at temperatures below 14º C, and remain viable when temperature was increased. This observation prompted the possibility that *A. ocellatum* could be stored through the process of cryopreservation, which has been widely used and originated in the late 1940's and 50's (Hubalek, 2003).

In most cases, organisms require a cryoprotectant in the medium in which they are to be frozen to minimise cellular damage. Cryoprotectants act to depress the freezing point of a solution via colligative action, which reduces salt concentration to avoid osmotic shock (Hubalek, 2003). Additionally, by permeating a cell, a cryoprotectant can decrease the water volume within the cell and reduces osmotic

injury inflicted on a cell during freezing by reducing water available for expansion. Although some microorganisms are able to survive cryopreservation without cryoprotectants, survival rates are significantly improved with their addition (Hubalek 2003). The most widely utilised cryoprotectants are dimethyl sulfoxide (DMSO) and glycerol. However, the range of other useful substances is substantial, and includes: methanol, sorbitol, glucose, sucrose, lactose, skim milk and blood serum (Hubalek, 2003). Glycerol and DMSO have an established history of cryoprotecting protozoans including some dinoflagellates (Hubalek, 2003) and were selected as candidates to test cryopreservation of *A. ocellatum*.

2. MATERIALS AND METHODS

2.1 Specimen collection

Amyloodinium ocellatum was isolated from Australian Bass, *Macquaria novemaculeata,* brood-stock held at Port Stephens Fisheries Centre (PSFC), central coast of New South Wales, Australia. Tomonts were collected by bathing infected fish in fresh water for ten minutes. Bath contents were subsequently screened through 150 µm and 47 µm screens, the former to remove debris and the latter to catch all tomonts larger than 47 µm in diameter. Salinity was increased in two stages through the addition of thiosulphate-sterilised seawater, first to 15 ppt for 10 minutes, then to 30 ppt, in which the tomonts were maintained.

2.2 Cryoprotectant addition

Samples of at least 300 tomonts were collected with a pipette and placed in individual micro-centrifuge tubes (2 ml volume). Concentrations of glycerol (7 concentrations (v/v) at 2 temperatures, -20º C and -80º C), DMSO (5 concentrations (v/v) at 2 temperatures, -20º C and -80º C), methanol (at 5% concentration (v/v), at 2 temperatures, -20º C and -80º C) and controls in 30 ppt sterilised seawater (at 2 temperatures, -20º C and -80º C) were added to each tube (see Tables 1 and 2). A second control was retained in 30 ppt sterilised seawater and maintained at 20º C, to assess the viability of that isolate of *A. ocellatum* under a normal temperature regime. Each cryoprotectant treatment was allocated equilibration times of 15 mins, 1 hour, 4 hours and 10 hours to allow diffusion of cryoprotectants through the tomont wall. Taking into account treatments and temperatures, a total of 112 micro-centrifuge tubes (containing at least 300 tomonts each) were used in this experiment. Equilibration times, cryoprotectants and cryoprotectant concentrations are shown in Tables 1 and 2.

2.3 Post-freezing observations

Samples remained frozen for 7 days. Tubes were removed from cryostorage and allowed to thaw at room temperature (20º C). Cryoprotectant solution was removed and parasites washed 3 times with sterilised seawater in an excavated glass block. Each sample was divided into thirds and placed in labelled, sterilised, sealed containers. Containers were placed in a tray with 0.5cm of water in the bottom to maintain even temperature. Every 4 hours for a period of 3 days, tomonts were checked for evidence of viability (i.e. palintomic divisions) and 75% of total water was changed with fresh sterilised seawater in each container.

3. RESULTS

Bacterial contamination had overgrown tomonts in sample containers after approximately 48 hours. No divisions were observed in any of the trial cryoprotectant media or controls at either cryopreservation temperature. Records are summarised in Tables 1 and 2.

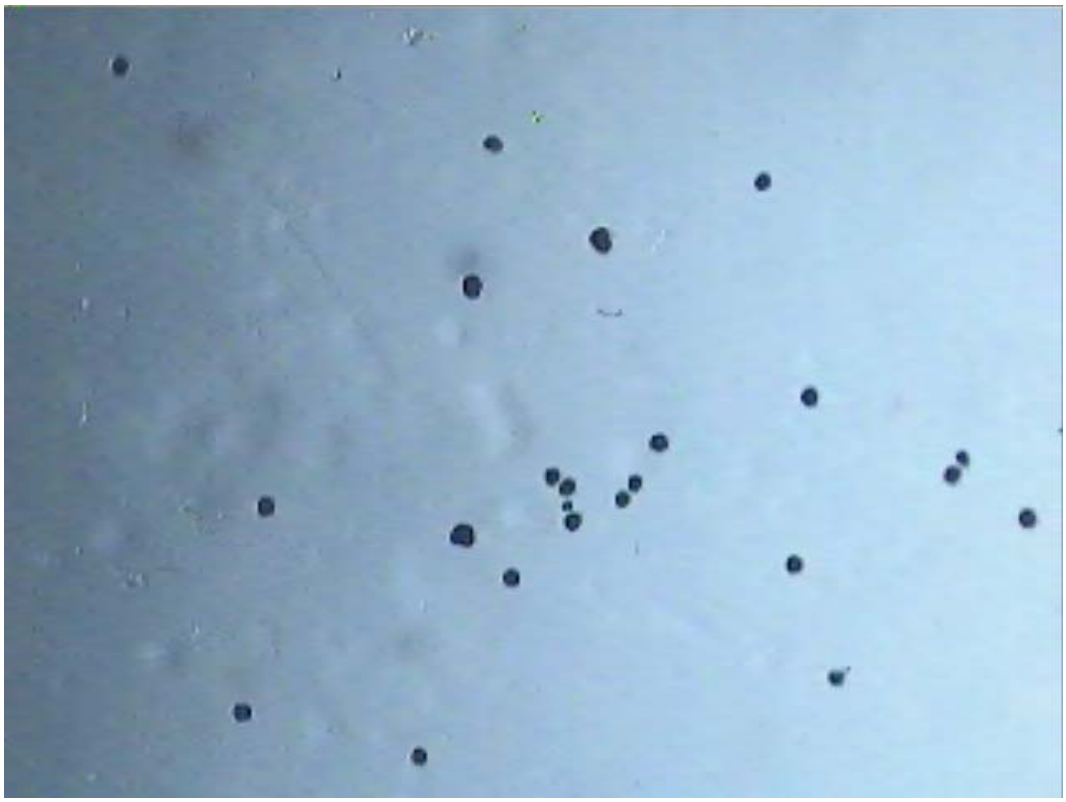

FIGURE 1

Thawed tomonts from the -80° freeze cycle including glycerol at 10%(v.v) after 36 hours post-thaw

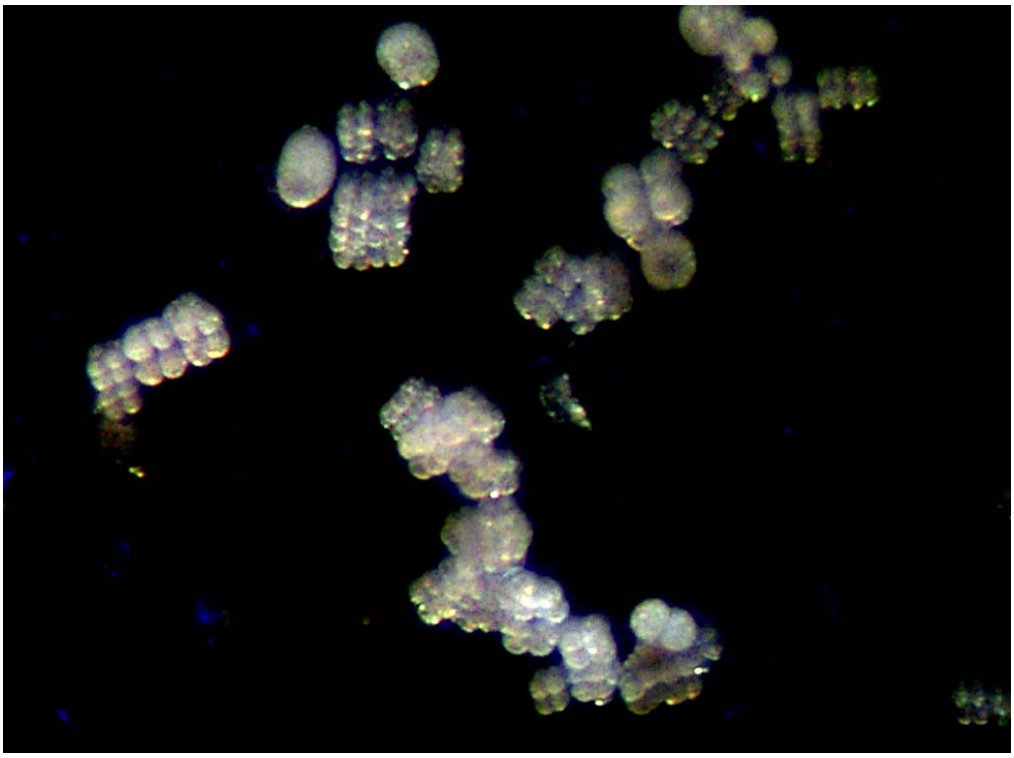

FIGURE 2

Non frozen control sample of the PSFC isolate tomonts after 36 hours

4. DISCUSSION

The cryoprotectants and cryopreservation methods used in this study have proven unsuccessful for storage and then retention of viability of *A. ocellatum*.

It is possible to speculate on some of the parameters that may have contributed to this result. The first is related to the transfer of cryoprotectant through the external wall of the tomont. It is well recognised that for environmentally-resistant stages (such as the tomont), diffusion or active uptake through the external membrane may be limited. As such, higher concentrations of cryoprotectants may have been required to provide protection, but some of these compounds have also been shown to be toxic to cells in high concentration. Furthermore, a combination of cryoprotectants may have been more efficacious. For example, DMSO can be used as a transport medium to allow other cryoprotectants into the cell.

The second possible parameter for future investigation is the method of thawing that is used following frozen storage. Cryopreservation blocks can reduce or increase temperature at an even rate and thawing techniques may be amended to rapidly reduce the concentration of cryoprotectant in samples. Additionally, examination of the suitability of the other life stages of the parasite towards cryopreservation may provide an option for successful storage and reanimation.

It appears that currently the only option for storage with retention of viability in *Amyloodinium ocellatum* is of short-term duration (circa 7 weeks) and invoked by dropping environmental temperature below 14º C but remaining above freezing. It may prove valuable in the future to investigate the correlation between retention of *A. ocellatum* viability and a regime of temperatures between 0º and 14º C.

TABLE 1

Cryopreservation experimentation concentrations and contact times for -20°C where *n*=number of observed divisions after 48 h observation and – indicates no observed divisions

Equilibration time	Control (Sterilised Seawater)	Glycerol Concentration (%)							Dimethyl sulfoxide (DMSO) concentration (%)					Methanol Concentration (%)
		2	3	10	20	30	40	50	1	2	3	4	10	5
10 Hours	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4 Hours	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 Hour	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15 Minutes	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 2

Cryopreservation experimentation concentrations and contact times for -80°C where *n*= number of observed divisions after 48 h observation and – indicates no observed divisions

Equilibration time	Control (Sterilised Seawater)	Glycerol Concentration (%)							Dimethyl sulfoxide (DMSO) concentration (%)					Methanol Concentration (%)
		2	3	10	20	30	40	50	1	2	3	4	10	5
10 Hours	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4 Hours	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 Hour	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15 Minutes	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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4.10 *Amyloodinium ocellatum* taxonomic investigations using ITS (internal transcribed spacer region)

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1. INTRODUCTION

Parasitic dinoflagellates are a diverse group within the phylum Dinoflagellata. The zoological tribe Blastodinida are dinoflagellates found on a wide range of hosts from copepods to teleosts and grouped together due to their extracellular parasitic life style (Cachon & Cachon, 1987). Within the tribe Blastodinida, the Oodinidae are known to cause disease in both freshwater and marine fish (Cachon & Cachon, 1987).

Amyloodinium ocellatum Brown, 1931, is currently the only member of the genus within the Oodinidae and is a significant disease agent in the marine aquaculture industry (Noga, Smith & Landsberg, 1991; Ellis & Watanabe, 1993, Colorni, 1994, Jenkins, Heyward & Smith, 1998, Rigos, Christofilogiannis, Giahnishi, Andriopoulou, Koutsodimoy, Nengas & Alexis, 1998). It is considered ubiquitous to tropical and subtropical marine waters. Three stages are characterised in the life-cycle of *A. ocellatum*: A trophont, that attaches to and feeds on fish gill and epithelial tissue; a tomont that undergoes division on the substrate; and a free-swimming, infective dinospore (Brown & Hovasse, 1946).

The host range of *A. ocellatum* is reportedly broad (see Table 1). In Mississippi Sound, Lawler (1980) examined 43 species of fish and found that 37% were naturally infected with *A. ocellatum*. Dinospores of the Mississippi isolate were then exposed to 78 species of teleost fish and one species of elasmobranch, *Dasyatis sabina,* in recirculating systems and found to infect 90% of these species including the elasmobranch (Lawler, 1980). Furthermore, the parasite is capable of attachment and perpetuation of infection using monogenean helminths as hosts (Colorni, 1994).

Members of the Oodinidae are distinguished by their elaborate host attachment organelle sometimes including root-like processes (Lom & Dykova, 1992). *Amyloodinium* is further distinguished by a special organelle in the trophont phase of the lifecycle. A tentacular process known as the stomopode, extends from the base of the trophont and is thought to assist in feeding (Brown & Hovasse, 1946; Cachon & Cachon, 1987; Lom & Dykova, 1992). This feature is unique within the Blastodinida to *Amyloodinium*, although possible modification of this organelle exists within *Protoodinium* species (Cachon & Cachon, 1987).

Sequencing of dinoflagellate small sub-unit (SSU) ribosomal RNA gene has lent support to major subclass level groupings based on morphological characters (Litaker *et al.* 1999). However, within the rRNA gene repeat, the SSU region is relatively conserved (Diggles & Adlard, 1997). This may make species or sub-species level distinction imprecise. The internal transcribed spacer region of the rRNA gene is known to evolve at a higher rate and has proven useful in species and sub-species level comparison (Diggles & Adlard, 1997). Determination of species boundaries is often based on percentage sequence differences in closely related taxa (see Litaker, Vandersea, Kibler, Reece, Stokes, Lutzoni, Yonish, West, Black & Tester, 2007). While appearing arbitrary, the threshold figure is generally determined with reference to differences in other known species within the family.

Ubiquitous parasites with broad host and geographic ranges are often found to be species complexes after scrutiny of ITS sequences. *Cryptocaryon irritans* was considered ubiquitous until internal transcribed spacer region (ITS1) sequencing and detailed morphological examination of a number of global isolates revealed that it was in fact a species complex (Diggles & Adlard, 1997). In contrast,

evidence for a species complex within *Kudoa thrysites* has not been found in the rRNA region of its genome despite observed morphological and geographical variation (Whipps, Adlard, Bryant, Lester, Findlay & Kent, 2003). Whipps *et al.* (2003) took the conservative view (to retain the status quo) because genetic variation was not correlated with other observed characters.

Variation exists in the morphological, behavioural and physiological characteristics of *A. ocellatum*. Intra-specific morphological and developmental variation of *A. ocellatum* has been noted in a number of studies (Lom & Dykova, 1992; Abreu, Robaldo, Sampaio, Bianchini & Odebrecht, 2005; Levy, Poore, Colorni, Noga, Vandersea & Litaker, 2007). Maximum trophont size is reported from 150µm up to 350µm (Lom & Dykova, 1992). At least two dinospore forms have also been described in the literature (Lawler, 1980; Levy *et al*. 2007). The Red Sea isolate reported by Levy *et al*. (2007) was observed to have more laterally compressed dinospores than the other isolates used in that study. Most descriptions of the dinospore indicate a dorso-ventrally flattened shape whereas those described by Lawler (1980) were more anterio-posteriorly flattened. Development has been observed over a broad range of salinity (10 to 60 ppt) and temperature (16° C to 30° C) with optimum conditions found to differ dependent on the study in question (Paperna, 1984b; Noga, 1987; 1989; Fielder & Bardsley, 1999). Additionally, behavioural differences have been observed in at least two regional isolates of the parasite (Levy *et al.* 2007). The DC-1 (a cultured isolate for over 15years, originally sampled from a home aquarium) and Red Sea isolates were consistently observed to swim more rapidly and turn less than the Gulf of Mexico isolate. While the observed differences listed above may point towards the presence of a species-complex they could equally be the product of phenotypic variation within a plastic single species.

Thecal plate tabulation is a morphological feature oft used in species differentiation in free living dinoflagellates. Early references to *Amyloodinium ocellatum* describe dinospores as naked gymnodinioid type (Brown, 1934; Nigrelli, 1936; Brown & Hovasse, 1946). However, SEM has now been utilised to describe the thecal plate tabulation of isolates obtained in various locations about Florida, USA (Landsberg, Steidinger, Blakesley & Zondervan, 1994). The tabulations were almost identical across all dinospores examined. Differences were observed in postcingular/precingular plate numbers. The study by Landsberg (1994) did not describe tabulation patterns from any other geographically distant strains. Results of the study by Landsberg (1994) have been interpreted as evidentiary for or against multiple species of *Amyloodinium*, interestingly, by the same authors (Levy & Noga, 2005, Levy *et al.* 2007).

Levy *et al*. 2007, published a study based on five northern hemisphere isolates of *A. ocellatum*. ITS sequence data was used to examine relationships of the isolates, finding no significant genetic variation, despite distinct morphological and behavioural differences in three strains. In this study, we have sequenced partial SSU, ITS1, 5.8S, ITS2 and partial large sub-unit (LSU) of *Amyloodinium ocellatum* isolated from NSW DPI Fisheries, Port Stephens Fisheries Centre (PSFC isolate) as well as a new isolate obtained from Louisiana, USA (LUA isolate). These sequences were then compared with previously published sequences of *A.ocellatum,* to further explore whether reported morphological and developmental discrepancies between geographically distinct isolates, are reflected by genetic differences.

2. MATERIALS AND METHODS

2.1 Specimen collection and genomic DNA extraction

Amyloodinium ocellatum was isolated from Australian Bass, *Macquaria novemaculeata,* brood-stock held at Port Stephens Fisheries Centre (PSFC) and Barramundi, *Lates calcarifer*, used for *in vivo* parasite culture at the University of Queensland.

Tomonts were collected by bathing infected fish in 200 ppm formalin for ten minutes. Bath contents were subsequently screened through 150 µm and 47 µm screens, the former to remove debris and the latter to catch all tomonts larger than 47 µm diameter. These were then washed with clean sea-water three times to remove any remaining formalin solution. Tomonts were fixed in 100% ethanol.

DNA of tomonts was extracted using the DNeasy tissue kit (QIAGEN) according to manufacturer's instructions.

2.2 Primer design

Initial primers were designed from *A. ocellatum* DC-1 strain ITS sequences determined by Noga *et al*., 1991 (GenBank accession numbers, AF352362, AF352361, AF352360 and AF352359). Internal primers were designed from the PSFC isolate sequence using the web-based software Primer3 (Rozen & Skaletsky, 1996) (Table 1). Sequences generated in this study and those from previous studies are listed in Table 2.

2.3 PCR amplification

DNA extracts were amplified by polymerase chain reaction (PCR) in 25µl volumes using 2U Hotmaster taq (Eppendorf), 10µM dNTP, 3.2µM of each primer and 2.5mM Hotmaster taq buffer and template DNA. PCRs were run under the following cycling conditions using a thermocycler (Corbett Research, Sydney): initial denaturation 94°C for 2min then 35 cycles at 94°C for 20s (denaturation), 48°C for 10s (annealing) and 65°C for 20s (extension) followed by a final extension at 65°C for 5 min.

Initial DNA amplification for cloning was performed on an Eppendorf Mastercycler EP Gradient S using the above described cycling parameters and primer concentrations. A high fidelity enzyme was used (Takara PRIMEstar) to allow any intragenomic variation to be detected with accuracy. Amplification was confirmed following PCR by agarose gel electrophoresis.

2.4 A tailing

The initial PCR products for cloning were cleaned using a PCR cleanup kit (Eppendorf) according to manufacturers instructions. Cleaned amplicons were then A-tailed by addition of 1 unit of Taq DNA polymerase (Bioline), 1X Taq buffer and 20µM dATP followed by incubation in the thermocycler at 70°C for 1 h. Samples were then re-purified using a PCR cleanup kit (Eppendorf).

2.5 Cloning reaction

Cleaned, A-tailed amplicons were then TA-ligated into PCR4-TOPO (Invitrogen) before cloning by transformation into TOP10 chemically competent kit *E. coli* (Invitrogen) in accordance with the manufacturer's instructions.

2.6 Sequencing

Following purification, PCR products were prepared for sequencing by the dideoxy chain termination method as described previously (see Adlard, Barker, Blair & Cribb, 1993). Sequencing from purified plasmids was performed by Australian Genome Research Facility (www.agrf.org.au).

2.7 Phylogenetic analyses of Amyloodinium ocellatum isolates

The entire ITS1 and ITS2 from *Amyloodinium ocellatum* isolates sequenced here were aligned with those acquired from GenBank (Table X) by eye using MacClade version 4.08 (Maddison & Maddison, 2005). The resulting ITS1 and ITS2 rDNA dataset was then analysed with minimum evolution (ME), maximum likelihood (ML) and maximum parsimony (MP) using PAUP* version 4.0b10 (Swofford 2003) and with Bayesian inference analysis (BI) using MrBayes version 3.1.1 (Ronquist & Huelsenbeck, 2003).

Minimum evolution analyses were based on total genetic distance and estimated by total distance, with heuristic searches employing tree bisection-reconnection (TBR) swapping. Maximum parsimony

analyses used heuristic searches employing TBR branch-swapping with all characters equally weighted and unordered, and separate analyses conducted with gaps treated as missing data (uninformative) or as a fifth state. Nodal support for MP analyses was conducted using 10,000 bootstrap replicates.

Modeltest version 3.7 (Posada & Crandall, 1998) was used to estimate the best nucleotide substitution model for the combined ITS1 and ITS2 dataset. Maximum likelihood analyses were conducted for the combined dataset twice, using both of the nucleotide substitution models and parameters predicted by the hierarchical likelihood ratio test (hLRT) and the Akaike Information Criterion (AIC) methods of model prediction in Modeltest.

Bayesian inference analysis was conducted on the combined dataset using the nucleotide substitution models predicted by Modeltest. The ITS1 and ITS2 regions were partitioned in the combined dataset to allow estimates of each model parameter for each gene region. The Bayesian inference analysis was run over 1,000,000 generations (nge*n*=1000000) via four simultaneous Markov Chain Monte Carlo (MCMC) chains (nchains=4) and every 100th tree saved (samplefreq=100). Bayesian analyses used the following parameters: nst=6, rates=invgamma, ngammacat=4, and the MCMC parameters were left at the default settings, and the priors parameters of the combined dataset were set to ratepr=variable. Samples of substitution model parameters, and tree and branch lengths were summarized using the parameters 'sump burni*n*=1500', 'sumt burni*n*=1500'. These 'burnin' parameters were chosen because the log likelihood scores 'plateaued' well before 150,000 replicates in the BI analyses.

3. RESULTS

3.1 Phylogenetic analyses of Amyloodinium ocellatum isolates

Sequencing of the ITS1 yielded an average of approximately 500 base pairs (bp) for all isolates. After trimming the ends (both 5' and 3') of fragments in the ITS1 alignment, a total of 450 bases that included the entire ITS1 and small portion of 5' end of 5.8S region, were available for analysis. Sequencing of the ITS2 yielded an average of approximately 800 bp for all isolates. After trimming the ends of fragments in the ITS2 alignment, a total of 745 characters (included the entire ITS2 and a small portion of the 5' end of the 28S region) were available for analysis. The combined ITS1 and ITS2 dataset resulted in 1195 characters for analysis, with 1134 invariant, 27 parsimony uninformative and 34 parsimony informative characters.

Modeltest predicted the two transversion-parameters model 1 unequal frequencies (K81uf) incorporating estimates of invariant (I) sites with among-site variation (G), or K81uf+I+G was the best estimator using hLRT and the transitional model (TIM), TIM+I was predicted as the best estimator using the AIC.

Bayesian inference and maximum likelihood analyses of the combined dataset using the nucleotide substitution models predicted by Modeltest resulted in phylograms with identical topologies and branch lengths (Figure 1). Relatively high posterior probabilities were observed for all clades of isolates observed in the analyses. Minimum evolution analysis of the combined dataset resulted in a tree (ME score = 70.99) that was similar in topology to the BI/ML trees, but topology differed slightly in two branches (Figure 2). The Med Sea 4 and Adriatic Sea 4 isolates grouped slightly differently in the ME tree. Med Sea 2, Adriatic Sea 2, Gulf of Mexico 21, Gulf of Mexico 16 and DC1 3 isolates also had slightly rearranged topology in the ME tree. Maximum parsimony analysis of the combined dataset with both gaps treated as missing data and gaps treated as a fifth state yielded six equally parsimonious trees. The resulting strict consensus tree from the MP analyses showed very similar topology to the BI/ML trees (Figure 3). However, Red Sea isolates and PSFC isolate grouped distinctly in MP phylograms compared to Bayesian/ML phylograms.

4. DISCUSSION

Amyloodinium ocellatum has previously been identified in disease outbreaks in Europe, Asia and across the Pacific. The alignment and comparison of ITS sequences of the PSFC isolate with previously

submitted sequences confirms that this isolate belongs to the genus *Amyloodinium*. The discovery of this parasite at the Port Stephens Fisheries Centre (Fielder & Bardsley, 1999) is the first literature reported occurrence in an Australian aquaculture facility. Port Stephens would be expected to be near the southernmost limit of *A. ocellatum*'s range, given the parasite's apparent preference for tropical climates. Further reports are to be expected in more northern Australian locations as tropical finfish aquaculture increases.

Observed values of $p \geq 0.04$ are proposed as the benchmark for species delineation in freeliving dinoflagellates (Litaker *et al*. 2007). Variation at an intra-secific level was proposed at values between 0.0 and 0.021. Levy *et al* (2007) reported $p<0.03$ for *A. ocellatum* ITS sequences. While this is still below the proposed 0.04 substitutions per site for species level differences, it should be noted that this would place it above an intra-specific level of 0.02 substitutions per site. Analysis in the current study revealed a mean *p* value of 0.012 and a maximum of 0.0202 for the currently sequenced *A. ocellatum* isolates. This is clearly within the range of intra-specific variation proposed by Litaker (2007). *Symbiodinium* species are delineated at values of $p<0.04$ due to highly varied host species selection and the resultant rapid genetic isolation. This may be a result of unique selection pressures dependent on host environmental preference (Litaker *et al*. 2007).

Dominant ITS alleles in free-living species of dinoflagellates are proposed to be characteristic of species (Litaker *et al.* 2007). This is important where more than one ITS population exists within a species. It is likely that dominant ITS allele determination is a more informative character for species level variation within *Amyloodinium* given the observed ITS variation within individual isolates.

Issues that confound the results of protozoan genetic studies can present themselves when isolates are cultured in a laboratory. An example of this was observed in a study of *Cryptocaryon irritans* (see Diggles & Adlard. 1997) where more variation was found in a cultured isolate than the combined variation from geographically separate isolates. This is especially important for protozoans with short generation times. Additionally, in *A. ocellatum* cell culture protocols, the culture medium represents a compromise between physiological needs of the host cell layers/aggregates and the parasite life stages (Oestmann & Lewis. 1996b). Indeed, the PSFC isolate in this study and a subculture maintained in vivo for 12 months were found to differ by 1.43% over combined ITS 1 and 2 sequences. However, only one clone each of these isolates was used in analysis and as such may not truly be representative of variation. Observed variation in this study and others may well be an artefact of the culture process.

Convergent evolution is the mechanism proposed to homogenise tandemly repeated gene regions, such as the rDNA region, within an organism (Dover, 1982). The ITS region of *A. ocellatum* would therefore be expected to retain the same sequence within a given isolate. Our results and those of Levy et al (2007) identify multiple ITS copies within *A. ocellatum* isolates. Similar results have been observed in *Dientamoeba fragilis*, where ITS heterogeneity was deemed to make the region an unsatisfactory marker for strain or sub-species identification (Windsor, Macfarlane & Clark, 2006). Our results would indicate that using ITS regions to discern inter-specific variation within *A. ocellatum* does not offer adequate resolution.

Our results are consistent with those of Levy *et al*. 2007. However, given the level of intra-specific or intra-isolate variation observed in our analyses it would seem prudent that another gene may offer more clarity to the issue of species-level variation within *Amyloodinium*. Variable environmental tolerances, behavioural characteristics and morphological characters lead to questioning whether this species should be considered ubiquitous. However, adaptability of the parasite may also be the reason for its global success. Further studies to relate the morphological and behavioural variation to a gene without multiple copies present in the genome and *A. ocellatum'*s biology are necessary to evaluate the scale of variation within the genus. This should include dominant allele determination and dinospore thecal plate tabulation of all available isolates.

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TABLE 1

Amyloodinium ocellatum hosts recorded in literature

Host Family	Host Species	Location	Reference
Atherinidae	*Atherina harringtonensis*	Bermuda	Rand, 1988 (EN#11)
	Morone saxitalis	Chesapeake Bay, United States	Paperna, 1976 (EN#45)
Cichlidae	*Oreochromis aureus*	Experimental	Smith, 1993 (EN#33)
Poecilidae	*Poecilia reticulata*	Experimental	Noga, 1987 (EN#8)
	Morone saxitalis X Morone chrysops	Experimental	Noga, 1987 (EN#8)
Pomacentridae	*Amphiprion ocellaris*	Experimental	Noga, 1987 (EN#8)
Cichlidae	*Oreochromis aureus*	Experimental	Landsberg, 1992 (EN#42)
Pomacentridae	*Amphiprion frenatus*	Experimental	Cobb, 1998 (EN#30)
	Morone saxitalisX Morone chrysops	North Carolina, United States	Noga, 1991 (EN#37)
	Red drum	Florida	Landsberg, 1994 (EN#40)
Pomacentridae	*Amphiprion clarkii*	Florida	Landsberg, 1994 (EN#40)
Pomacanthidae	*Pomacanthus imperator*	Florida, United States	Landsberg, 1994 (EN#40)
Pomacanthidae	*Centropyge loriculus*	Florida	Landsberg, 1994 (EN#40)
Dragonette	*Pterosynchiropus splendidus*	Florida	Landsberg, 1994 (EN#40)
Sparidae	*Puntazzo puntazzo*	Central Greece	Rigos, 1998 (EN#38)
Polynemidae	*Polydactylus sexfilis*	Hawaii	Montgomery-Brock, 2001 (EN#28)
Sparidae	*Sparus aurata*	Italy	Barbaro, 1985
	Pterois volitans	London, England	Brown, 1946 (EN#27)
	Chilomycterus schoepfi	London, England	Brown, 1946 (EN# 27)
Ophichthidae	*Ophichthus gomesi*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Synodontidae	*Synodus foetens*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Cyprinodontidae	*Cyprinodon variegatus*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Lutjanidae	*Lutjanus analis*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Gerreidae	*Euchinostomus argenteus*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Sparidae	*Archosagrus probatocephalus*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Sparidae	*Lagodon rhomboides*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Sciaenidae	*Cynoscion nebulosus*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Sciaenidae	*Menticirrhus americanus*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Sciaenidae	*Micropogonias undulatus*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Mugilidae	*Mugil cephalus*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Blenniidae	*Hypsoblennius ionthas*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Gobiidae	*Gobiosoma bosci*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Triglidae	*Prionotus rubio*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Ostraciidae	*Lactophrys quadricornis*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Diodontidae	*Chilomycterus schoepfi*	Mississippi sound, United States	Lawler, 1980 (EN#45)
Dasyatidae	*Dasyatis sabina*	Experimental	Lawler, 1980 (EN#45)
Ophichthidae	*Myrophis punctatus*	Experimental	Lawler, 1980 (EN#45)
Ophichthidae	*Ophichthus gomesi*	Experimental	Lawler, 1980 (EN#45)
Clupeidae	*Harengula jaguana*	Experimental	Lawler, 1980 (EN#45)
Engraulidae	*Anchoa mitchilli*	Experimental	Lawler, 1980 (EN#45)
Synodontidae	*Synodus foetens*	Experimental	Lawler, 1980 (EN#45)
Ariidae	*Arius felis*	Experimental	Lawler, 1980 (EN#45)
Ariidae	*Bagre marinus*	Experimental	Lawler, 1980 (EN#45)
Tetraodontidae	*Anisotremus virginicus*	New York Aquarium, United States	Cheung, 1981 (EN#90)
Acanthuridae	*Zebrasoma flavescens*	New York Aquarium, United States	Cheung, 1981
	Chilomycterus schoepfii	New York Aquarium, United States	Nigrelli, 1936 (EN#24)
Tetrodontidae	*Spheroides maculatus*	New York Aquarium, United States	Nigrelli, 1936 (EN#24)
Lutjanidae	*Lutjanus argentimaculatus*	Phillipines	Cruz-Lacierda 2004
	Chanos chanos	Phillipines	Cruz-Lacierda 2004
Sparidae	*Pagrus auratus*	Port Stephens, Australia	Stopford, 2000 (EN#5)
Sillaginidae	*Sillago ciliata*	Brisbane, Australia	Stopford, 2000 (EN#5)

TABLE 1 Continued

Tetraodontidae	*Tetractenos hamiltoni*	Brisbane, Australia	Stopford, 2000 (EN#5)
Tetraodontidae	*Tetractenos pleurostictus*	Brisbane, Australia	Stopford, 2000 (EN#5)
Agryosomatidae	*Agryosomus japonicus*	Port Stephens, Australia	Fielder, 1999
Latidae	*Lates calcarifer*	Experimental	Roberts-Thomson, 2006
Sparidae	*Sparus auratus*	Eilat, Red Sea	Paperna, 1984 (EN#39)
Capsalidae	*Neobendenia melleni*	Red Sea	Colorni, 1994 (EN#3)
Sparidae	*Sparus aurata*	Eliat, Red Sea	Paperna, 1984
Cichlidae	*Oreochromis mossambicus*	Salton Sea, United States	Kuperman, 1999 (EN#25)
Rachycentridae	*Rachycentron canadum*	Taiwan	Liao, 2004
	Trachinotus carolinus	Mississippi, United States	Lawler, 1977a (EN#93)
	Sciaenops ocellatus	Texas, United States	Oestmann, 1995 (EN#10)
	Sciaenops ocellatus	Experimental	Lewis, 1988 (EN#32)
Pomatomidae	*Pomatomus saltatrix*	New York Aquarium	Nigrelli, 1936
Carangidae	*Caranx hippos*	New York Aquarium	Nigrelli, 1936
Carangidae	*Caranx crysos*	New York Aquarium	Nigrelli, 1936
Carangidae	*Trachinotus falcatus*	New York Aquarium	Nigrelli, 1936
Carangidae	*Naucrates ducator*	New York Aquarium	Nigrelli, 1936
Pomatomidae	*Roccus lineatus*	New York Aquarium	Nigrelli, 1936
Pomatomidae	*Centropristis striatus*	New York Aquarium	Nigrelli, 1936
Sparidae	*Stentomus chrysops*	New York Aquarium	Nigrelli, 1936
Sciaenidae	*Cynoscion regalis*	New York Aquarium	Nigrelli, 1936
Scianidae	*Leiostomus xanthurus*	New York Aquarium	Nigrelli, 1936
Sciaenidae	*Menticirrhus saxatilis*	New York Aquarium	Nigrelli, 1936
Triglidae	*Prionotus carolinus*	New York Aquarium	Nigrelli, 1936
Triglidae	*Prionotus evolans*	New York Aquarium	Nigrelli, 1936
	Chaetodipterus faber	New York Aquarium	Nigrelli, 1936
Pomacanthidae	*Pomacanthus paru*	New York Aquarium	Nigrelli, 1936
	Fundulus heteroclitus	New York Aquarium	Nigrelli, 1936

TABLE 2

List of Sequences

Isolate	Origin	Host/ Culture	Reference (Accession number)
AO1	Port Stephens, Australia	Culture	Present study
PSFC	Port Stephens, Australia	*Macquaria novemaculeata*	Present study
LUA	Mississppi sound, USA	Culture	Present study
DC1 1	Aquarium specimen	Culture	Levy et al, 2007 (AF352359)
DC1 2	Aquarium specimen	Culture	Levy et al, 2007 (AF352360)
DC1 3	Aquarium specimen	Culture	Levy et al, 2007 (AF352361)
DC1 4	Aquarium specimen	Culture	Levy et al, 2007 (AF352362)
Med Sea 1	Mediterranean coast, Israel	Culture	Levy et al, 2007 (DQ490264)
Med Sea 2	Mediterranean coast, Israel	Culture	Levy et al, 2007 (DQ490265)
Med Sea 3	Mediterranean coast, Israel	Culture	Levy et al, 2007 (DQ490266)
Med Sea 4	Mediterranean coast, Israel	Culture	Levy et al, 2007 (DQ490267)
Red Sea 1	Gulf of Eilat, Israel	Culture	Levy et al, 2007 (DQ490268)
Red Sea 5	Gulf of Eilat, Israel	Culture	Levy et al, 2007 (DQ490269)
Red Sea 6	Gulf of Eilat, Israel	Culture	Levy et al, 2007 (DQ490270)
Red Sea 7	Gulf of Eilat, Israel	Culture	Levy et al, 2007 (DQ490271)
Adriatic Sea 2	Italy	Culture	Levy et al, 2007 (DQ490261)
Adriatic Sea 4	Italy	Culture	Levy et al, 2007 (DQ490262)
Adriatic Sea 7	Italy	Culture	Levy et al, 2007 (DQ490263)
Gulf of Mexico 16	Florida, USA	Culture	Levy et al, 2007 (DQ490259)
Gulf of Mexico 21	Florida, USA	Culture	Levy et al, 2007 (DQ490260)

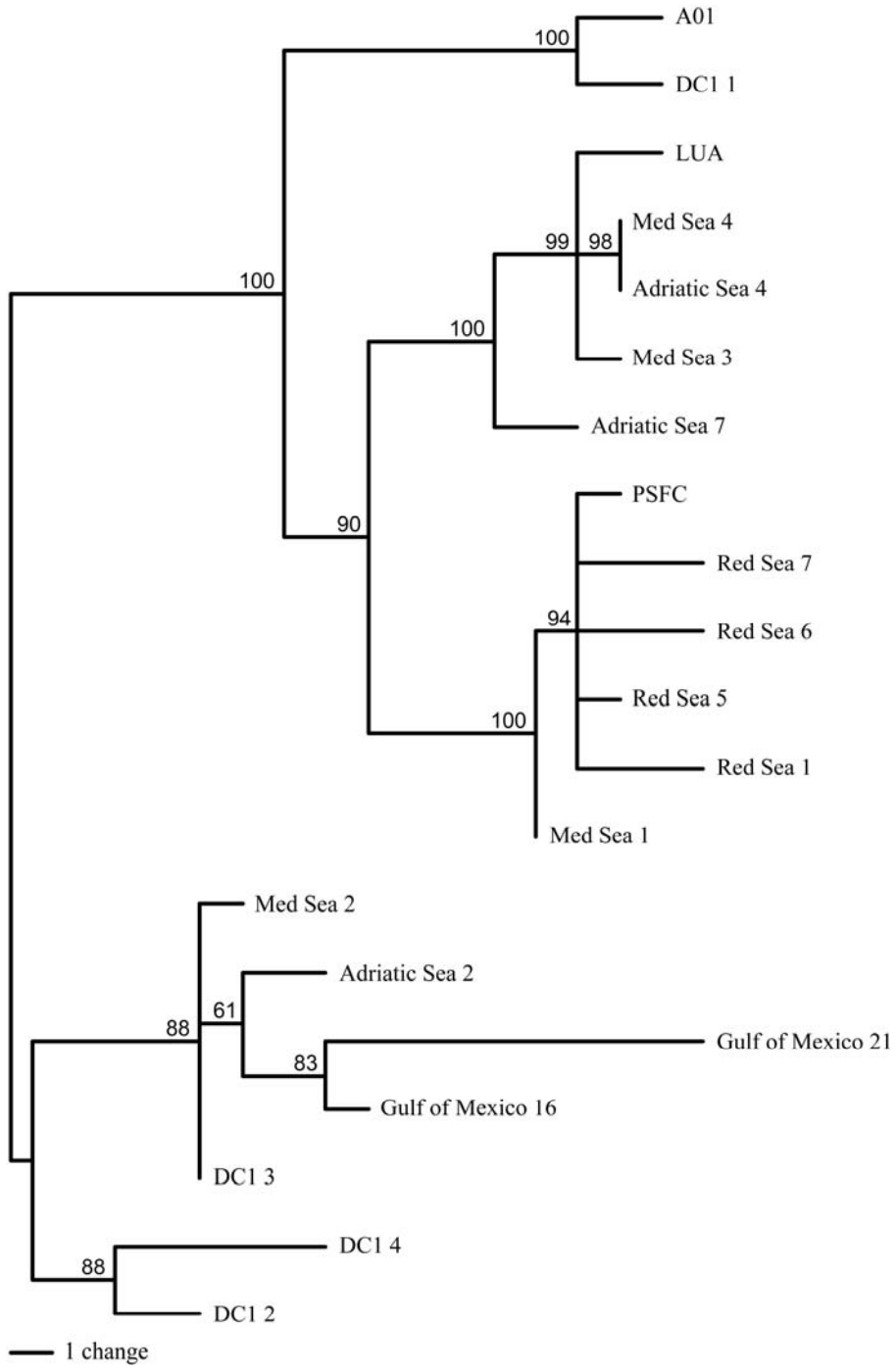

FIGURE 1

L/Bayesian

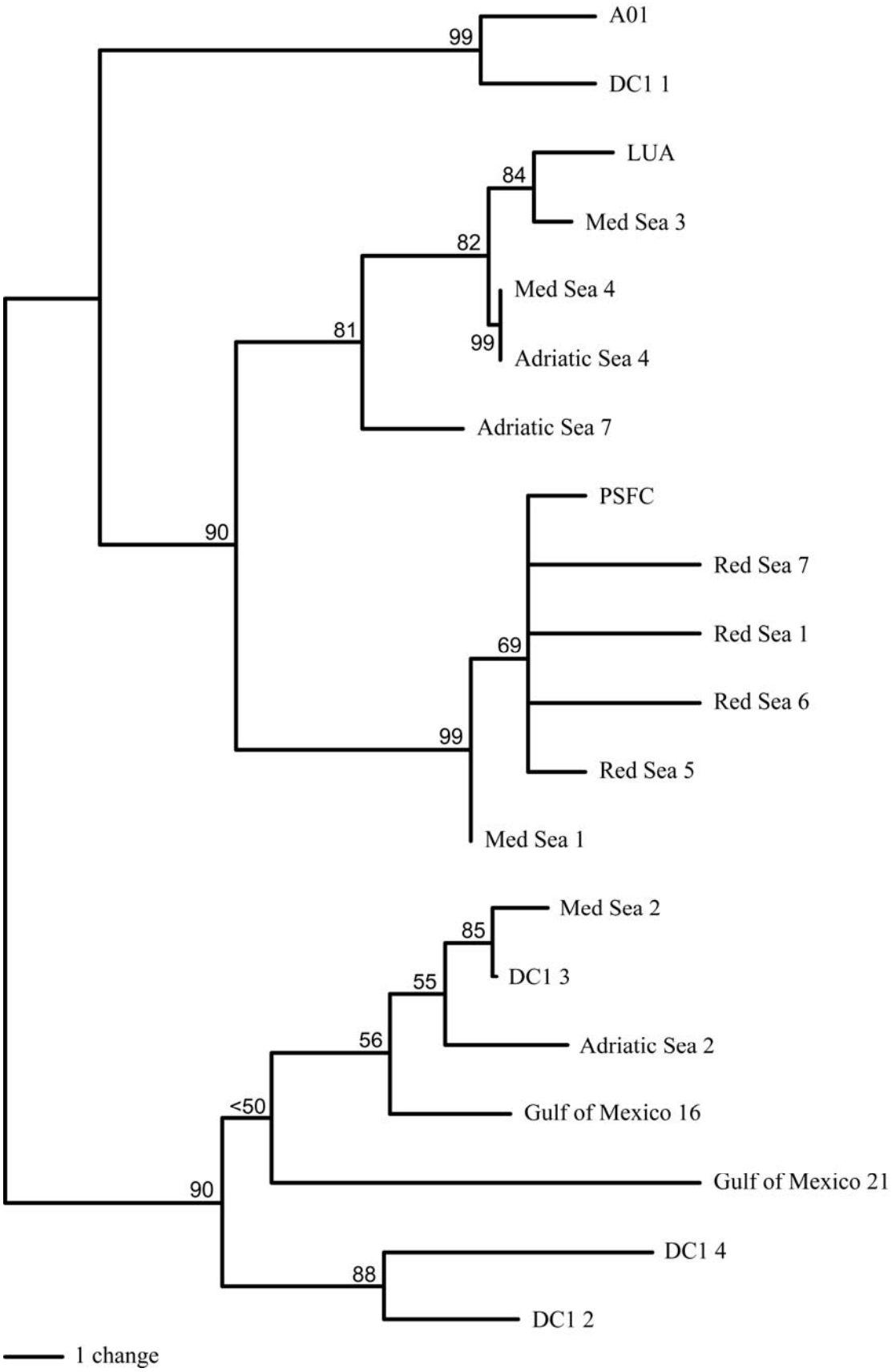

FIGURE 2

Minimum evolution tree

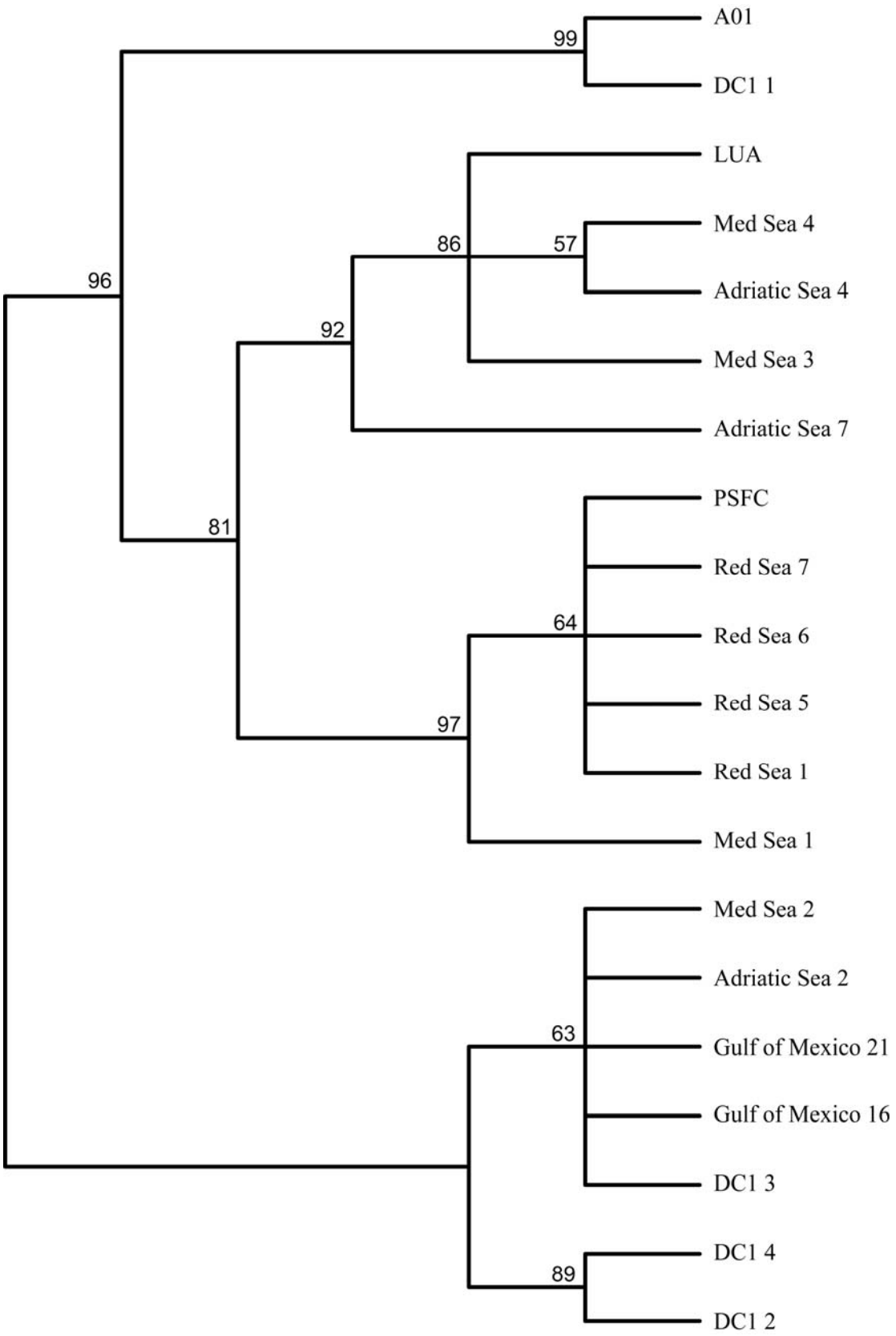

FIGURE 3

Consensus tree maximum parsimony

4.11 Aerosol dispersal strategies of the fish pathogen, *Amyloodinium ocellatum*

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ABSTRACT

Amyloodinium ocellatum, a frequently encountered parasite in marine aquaculture, was investigated to determine if infective dinospore stages could be transported in aerosol droplets. We used an *in vivo* model incorporating static and dynamic airflow systems and found dinospores of *A. ocellatum* could travel in aerosol droplets: up to 440 mm in a static system and up to 3 m in a dynamic one. This is a novel transmission pathway for a marine protozoan parasite. It is possible that other marine protozoans can transfer via the aerobiological pathway. Management of *A. ocellatum* infections in aquaculture facilities could be affected, particularly where tanks and ponds are situated in close proximity.

1. INTRODUCTION

Amyloodinium ocellatum Brown, 1931, Oodinidae, is considered a ubiquitous, protozoan parasite found in tropical and subtropical marine waters. It is currently the only known representative of this genus. The life stages of *A.ocellatum* include a trophont that attaches to and feeds on fish gill and epithelial tissue, a tomont that undergoes division on the substrate and a free-swimming, infective dinospore (Brown & Horvasse, 1946).

Amyloodinium ocellatum infections in wild fish populations rarely cause mortality or even present symptoms of acute amyloodiniosis because the dilution factor in the wild prevents repeated infections of the same host individual (Kuperman & Matey, 1999; Lawler, 1980). Fish maintained at high densities, such as those in cage or pond aquaculture systems, are constrained to a finite area, providing ideal conditions for fulminating infections once *A. ocellatum* has been introduced into the system. Land based culture facilities can also be significantly affected by *A. ocellatum* (Fielder & Bardsley, 1999). Inadequate quarantine and biosecurity procedures are typically blamed for parasites gaining access to land based facilities. Entry pathways probably include introduced broodstock, food or through inefficiently filtered influent water.

Of the possible methods of entry into aquaculture systems, one pathway that has generally been overlooked is via aerosol. Common fish pathogens such as the ciliate *Ichthyophthirius multifiliis* Fouquet, 1876 and the bacterium *Aeromonas salmonicida* Emmerich & Weibel, 1894 have been found to disperse in aerosols generated from infected water (Bishop *et al.* 2003; Wooster & Bowser, 1996).

The aerobiological pathway consists of launch, aerial transport and deposition (Bishop, *et al.* 2003). Natural aerosol droplets can range in size from 0.5µm up to 100µm in diameter Bishop, *et al.* 2003. Droplets larger than 100 µm require strong air currents to carry them any distance Bishop, *et al.* 2003. Persistence of natural aerosol particles is strongly dependent on ambient temperature and relative humidity at the time of aerial transport. These factors allow aerosol dispersal of parasites over only small distances in static systems as they significantly affect the internal environment of the aerosol rendering the pathogen non-viable through either dessication or hyper-salinity (Bishop, Smalls, Wooster & Bowser, 2003; Wooster & Bowser, 1996). For *I. multifiliis* this distance has been found to be up to

91.4cm in a static airflow experiment Bishop, *et al.* 2003. The infective stage (theront) of *I. multifiliis* measures approximately 30x50 µm (Dickerson & Dawe, 1995), while the diameter of dinospores of *A. ocellatum* are significantly smaller (range from 11.6 –15.4 µm x 10.4- 14.5 µm (Lom & Dykova, 1992)), suggesting that on this parameter alone, *A. ocellatum* is a prime candidate for aerosol transport.

Here we examine the ability of *A. ocellatum* dinospores to disperse via aerosols and determine the effective range in static and dynamic systems using an *in vivo* model.

2. MATERIALS AND METHODS

2.1 Assessment of infection

Trophonts of A. ocellatum detach from host marine fish within minutes when exposed to freshwater (Montgomery-Brock, Sato, Brock & Tamaru, 2001). Infection of experiment fish with *A. ocellatum* was assessed by bathing fish in fresh water for 5 minutes and passing the bath contents through a 47µm screen. Captured tomonts were placed into a Petri dish in clean seawater and counted. Tomonts were then monitored over a 24 hour period to determine whether division occurred, as a measure of viability (Paperna, 1984a).

2.2 Fish

Fish used in aerosol experiments were juvenile barramundi, *Lates calcarifer* Bloch, 1790 of 5-11cm in total length obtained from a commercial barramundi growout facility (Barramundi Australia, Brisbane). Barramundi were used because they are euryhaline and thus could be sourced from freshwater farms, able to be fed commercial pellet diet, and easy to maintain in captivity. This system provided a ready supply of fish which were not infested with *A. ocellatum* (naïve). Confirmation that experiment fish were not infested with *A. ocellatum* prior to experimentation, was achieved by bathing fish in fresh water for five minutes and then observing the fish behaviour for 30 minutes. At no time did barramundi in the experiment tanks exhibit typical flashing behaviour indicative of infection (Montgomery-Brock, *et al.* 2001) nor did fresh water bathing yield tomonts of *A. ocellatum* before the commencement of any trial. Fish were acclimatised to filtered (10 um?) seawater (35ppt) in experiment tanks for 3 days before commencement of each trial. At the end of each trial experiment tanks were washed with fresh water and allowed to dry completely prior starting the next trial.

2.3 Pathogen

Amyloodinium ocellatum tomonts were originally collected from Juvenile snapper *Pagrus auratus* Forster, 1801 at the New South Wales Department of Primary Industries, Port Stephens Fisheries Centre (PSFC) then transported in bags containing oxygen and seawater to the experiment aquarium system at the University of Queensland Veterinary Science Farm, Pinjarra Hills. An *in vivo* infection was then maintained in two 100L, aerated, plastic tanks, which were each stocked with three teleost fish of either *Acanthopagrus australis* Gunther, 1859 (Sparidae), *Sillago ciliata* Cuvier, 1829 (Sillaginidae) and *Tetractenos hamiltoni* Richardson, 1846 (Tetraodontidae). Approximately 25% of the tanks volume was exchanged each week with filtered seawater. Infected fish were maintained in an infection room which was discrete from the experiment room and strict quarantine procedures including foot baths, no equipment transfer and hand washing, were put in place to minimise possible cross contamination of the experiment room. The experiment and infection room were both maintained at 19°C-20°C with a relative humidity of 75%-80%. *A. ocellatum* tomonts were obtained for experimental use by removing infected fish from the 100 L tanks and placed into 10 L tanks filled with fresh water for 5 minutes to detach parasites from body surfaces. Collected bath water was then passed through a series of 150 µm and 47 µm nylon filter screens (nytex) to remove organic debris and collect tomonts, respectively. Tomonts were transferred to a Petri dish containing seawater at room temperature and held for 3 days until sporulation and dinospore emergence.

2.4 Static Airflow Trials

Six replicated trials were undertaken in 10 L aquaria filled with filtered seawater to 200 mm from the top to determine the effect of aerosol on transfer of A. ocellatum under windless (static) conditions (Figure 1). Tanks were placed in a spiral arrangement increasing in distance from the aerosol source. Experiment tanks were not aerated in order to stop secondary aerosol generation. Two *A. ocellatum* naïve *L. calcarifer* were placed into each of the eight experiment tanks (0, 110, 180, 270, 440, 560, 720 and 950 mm from aerosol source). To minimise direct tank-to-tank contamination from fish splashing, tanks were half filled with seawater. The tank system was completely enclosed within a level, rectangular box (2 m x 1 m x 0.5 m) made from opaque fibreglass sheeting to minimise the influence of extraneous air movement within the system.

In each trial approximately 100 000 dinospores were placed into a dispersing tank (Figure 1) filled with seawater. The number of dinospores was estimated by allowing 500 tomonts from the *in vivo* infection to sporulate then assuming that 256 dinospores (Brown & Hovasse, 1946) emerged from the majority of tomonts. In the first three trials the aerosol was created by diffusing air through an air stone with an air pump at L/min, which was placed on the bottom of the dispersal container. The diffuser ran continually for the first three days of each trial and was then turned off. The static system was then maintained for a further three days before the status of infection of each fish was assessed allowing for any infection to build within the individual experiment tanks if present. After a further 3 days another infection assessment was undertaken to confirm that any low intensity infections were not overlooked.

Trials four to six used a modified aerosol dispersing apparatus. A misting nozzle was fitted to a pressurised garden sprayer (Pope Australia Pty Ltd). The canister was filled with 2L of seawater and 100 000 dinospores added. The canister was pressurized using the hand pump to deliver the whole volume through the spray nozzle in 2 mins (i.e. 1 L/min flow rate at nozzle). All other parameters were identical to those used in trials 1-3.

2.5 Dynamic Airflow Trials

A dynamic airflow model was used in 3 replicated trials (Figure 2) to determine the effect of aerosol on transfer of *A. ocellatum* under dynamic airflow conditions. Five experiment tanks (described for trials 1-6) were placed at 1 m intervals, in a single line (1, 2, 3, 4 and 5 m) from a pressurised canister (as described for trials 4-6) filled with 100,000 dinospores. A pedestal fan of 1 m elevation was placed 60 cm behind the aerosol source, which was also elevated to 1m. This configuration minimised downward air currents while allowing upward and horizontal airflow past the aerosol nozzle thus maximising the distance that droplets were broadcast. The fan was run until all liquid was sprayed from the pressurised canister containing the source of dinospores, after which the system was returned to stasis. Tanks were half filled to reduce the possibility of splash contamination and spaced such that the distance was greater than the maximum travelled distance of infection in the static trials. Assessment of infection occurred after 6 days followed by a second screening at 9 days.

3. RESULTS

3.1 Static Airflow

Trials one to three, in which a diffuser was used as the aerosol source showed no transfer of infection. Tanks were allowed to stand for up to 12 days to allow potential infections to propagate to detectable levels. However, no *A. ocellatum* infection was detected using freshwater baths and none of the fish displayed any behavioural signs consistent with infection.

In trials four to six, fish in tanks up to 440 mm from the aerosol source were infected with *A. ocellatum.* Tomont numbers collected from the varying tank distances are shown in Table 1. The intensity of infection decreased as distance from the aerosol source was increased.

3.2 Dynamic Airflow

All trials demonstrated infection transferring into all tanks up to and including 2 m from the aerosol source, albeit at a low intensity (Table 2). However, Trial 8 yielded a single dinospore from one barramundi in a tank 3 m from the source of infection. Intensity of infection decreased as distance was increased.

4. DISCUSSION

Our results demonstrate that *Amyloodinium ocellatum* dinospores are capable of travelling in aerosol droplets and infecting fish to a distance of at least three metres from their origin. This is only the second protozoan fish pathogen found capable of transferring infection via this pathway Bishop, *et al.* 2003. More importantly it is the first marine species found capable of such an infection pathway. Aerosols derived from seawater can be considered a hostile environment due to a rapidly increasing salt concentration as evaporation and droplet volume reduction occurs (Song, 1999). However, the broad environmental tolerance of *A. ocellatum* is well recognised, in particular salinity tolerances from 1ppt to 60ppt in which motile dinospores have been observed (Paperna, 1984b) would contribute significantly to maintaining viability of this pathogen and allowing it to exploit such a transmission pathway. It should be noted that little information exists on aerosol transmission of other aquatic pathogens but those with broad halotolerance or those that possess environmentally resistant stages, should be considered potential candidates for such a pathway.

Identification of this transmission pathway may explain why *A.ocellatum* and other infections are able to bypass even the strictest biosecurity protocols, which focus typically on transmission through either contaminated seawater, equipment or staff. The need for marine fish hatcheries to maximise fish production often results in increased culture intensity and placing ponds or tanks close together in order to fit more in to a given space is a common scenario. The demonstrated distances in our experiments are certainly enough for infection transfer to occur in closely placed tanks or ponds.

No transfer of infection occured using air diffusers and may be due to air bubble size or aerosol particle size. Air bubble size may have been too large for sufficient aerosol generation. If aerosol particles generated from these bubbles were in the larger part of the range then they may not have been able to carry far enough from the aerosol source. The pressurized canister was able to deliver all the dinospores into roughly uniform particles and dispersed them over a short period of time.

For obvious reasons, many aquaculture facilities are located close to a body of seawater. Often they consist of a number of outdoor ponds, roofed tanks, polyhouses and hatchery sheds. Most of these structures do not provide barriers to airborne infection and are thus at risk of within-facility parasite distribution.

At the Port Stephens facility from which the *A. ocellatum* was originally sourced, broodstock are maintained in an outdoor pond in an unfiltered flow through system using estuarine water. Examination of fish from this pond revealed no physical signs of disease. However, it is possible that a latent infection below detectable levels exists within this pond. Ponds are only 1-2 metres apart, well within distances that aerosols of *A. ocellatum* dinospores were found to travel under experimental conditions reported in our study. Zooplankton blooms are often present and can lead to a foamy build-up on pond surfaces. All ponds are constantly aerated and also exposed to prevailing winds, which regularly reach up to 30 knots. These circumstances provide ideal conditions for infection transfer via aerobiological pathways. Ponds are used for juvenile grow out and experimental work. After use they are drained and the liners are allowed to dry in attempts to kill any infection present previously. While biosecurity in ponds is unable to be as strict as in the hatchery, all equipment is chlorine sterilised after each use and transfer of animals between ponds rarely occurs. Despite this, recurrent infections of *A. ocellatum* still occur, lending support to our hypothesis that *A. ocellatum* is transferred by aerosol. The likelihood that infections are being transferred from pond to pond is high. Unfortunately, ponds are particularly hard to treat due to large volumes and high associated treatment costs.

5. CONCLUSION

This study has identified aerosols as a novel transmission pathway for a marine protozoan pathogen. This result shows that traditional biosecurity measures may be inadequate to exclude this pathogen from aquaculture facilities and certainly inadequate to control spread within a facility. Control measures for *A. ocellatum* should include minimisation of airflow between tanks in hatcheries and broodstock facilities where possible.

Grow-out ponds are likely to be susceptible to contamination via aerosols. High levels of aeration, zooplankton blooms that form foamy build-ups on pond surfaces combined with strong winds across ponds make transfer between ponds likely. Airflow in these situations is uncontrollable. Similar parasites should be subjected to this testing to identify the extent to which aerosol contamination occurs.

ACKNOWLEDGEMENTS

The authors wish to thank G. Wooster and P. Bowser for their comments on experimental design, T. Cribb and M. Nolan for their critical review of this manuscript. This study was part of the Cooperative Research Centre for Sustainable Finfish Aquaculture (Aquafin CRC) snapper project 2001/208 with financial support from the Aquafin CRC, Fisheries Research and Development Corporation and University of Queensland gratefully acknowledged.

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TABLE 1

Aerosol dispersal of *Amyloodinium ocellatum* to infect barramundi in a static airflow system. Data indicates the number of tomonts recovered by freshwater bathing both fish at each distance.

	Tomonts Recovered		
Distance (mm)	Trial 4	Trial 5	Trial 6
0	89	110	107
110	80	62	74
180	14	35	28
270	22	8	8
440	5	3	0
560	0	0	0
720	0	0	0
950	0	0	0

TABLE 2

Aerosol dispersal of *Amyloodinium ocellatum* to infect barramundi in a dynamic airflow system. Data indicates the number of tomonts recovered after freshwater bathing both fish at each distance.

	Tomonts Recovered		
Distance (m)	Trial 7	Trial 8	Trial 9
1	9	17	15
2	8	7	8
3	0	1	0
4	0	0	0
5	0	0	0

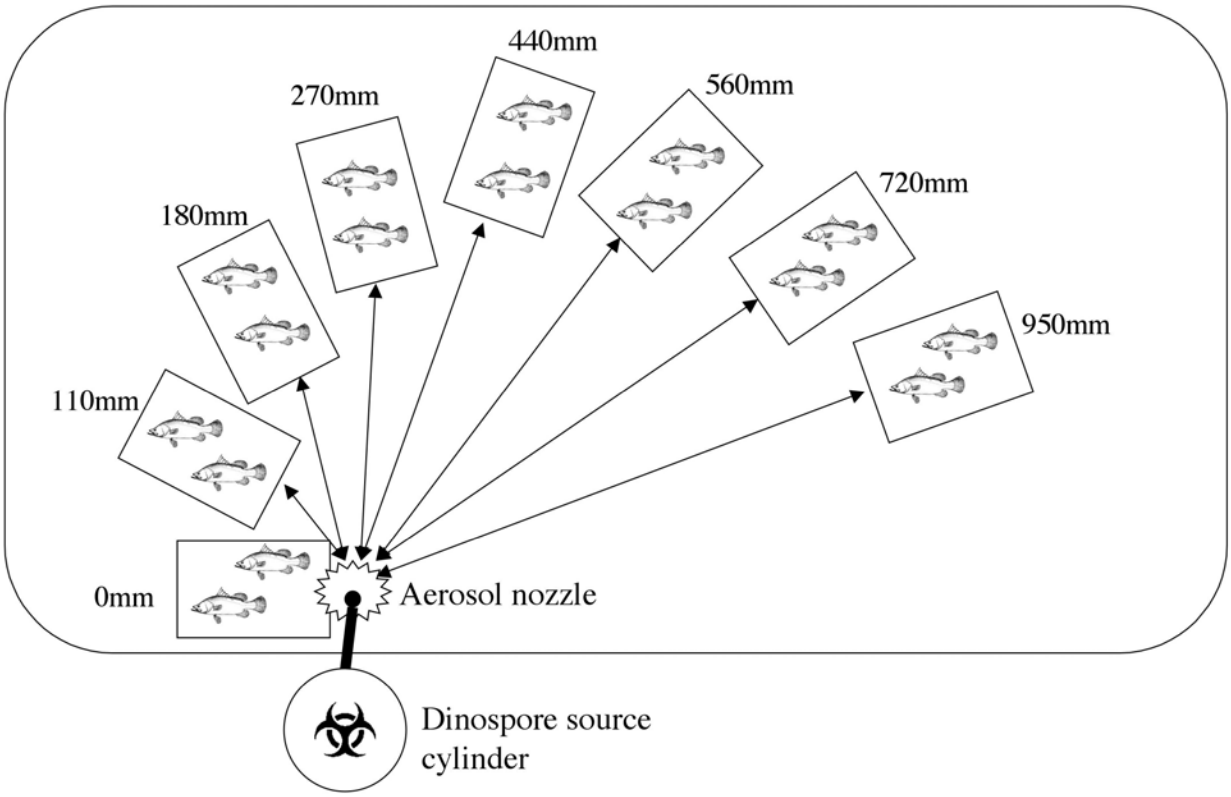

FIGURE 1

Overhead view of static airflow experimental design. Arrows indicate the closest distances of tanks to the aerosol source.

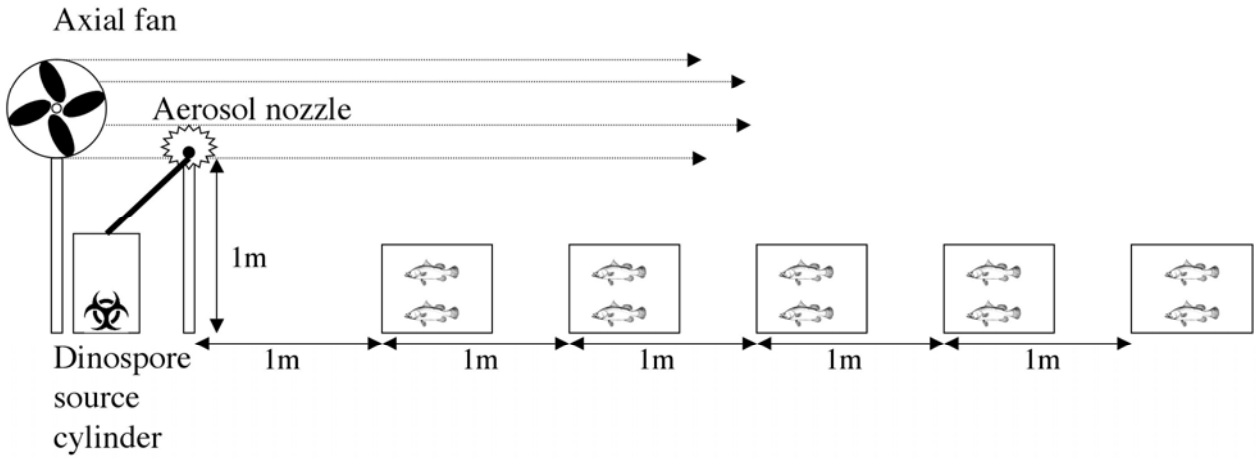

FIGURE 2

Dynamic airflow experimental design. Dashed lines indicate air currents created by the pedestal fan.

4.12 Detection of the fish pathogen, *Amyloodinium ocellatum* using PCR based diagnostic assays

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1. INTRODUCTION

Amyloodinium ocellatum is a dinoflagellate parasite commonly found in marine aquaculture facilities. The life cycle comprises three distinct stages. A feeding stage called a trophont attaches to host epidermal cells and feeds on the host. The trophont detaches and forms an environmentally resistant cyst stage known as the tomont. The tomont stage undergoes palintomic division up to 8 times and then releases up to 256 free-swimming, host-seeking dinospores. The life cycle can be completed in as little as 6 days, with feeding on the host for three days and division on the substrate for three days, under optimum conditions. The parasite is able to reproduce in salinities from 1ppt to 78ppt and temperatures from 15°C to 35°C (Paperna, 1984b), making it incredibly resilient with regard to environmental fluctuations, often more so than its host.

Closed culture systems with high stocking densities are particularly affected by *A. ocellatum* infections. Due to the proliferative nature of the life cycle, infection intensity can build quickly in closed systems, causing significant mortality events. Large numbers of trophonts on the gills may compromise the hosts ability to osmoregulate and can lead to secondary bacterial infections. Signs of *A. ocellatum* infection include flashing, rapid opercular ventilation and loss of appetite. However, none of these are exclusive indicators. Indeed, numerous infections in aquaculture present similar signs (e.g. *Cryptocaryon irritans*, *Vibrio* sp., monogeneans) and even poor water quality can lead to similar behaviour.

Prior to 2007, diagnosis of *A. ocellatum* relied on microscopy. This remains useful when examining gills of infected fish. However, without utilising a fast acting fixative (e.g. Bouin's fixative) on samples, trophonts may retract their rhizoids, drop from the host tissue and form the cyst-like tomont soon after host death. Tomonts are character poor with respect to morphology, however, with the onset of palintomic division, the dividing form is more recognisable. Nevertheless, up to 24 hours away from the host is necessary before this is visible. The dinospore life-stage does offer numerous morphological characters. However, it is analogous to other free-living dinoflagellate forms and are difficult to distinguish from these species under light microscopy. Further examination using SEM and a technique called 'suture swelling' to reveal the thecal plate tabulation (Landsberg, Steidinger, Blakesley & Zondervan, 1994) of the dinospore was helpful in diagnosis of *A. ocellatum*. All the above methods of diagnosis can be too slow to enable effective use of *A. ocellatum* specific chemotherapeutics and management practices.

PCR approaches to diagnosis of protozoan pathogens have proven rapid and sensitive in numerous instances (Carnegie, Barber, Culloty, Figueras & Distel, 2000, Kleeman & Adlard 2000, Grueble, Frischer, Sheppard, Neumann, Maurer & Lee, 2002). Their usefulness is not limited by life-stage and their sensitivity surpasses traditional microscopy. The precision and suitability of PCR-based diagnostics for *A. ocellatum* is already established (Levy, Poore, Colorni, Noga, Vandersea & Litaker, 2007). Correct design of probes and primers as well as thorough testing for cross-reaction mean that that PCR diagnostic can quickly and accurately provide information enabling decisions on the best method of managing *A. ocellatum* in aquaculture facilities.

In this chapter, a PCR diagnostic based on the ITS 2 region of the rDNA of *Amyloodinium ocellatum* is presented and its applications are discussed.

2. MATERIALS AND METHODS

2.1 Specimen collection

Amyloodinium ocellatum was isolated from Australian Bass, *Macquaria novemaculeata,* brood-stock, held at Port Stephens Fisheries Centre (PSFC). This isolate was subsequently cultured *in vivo* at the University of Queensland for a period of 12 months using barramundi, *Lates calcarifer*. Additional cultured isolates were obtained from laboratories in the USA. One isolate from Louisiana, isolated from *Mugil cephalus* in the Gulf of Mexico, USA and one collected from the Red Sea, Israel.

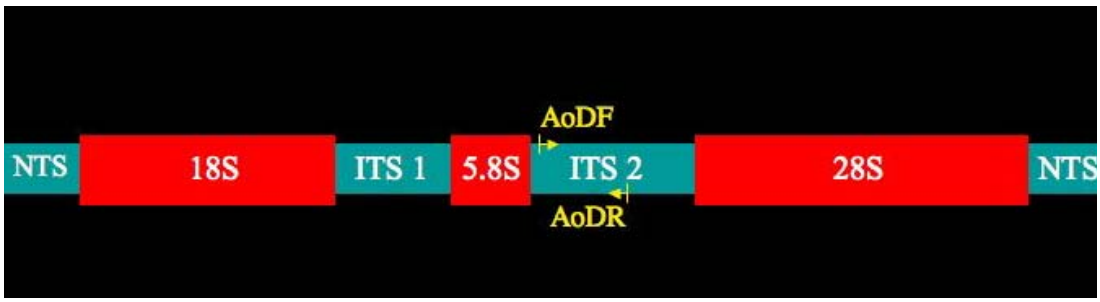
Isolate	Parasite	Origin	Host/ Culture
AO1	*Amyloodinium ocellatum*	Port Stephens, Australia	Culture
PSFC	*Amyloodinium ocellatum*	Port Stephens, Australia	*Macquaria novemaculeata*
LUA	*Amyloodinium ocellatum*	Mississippi sound, USA	*Mugil cephalus*
RS	*Amyloodinium ocellatum*	Red Sea, Israel	Culture
CA1	*Crepidoodinium australe*	Moreton Bay, Australia	*Sillago ciliata*
CA2	*Crepidoodinium australe*	Moreton Bay, Australia	*Tetractenos hamiltoni*
S. inniae	*Streptococcus inniae*	Bowen, Australia	*Lates calcarifer*
DNA2	*Kudoa* sp.	Moreton Bay, Australia	*Abudebduf bengalensis*

2.2 DNA extraction

DNA of tomonts from each of the isolates was extracted using DNeasy tissue kit (QIAGEN) according to manufacturers instructions and then eluted into 100µl ultra pure water.

2.3 Primer synthesis

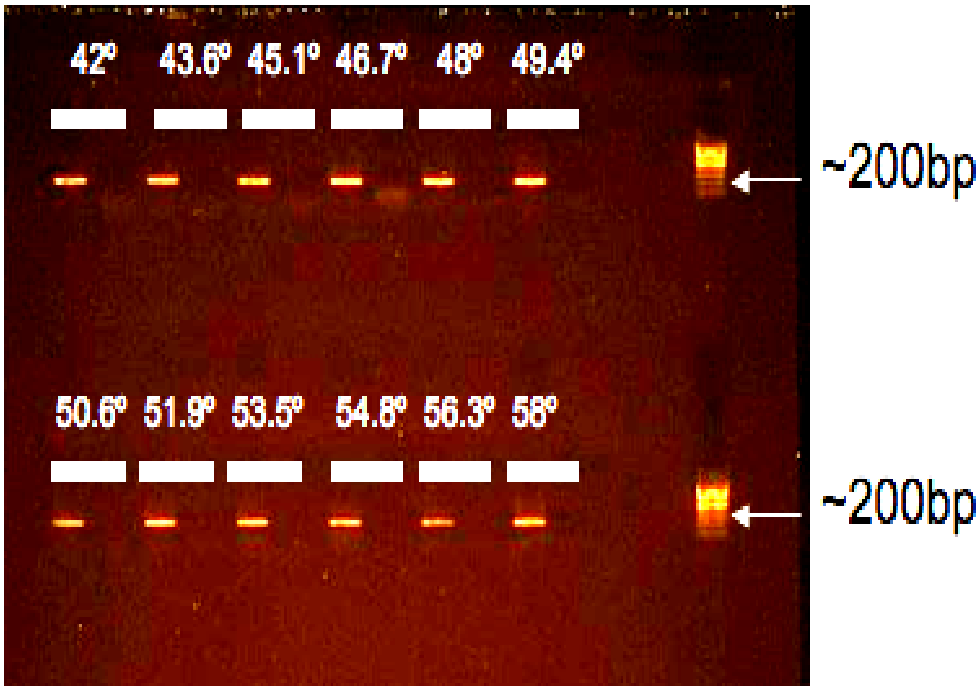
Diagnostic primers were designed based on PSFC isolate sequences and published sequences of the cultured isolate, DC-1, using the web-based software Primer3 (Rozen & Skaletsky 1996). Primers were positioned approximately 200bp downstream from the 5' end of the *A. ocellatum* ITS 2 region. The primer sites were chosen because they were homogenous between clones AF352362, AF352361, AF352360 and AF352359 (Genbank) and the PSFC isolate. BLAST searches of the individual primers were performed to explore possible cross reactivity. These primers were designated AoDF (5'-tcgtgttacgcacctgagtc – 3') and AoDR (5' - gttaattgcgaggaggagga - 3'). Amplified fragment size was ~200bp.



2.4 PCR

Extracted DNA was amplified by polymerase chain reaction (PCR) in 25µl volumes using 2U Hotmaster taq (eppendorf), 10µM dNTP, 3.2µM of each primer, 1x Hotmaster taq buffer (with Mg^{2+}) and approximately 100ng template DNA. Cycling parameters were optimised by running a sample of PSFC isolate DNA in a gradient thermocycler (Corbett Research, PCR gradient thermocycler). Annealing temperatures were incrementally increased over 12 wells from 42° C to 58° C. Due to Mg^{+} concentration being automatically optimised through the buffer mix, a MgCl gradient was not necessary

in the optimisation of the protocol. Validation PCRs were run under the following cycling conditions (using a Corbett Research, PCR thermocycler): initial denaturation 94°C for 2min then 35 cycles at 94°C for 20s, 48°C for 10s and 65°C for 30s followed by a final extension at 65°C for 5 min.



2.5 PCR sensitivity

Sensitivity of the PCR diagnostic was assessed by serial dilution of extracted rDNA of the PSFC isolate from a known quantity of tomonts and a known quantity of dinospores. Quantification was performed using a haemocytometer. Tubes containing dinospores and tomonts and then made up to 2 ml with ethanol were vortexed to homogenise the parasite. A pipette was then used to remove 50 µl of the sample which was placedin a haemocytometer. After three repeats of this procedure, the average of the three counts was multiplied by a factor of 40 to give an estimate of parasite concentration in each tube. The amount of solution necessary to give 1000 dinospores and 100 tomonts was then placed into tubes. Extractions were performed using a DNeasy extraction kit and eluted into 100 µl of ultrapure water. One in ten dilutions were then performed to achieve the desired amount of dinospore and tomont DNA equivalents, for tomonts 10, 1, 0.1, and for dinospores 100, 10, 1, 0.1, 0.01). PCR was then performed using the specific primers (AoDF and AoDR).

2.6 PCR specificity

The diagnostic PCR was tested for cross reactivity against the following species: *Streptococcus inniae* (bacteria); *Lates calcarifer* (teleost); *Crepidoodinium australe* (dinoflagellate); and *Kudoa* sp. (Myxosporea) using the same extraction proceedures as *A. ocellatum* isolates. All specificity testing was performed using the same optimised PCR conditions described earlier.

3. RESULTS

3.1 Specificity

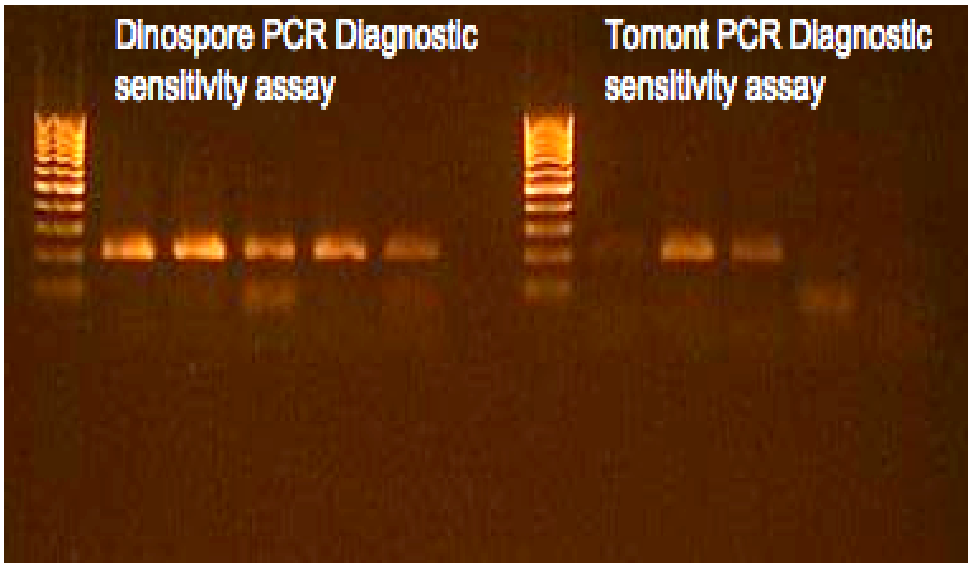
The optimal annealing temperatures for AoDF and AoDR primers proved to be 48° C. This selection was based on allowing sufficient stringency in the reaction to maintain host specificity but to retain

amplification of minor variations in sequence. All BLAST searches of primer site sequence confirmed *A. ocellatum* as the highest matching score.

Even in optimal conditions for PCR, there was no amplification of any other species tested. *Crepidoodinium australe*, a co familial member of the Oodinaceae was the closest available species to *A. ocellatum* taxonomically and yet was not amplified using *A. ocellatum* specific primers.

3.2 Sensitivity

PCR amplification was successful at all dilutions (100-0.01 equivalent to 100- 0.01 dinospores) in the dinospore assay. Band intensity decreased with increasing dilution. However, the tomont assay was not as sensitive, being unable to detect the highest dilutions. The 100 tomont equivalent reaction showed only very faint amplification. The 10 and 1 tomont DNA equivalent reactions showed banding, which did decrease with increasing dilution. However, the 0.1 tomont DNA equivalent reaction did not reveal any amplification.



4. DISCUSSION

Molecular detection of *Amyloodinium ocellatum* is potentially a more useful means of identification than current histological methods. The PCR diagnostic was able to detect unambiguously as little as 0.1 dinospore DNA equivalents and 1 tomont DNA equivalents.

The lack of reactivity in the 100 tomont equivalents may be due to higher concentrations of PCR inhibitors, such as starches, being present in the tomont. Other extraction methods which remove inhibitors could overcome such problems. Indeed, a soil kit is used in the other published *A. ocellatum* PCR diagnostic (Levy *et al.* 2007).

The lack of amplification in the 0.1 tomont DNA equivalent reaction indicates the limit of detection for that life stage. First stage tomonts were purposely chosen for this study because they would have the least DNA. Tomont diagnostic results indicate an absolute minimum to the detection limits, when compared to facility samples that would contain tomonts in varied stages of division. Additionally, the process of division uses up energy reserves in the cell, reducing the amount of inhibitors in the cell. This is very helpful for a diagnostic, however, if the results are used to quantify tomonts, these confounding factors must be accounted for before quantification is assumed accurate. Moreover, using the rDNA region in quantification also assumes homogeny of the number of times that this is repeated within the genome. Actually, the number of repeats of rDNA can vary greatly between cells (Levy, 2007), confounding efforts at quantification. Even with methods more suited to quantification, (e.g. real-time PCR) care would be necessary to choose a suitable region of the genome and targeting of a suitable life stage of the parasite to make the test accurate.

The diagnostic assay presented here is sensitive and specific to *A. ocellatum*. Positive results were returned from testing available isolates against this diagnostic. Unlike the other diagnostic published (Levy *et al*. 2007), this study presents a test from the ITS 2 region of the A. ocellatum genome. The ITS 2 is recognised to be more genetically variable than the18S rDNA region (Litaker, 2007). To this end, a number of different ITS 2 alleles are show to be present across the global distribution of *A. ocellatum* (see Section 4.9). Situating a diagnostic within a variable region may offer added advantages by discerning intra-genomic or inter-specific variation. A number of methods could be employed to exploit variation within the diagnostic region. These include restriction fragment length polymorphism (RFLP) and single strand confirmation polymorphism (SSCP), both of which can be undertaken after a positive result is found in the initial diagnostic. Alternatively, performing a multiplex real-time PCR assay with the addition of multiple probes situated on the variable regions within the diagnostic fragment of *A. ocellatum* could not only give a positive or negative result, but also allelic discrimination and even dominant allele determination.

Rapid and accurate diagnostics are a necessary step in marine fish hatcheries to allow management of *A. ocellatum*. The sensitivity of this PCR diagnostics allows detection of the parasite before major mortality events can occur. The specific and sensitive nature of the diagnostic means that a monitoring program and biosecurity assessment in respect to *A. ocellatum* is possible.

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4.13 Contemporary chemotherapeutic management of *Amyloodinium ocellatum* in aquaculture

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1. INTRODUCTION

The pathogenic dinoflagellate, *Amyloodinium ocellatum*, has been recorded commonly from aquaria and aquaculture facilities around the world. It is currently considered ubiquitous and to display little host specificity (Lawler, 1980; Levy, Poore, Colorni, Noga & Vandersea, 2007) while remaining a prominent cause of morbidity and mortality in marine aquaculture systems. Common signs of disease in infected fish include the refusal of food, flashing against the substrate and other fish, and gasping at the surface (Lom & Dykova, 1992). Under high stocking densities, fulminating infections such as *A. ocellatum* are able to infect host fish at high intensities and cause substantial mortality and consequent economic loss. Moreover, infection with *A. ocellatum* has further implications for the safe movement of stock for on-growing in commercial fish facilities.

In the present study, bronopol, a potential new chemotherapeutic treatment for *A. ocellatum* is investigated in barramundi, *Lates calcarifer*. Therapeutants previously found to be effective in treating the parasite (e.g. formalin, hydrogen peroxide and malachite green) are also examined for comparative purposes (Paperna, 1984a; Montgomery-Brock, Sato, Brock & Tamaru, 2001; Fajer-Avila, Abdo-de la Parra, Aguilar-Zarate, Contreras-Arce, Zaldivar-Ramirez & Miguel, 2003). In contrast to some of the previous investigations, which have only examined trophont removal (see Montgomery-Brock, *et al*. 2001; Fajer-Avila, *et al.* 2003), the present work evaluates the efficacy of each of the previous and the new treatment against each of the life-stages of *A. ocellatum*. The results are discussed in terms of the relative applicability of these various treatments in the management of marine *A. ocellatum* infections in commercial operations.

2. MATERIALS AND METHODS

2.1 Fish

Fish used in chemotherapeutic trials were juvenile barramundi, *Lates calcarifer*, of 5-11 cm in total length, obtained from a commercial barramundi growout facility (Barramundi Australia, Brisbane).

2.2 Pathogen

Amyloodinium ocellatum tomonts were collected originally from juvenile snapper *Pagrus auratus* at the New South Wales Department of Primary Industries, Port Stephens Fisheries Centre (PSFC) and transported in bags containing oxygenated seawater to the University of Queensland Veterinary Science Farm, Pinjarra Hills, Brisbane, Australia. An *in vivo* infection was then maintained in two 50 L, aerated, tanks, each of which were stocked with juvenile barramundi, *L. calcarifer*. The experiment and infection room were both maintained at 22°C.

Amyloodinium ocellatum tomonts were obtained by adding experiment fish to the *in vivo* infection tanks one week prior to use. Once heavily infected these fish were then removed from the 50 L tanks and placed into 10 L tanks filled with fresh water for 10 minutes to detach parasites from body surfaces. Collected bath water was then passed through a series of 150 µm and 47 µm nylon filter screens to remove organic debris and collect tomonts, respectively. Tomonts were then washed from screens using filtered sea-water and collected in Petri dishes for experimental use.

2.3 Assessment of infection

Fish were removed from the in vivo infection tanks at the same time to keep differences in infection level at a minimum. Infection levels were assessed by non-lethal gill biopsy of all fish within the experiment and control tanks. The right operculum of each barramundi was raised and the distal tips of eight gill filaments from the first gill arch (on first and second biopsy) or second gill arch (for third and fourth biopsy), were removed from the mid-lateral gill with dissection scissors. Counts of trophonts using a compound microscope (Olympus) were then taken to assess initial infection levels. Post-treatment biopsies were undertaken at one, three and five days from a similar area of the first or second gill arch. Counts of post treatment infection levels were then compared statistically.

2.4 Chemotherapeutic agents

An aqueous solution of 50% bronopol (2-bromo-2-nitropropane-1, 3 diol) commercially available as Pyceze, (Novartis, Braintree, UK) was used as a treatment at a concentration of 50 mg/l in a 30 min bath treatment for *A. ocellatum* infected fish and also tomonts were exposed to this dose for 30 min and also over four days. 'Marine Oodinium and Whitespot Treatment' (Aquarium science, Loganholme, Australia) is a commercially available treatment for ornamental marine aquariums comprising, 30mg/ml quinine hydrochloride and 3.8 mg/ml Malachite green; this was used at a concentration of 15 mg/l in a chronic bath treatment of infected fish and exposure of tomonts over 4 days. An aqueous solution of 37% formaldehyde (Ajax Finechem, Sydney, Australia) was also tested at a treatment dose of 25 mg/l in a four day treatment and 200 mg/l in a 60 min treatment against tomonts and 200 mg/l in a 60 minute bath treatment for fish. Hydrogen peroxide with 30% active ingredient (Ajax Finechem, Sydney, Australia) was used at a dose of 75mg/l for 30 min bathing of fish and at 75 mg/l and 200 mg/l in 30 min and four day tomont treatments. Freshwater was sourced from local mains supply then aerated for a minimum five days to remove ammonia and chlorine. Salinity was determined with a hydrometer to be 0 ppt.

2.5 Trophont treatment trials

Fish maintained in the *in vivo* infection tanks were given an initial gill biopsy with the number of trophonts recorded. Four barramundi per tank were placed in six 10-L tanks containing the various treatments. The bronopol dose was 50 mg/l and the exposure period was 30 min, the hydrogen peroxide dose was 75mg/l and exposure time was 30 min, formalin dose was 200 mg/l with an exposure time of 60 min and freshwater (0 ppt) had exposure time of 15 min. Control animals were placed in the tanks containing only seawater. Three replicates of all control and treatment trials were performed. Upon removal from the treatment tanks each group of four fish was placed in 40-L aerated aquaria containing filtered sterile seawater. The exception to this was 'Marine Oodinium and Whitespot Treatment', which was left as a bath treatment for four days according to manufacturer's instructions. Further gill biopsies were taken for each fish at one, three, and five days after the beginning of treatment and trophont numbers recorded for each fish in each treatment group.

2.6 Tomont and Dinospore treatment trials

Three Petri dishes containing approximately 100 tomonts were filled for each of the treatment doses and times (see Figure 1) and then covered to prevent evaporation. At 22°C dinospores typically emerge from tomonts after 72 hours, in treatments that were shorter in duration, at the end of the treatment period, the solution was removed using a pipette and the Petri dish was flushed three times with sterile sea-water. Dishes were maintained in a room at 22°C. Dishes were examined using an inverted microscope (Olympus CKX41) each day for four days. Data were collected on the presence or absence of tomont division, the maximum number of divisions where appropriate, presence or absence of dinospore emergence, and presence or absence of dinospore motility.

2.7 *Statistical analysis*

Statistical analysis was undertaken using the computer software package R (R Development Core Team 2006). Normality of data was estimated using a Shapiro-Wilkson goodness of fit test. Normal datasets were compared using a standard ANOVA. For all other treatments, non-parametric analyses were used due to the large number of zeros and high levels of over-dispersion within the dataset. A quasi-Poisson regression model was utilised to perform χ squared analyses. Probability value of $P<0.01$ was considered significant.

3. RESULTS

3.1 Trophont treatment trials

Hydrogen peroxide, formalin, 'Marine Oodinium and Whitespot Treatment' and freshwater were all found to reduce significantly ($P<0.01$) the number of trophonts of *Amyloodinium ocellatum* in gill biopsies (Figure 1). One trophont was found on day three in one fish of one replicate of the formalin treatment. Six trophonts were found on day one on two fish in one replicate after the marine oodinium and whitespot treatment. Three trophonts were found on day three in one replicate and one trophont was found on day five in a second replicate of the freshwater treatment. These treatments reduced parasite load to almost nil.

Trophont numbers in the bronopol-treated fish were not significantly ($P<0.01$) different from the control except on day three (Figure 1). Following the bathing trial, bronopol-treated fish were the only ones to continue presenting numerous trophonts in biopsies.

3.2 Tomont and dinospore treatment trials

All short exposures of tomonts to treatments were followed by division, dinospore emergence and motility with the exception of 75 ppm and 200 ppm hydrogen peroxide (Table 1). Following 30 minute hydrogen peroxide treatments at either concentration examined, dinospores did emerge from tomonts but were non-motile.

Exposure of tomonts to all 4 day treatments resulted in no dinospore emergence with the exception of the formalin treatment at a concentration of 25 ppm. In that case, division of tomonts was generally low and the dinospores that did emerge in this trial exhibited sporadic motility only.

4. DISCUSSION

Current management strategies for *A. ocellatum* include the use of chemotherapeutics upon presentation of clinical signs of infection (Munday, 1992). Many of these chemicals have a broad-spectrum effect and can be utilised in prophylactic treatment regimes for the management of a number of ectoparasitic infections. Induction of a specific immune response has been proposed to be the best method of control for *A. ocellatum* (Levy & Noga, 2005), though whilst acquired immunity has been shown to occur, there is no vaccine currently available.

Treatment via chemotherapeutic means is often a 'necessary evil' for farmers; in the absence of preventive management tools such as vaccination, and since it is often impossible to exclude ectoparasites completely from production systems. Since ectoparasites can be pervasive within aquaculture systems, reactive chemotherapeutic treatment is a common solution. Often this method requires chemotherapeutics at high doses, which can have a negative impact on already sick fish. Prophylactic treatment of chronic sub-clinical infection may be able to reduce parasite load and avoid mortality events associated with *A. ocellatum*. However, many otherwise effective treatments are banned for use on food animals due to their high non-specific toxicity and/or the longevity of tissue residues. Moreover, many potential treatments await regulatory assessment for use in aquaculture.

Therefore, it should be noted that many of the treatments in this study may not yet be approved therapeutants in many countries.

High costs may also be associated with chemical treatments, not only through direct cost of chemicals, but also indirect costs associated with enforced with-holding periods before harvest in order to allow chemical residues to be metabolised. This can delay the time for stock to get to market further increasing production costs and potentially losing market position through lack of continuity of supply to key accounts.

It is highly likely that the more resistant life-stage of *A. ocellatum* (e.g. tomonts), are better able to withstand the stresses of chemical treatment than are compromised hosts. Chemical treatment may shock trophonts from the fish, but, in many cases, they are still able to develop into tomonts and undergo division even in the presence of chemotherapeutic agents. Since chronic bathing can sometimes be more detrimental to fish, most treatments are administered as a periodic bath, but when treatment is suspended, tomonts on the substrate are able to divide and release dinospores to continue the life cycle. Additionally, the adherence of the tomonts to most surfaces (Abreu, Robaldo, Sampaio, Bianchini & Odebrecht, 2005) makes *A. ocellatum* virtually impossible to eliminate from a tank or pond unless stock are removed and thorough sterilisation is undertaken. *A. ocellatum* dinospores have been found to travel in aerosols (Roberts-Thomson, Barnes, Fielder, Lester & Adlard, 2006) making reinfection from external sources a distinct possibility.

Manipulation of environmental parameters such as salinity and temperature are also potentially useful to manage *A. ocellatum* infections within facilities. The practicality of such strategies will clearly be determined by the environmental tolerances of the fish species being farmed: freshwater bathing may not be tolerated by some cultured species requiring stable salinity levels. Temperature reduction may also control *A. ocellatum* outbreaks since tomonts stop dividing below 15°C (Paperna, 1984b). However, reduction of temperature will bring a concomitant reduction in the growth rates of farmed fish. Moreover, cooling large volumes of water in tropical and sub-tropical aquaculture is prohibitively expensive and thus not practical in larger facilities. Indeed, many tropical fish species affected by *A. ocellatum* will not tolerate extended periods at low temperature. Perhaps more significantly, hypothermic stress induces dormancy in tomonts rather than death and subsequent increases in temperature allow the parasite to continue its lifecycle.

It is evident from these experiments that numerous treatments are effective in the short-term management of *A. ocellatum* infections. Freshwater is particularly effective for treating euryhaline fish species such as the barramundi used in the present study. However, freshwater did not eliminate all trophonts from the gills after the *in vivo* trials. Any remaining trophonts could potentially reinfect the system if the original salinity is restored, even if fish are moved to a different tank or pond.

Hydrogen peroxide was also effective in these experiments at both 75 and 200 mg/l for 30 minutes. This treatment regime had previously been found to be effective for controlling *A. ocellatum* infections of threadfin salmon, *Polydactylus sexfilis* (Montgomery-Brock *et al.,* 2001), with a significant reduction in trophont count observed after peroxide exposure for 30 minutes at concentrations ranging between 75 and 150mg/l. However effects on both tomonts and dinospores were not examined (Montgomery-Brock, *et al*. 2001). Following our experiments, and those of Montgomery-Brock *et al.* (2001), hydrogen peroxide could be considered most useful in treating *A. ocellatum* due to its ability to shock trophonts from fish and its effects on dinospore motility. Repeat dosing would need to be considered in chronic treatments due to the rapid break down of hydrogen peroxide in the environment. However, caution needs to be exercised when using hydrogen peroxide as negative impacts to some hosts have included reduced growth rates for up to three weeks, with moribund animals being most affected (Hirazawa, Ohtaka & Hata, 2000). Nevertheless, the costs involved with treatment mean that often farms are looking for the cheapest alternative that is effective. Our results would indicate that hydrogen peroxide is an ideal candidate.

Bronopol (2-Bromo-2-nitropane-1, 3-dial) is a broad-spectrum biocide commonly used in salmonid hatcheries for the treatment of fungal infections (Aller-Gancedo & Fregeneda-Gandes, 2007). Within these applications, it has been licensed in Europe as a safe replacement for formalin and malachite green (Pottinger & Day, 1999). However, in studies of cooling tower water treated with bronopol, no effects were observed on protozoan populations (Kasai, Ando & Kuwashimia, 1989). Use of 50 mg/l bronopol for 30 minutes against *A. ocellatum* infected fish showed no significant difference in trophont burden compared with control fish. However, the suppression of dinospore emergence in the continuous *in vitro* exposure merits further examination of the usefulness of bronopol as an agent in controlling *A. ocellatum* infections.

The overall efficacy of agents that simply remove trophonts from infected animals (e.g. freshwater baths) will ultimately be determined by biosecurity procedures in place within a facility. If treatment is not coupled with movement of treated fish into new tanks, or tank brushing and vacuuming to remove the released tomonts from the system, then the lifecycle of the parasite has not been effectively disrupted and reinfection is almost certain.

Ideally, specific vaccination of farmed fish against ectoparasites is the most practical solution for the control of infections. Immune response against *A. ocellatum* has previously been demonstrated following natural infection (Noga, 1992; Cobb, Levy & Noga, 1998b). Moreover, mucosal immune response in barramundi is particularly high in seawater and resulting antibodies are capable of binding marine pathogens under seawater salinity conditions (Delamare-Debouteville, Wood & Barnes, 2006), and thus vaccination would appear to be a reasonable approach. Until such a time, the most effective methods of *A. ocellatum* control should include regular monitoring of stock for the presence of infection. This would lead to precisely timed, reactive treatments with hydrogen peroxide and enable on-farm management of Velvet disease.

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TABLE 1

Tomont chemotherapeutic results including tomont division, dinospore emergence and motility from in vitro trials.

Treatment	Dosage (mg/l)	Treatment time (mins)	Tomont Division	Maximum divisions	Dinospore Emergence	Dinospore Motility
Bronopol	50	30	Y	6	Y	Y
Bronopol	50	4 days	Y	6	N	N/A
Hydrogen Peroxide	200	30	Y	7	Y	N
Hydrogen Peroxide	200	4 days	Y	2	N	N/A
Hydrogen Peroxide	75	30	Y	7	Y	N
Hydrogen Peroxide	75	4 days	Y	2	N	N/A
Formalin	200	60	Y	6	Y	Y
Formalin	25	4 days	Y	5	Y	Y
Marine Oodinium and Whitespot Treatment	150	4 days	Y	4	N	N/A
Control	-	4 days	Y	8	Y	Y

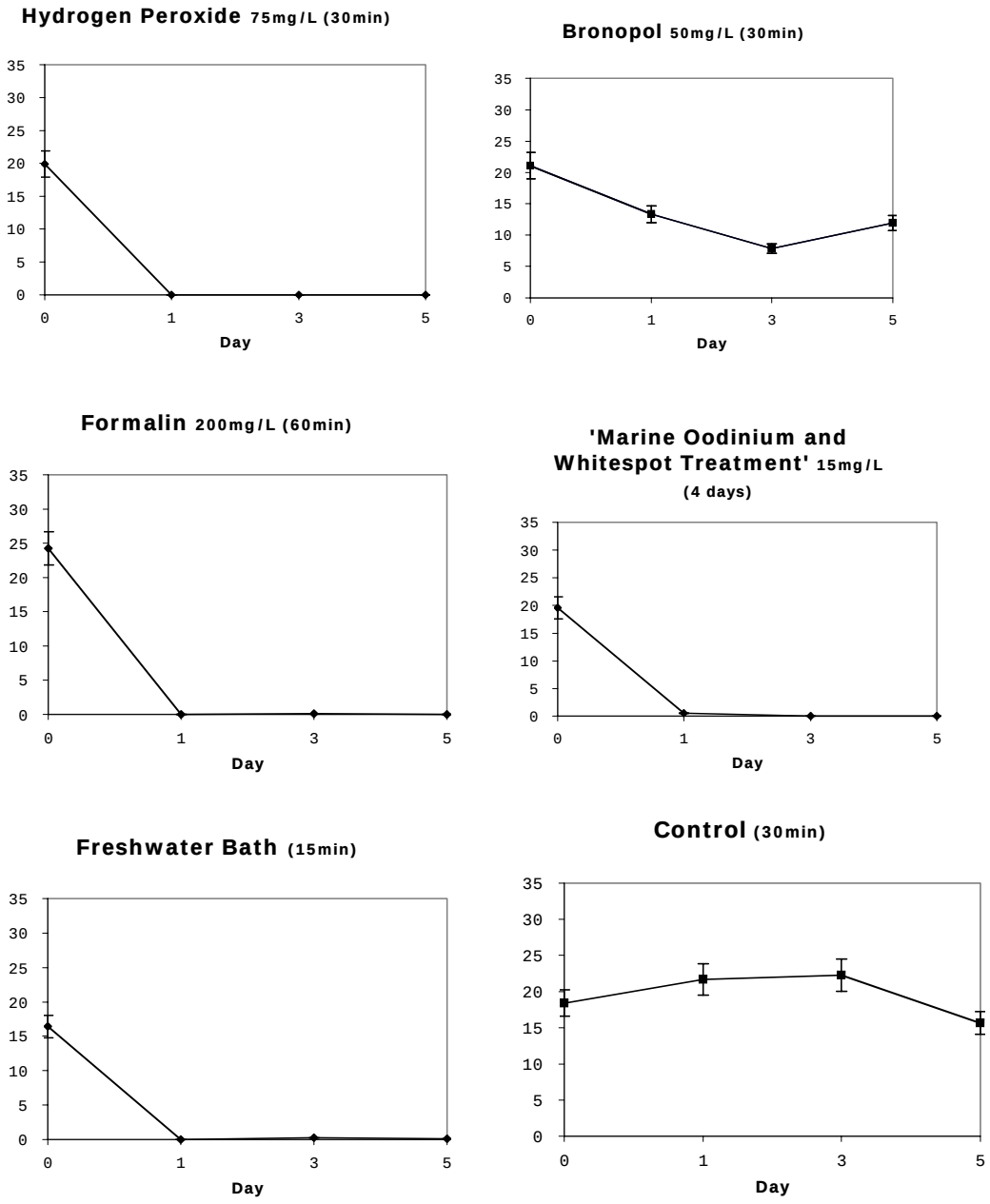

FIGURE 1

Trophont chemotherapeutic treatment trials of five chemotherapeutics and control barramundi infected with *A. ocellatum* showing dose concentrations and duration of treatment. Trophont counts are from non-lethal gill biopsy. Three replicates were performed with four fish per treatment tank.

5. BENEFITS AND ADOPTION

- The research strategy of Aquafin CRC has been developed explicitly to deliver the essential technologies needed by the Australian finfish farming industry. The industry partners agreed to invest in a CRC, defined the major goals which they believed a CRC could best achieve, and clearly indicated the weight of effort which should be applied to each of these goals. These goals and weightings were first defined at a workshop of potential CRC participants in December 1999.

- The industry participants have continued to refine their priorities during the subsequent development and industry participants and researchers have met regularly (several times per year) to discuss results, implications of results and how they might be used, and refined plans for subsequent experiments.

- While there is currently no commercial snapper farming in Australia (fish farmers have moved to other faster growing marine fish like mulloway and yellowtail kingfish) the research has contributed in several major ways to Australian marine fish aquaculture:

    - Diets developed for snapper have helped form the basis for formulations for other marine carnivorous species,

    - One of the most significant benefits is the confidence among feed manufacturers to use alternative ingredients to fishmeal in response to reductions in availability and/or increases in price. The progression towards least-cost formulation (as is industry practice for terrestrial monogastric animal feed formulation) relies on rigorous ingredient evaluation, accurate estimation of nutritional requirements and systematic validation of different formulations.

    - Hatchery practices and nursery technology has been refined providing a much clearer understanding of the importance of abiotic factors, larval feeding strategies and diets on the cost-effective production of snapper fingerlings. Low-cost technology for extensive production of snapper larvae in fertilised ponds has also been developed. The new technology has been applied to other marine fish species providing an excellent starting point from which to refine larval rearing techniques for those species.

    - A clear understanding of how to improve skin colour of farmed snapper through manipulation of the culture environment and optimisation of dietary pigments.

    - Although a "solution" has not been found, the project has leD to a much greater understanding of "velvet disease" caused by the parasitic dinoflagellate *Amlyoodinium occelatum* and best management practices to help avoid major problems.

    - The development of successful research methods cannot be underestimated as a benefit of this project. Research methods for diet development, larval rearing and nursery production and health management have all assisted in the design of new research to address similar problems for other species.

    - Research capacity at an institutional (NSW DPI and Ridley Aquafeeds) and personal level has been expanded.

- Adoption has been made possible because of the involvement of Ridley Aquafeeds throughout the project. The commercial fish farmers who were partners in the project are currently

represented by Anthony O'Donohue (Clear Water Mulloway) who is currently using the Ridley Marine Fish Diet for production of mulloway and yellowtail kingfish.

6. FURTHER DEVELOPMENT

Despite the commercial investment in snapper farming at the commencement of this project, snapper farming has not developed in Australia. This is mainly because the economics of snapper culture, when the species was farmed as the only species in sea cage operations, were sub-optimal and without exception, operators moved to faster growing species such as mulloway and yellowtail kingfish. However, even though the aquaculture production of snapper has not developed, marine fish farming in Australia has expanded considerably. The research conducted during this project has assisted with the development of culture techniques for mulloway and yellowtail kingfish. In particular, results have been adapted during the Aquafin CRC project "Feed technology for temperate fish species" for both the hatchery/nursery and the diet development components, and information gained is being used by feed manufacturers and hatchery operators.

Assuming commercial production costs are equivalent for different diets, use of ingredients similar to those tested in this study can reduce the levels of fishmeal and thus the cost of snapper (and marine fish) feeds. Due to the high protein requirements of marine fish, the fact that productivity improvements can be achieved by feeding high-protein feeds and the increasing demand on existing fishmeal supplies, replacement of fishmeal in diets will be increasingly important in the future. During the course of this project, the cost of fishmeal has increased by at least 100% and at times has been virtually unavailable in Australia because of reductions in production and massive increases in demand, particularly from China. This rapidly changing supply/demand relationship for fishmeal has major implications for marine fish farming, particularly in Australia, where low production reduces the ability to negotiate large volume purchases of fishmeal at globally attractive prices. This reinforces the priority for continued investment in fishmeal replacement research.

In addition, because the "formulation space" for other energy sources will be reduced in nutrient dense feeds, the challenge is to identify and test high protein ingredients that in combination have a similar nutritional quality to fishmeal but at a lower cost. The paradox for Australian marine fish farmers is that rather than lowering the cost of feeds, high-protein nutrient dense feeds will inevitably cost more per kilogram than those formulated with a lower nutrient specification. However, the increased growth and improved FCR of this feeding strategy should make the use of more expensive, nutrient dense diets economically sound. The imperative for future research is to ensure whole-farm economics is considered when modelling different nutritional and feeding strategies. A key aspect is to ensure research is conducted with large fish most relevant to farming operations.

This project has led to considerable improvements in hatchery technology for snapper. Hatcheries dedicated to producing snapper are now able to produce nearly twice as many batches of snapper juveniles as they could using the "best practice" technology available at the start of the project. As the emphasis has shifted to other temperate marine fish species, e.g. mulloway, yellowtail and even southern bluefin tuna, it will be very important that hatchery procedures for each species are optimised. The research capacity developed during the project will be invaluable. In the past, meetings of marine fish hatchery managers and technicians have been very effective as a means of sharing new technology and identifying bottlenecks in production. Further hatchery development for marine finfish in Australia would benefit greatly from an expansion of this concept.

As production intensifies, health management will become increasingly important. The research during this project has progressed knowledge of velvet disease, caused by the parasitic dinoflagellate *Amyloodinium occelatum*. However, other diseases will also emerge to limit aquaculture production This is a critical area of research for future marine fish aquaculture development.

7. PLANNED OUTCOMES

1. Profitable, expanding industry for snapper aquaculture in Australia. **Not met**. However, development of other marine fish farming industries has occurred and research methods developed during this project, as well as research results, have assisted with the development of the marine fish culture industry in Australia.

2. Viable hatcheries, breeding vigorous low-cost snapper fingerlings (for aquaculture or stock enhancement). Our goal is to reduce fingerling costs to around 25 cents/fish. (Specific outcome for Fingerling Production and Health Component). **Achieved.** Several marine fish hatcheries in Australia are now able to successfully produce snapper fingerlings and "commercial-scale" validation of new improved hatchery technology has occurred in South Australia, Western Australia and NSW.

3. Development of techniques for management exclusion of ectoparasites in marine fish hatcheries. *Amyloodinium ocellatum* infests many fish species in hatcheries throughout the world. All Australian marine fish hatcheries will potentially benefit from this technology. (Specific outcome for Fingerling Production and Health Component). **Achieved.** While a successful treatment for *Amyloodinium ocellatum* was not developed, methods for excluding the problem from hatcheries and nurseries were developed and successfully applied.

4. Commercially-available, cost-effective, high performance and low polluting diets for Australian snapper that help produce highly marketable fish of the desired colour. (Specific outcome for Diet Development and Skin Colour Component). **Achieved.** While there are no snapper being farmed at present, research results from the project have assisted the commercial feed manufacturer, Ridley Aquafeeds, with formulation of their Marine Fish Diet that is very effective for snapper. The Ridley Marine Fish Diet is recommended for grow out and the same diet with the addition of astaxanthin is recommended as a finisher diet to impart the desirable red pigment. A tank-based finishing treatment is recommended in addition to the pigmented finisher diet to ensure the best skin colour for farmed snapper.

5. Increased availability of snapper for domestic (and export) markets. **Not achieved**. There has, however, been considerable expansion in production of other temperate marine finfish species and research and commercial experience with snapper has helped lay the foundation for this expansion.

8. CONCLUSIONS

Before this project started, production of snapper larvae was done in hatcheries using intensive culture techniques. Intensive hatcheries are expensive to establish and operate as they require specialist facilities, highly qualified labour and often difficult to obtain and/or costly live feeds including *Artemia*. Extensive larval rearing on the other hand involves production of fingerlings in ponds. The ponds are filled with estuarine water and then fertilised to promote a succession of phyto- and zooplankton which are a food source for the fish larvae. Less sophisticated infrastructure and degree of technical expertise are required and the costs can be much lower.

Amyloodinium ocellatum continues to be a disease agent in marine hatcheries, despite study on managing the parasite since the 1930's. This study has examined a number of gaps in knowledge of the biology of the parasite. A number of the subsequent results may have important implications for management of *A. ocellatum* infections in aquaculture.

The major conclusions and findings of this research are:

- Techniques for successful culture of high quality juvenile snapper in outdoor, fertilised ponds were developed. Protocols for pond fertilisation and the time needed for production of suitable size and density of zooplankton were identified. The optimal age of larvae for stocking into ponds is approximately 16 d after hatching and is likely due to the facts that the larvae are advanced in development, more robust than newly-hatched larvae and are capable of consuming a wide range of live feeds.

- The water quality, in particular temperature, can fluctuate widely in outdoor ponds and this has a major influence on not only the success of an individual larval rearing run but also on the seasonality of pond culture. Optimal water temperature for snapper is 18-24^{o}C therefore pond culture in ambient ponds will be restricted to spring and autumn months in temperate, coastal NSW. Covering the ponds with a polyhouse however is a highly effective method to increase mean water temperature by 5^{o}C, to reduce daily water temperature fluctuation and to ultimately extend the culture period in ponds through winter. High summer water temperatures make pond culture unfeasible.

- The optimal strategy to produce high-quality juvenile snapper in ponds consists of a combination of early stage larval rearing for about two weeks in controlled, intensive hatchery conditions followed by stocking and culture for 3 to 4 weeks in extensive fertilised ponds.

- Feeding of *Artemia* remains one of the major operating costs in intensive fish hatcheries. *Artemia* availability and price varies widely on the world market from year-to-year and development of methods to reduce or exclude their use in hatcheries is a global priority. Protocols were developed to completely replace *Artemia* with either copepods which were cultured in fertilised ponds or with commercially available weaning pellet diet. Several commercial weaning diets were also compared and although they varied in purchase price (almost 10-fold range) and physical qualities there was no difference in larval snapper performance. This allows hatchery technicians the opportunity to select diets based on personal preference e.g. some diets are easier to obtain than others, and provides scope for hatchery management.

- Optimising feeding efficiency of fish is essential for efficient and cost-effective production and can be influenced by factors including fish size, photoperiod and feeding frequency. For juvenile snapper (1 to 60g), performance was best when the photoperiod was 18L:6D. For snapper within the same size range however, optimal feeding frequency changed as fish

increased in size i.e. small fish (1 to 5 g) require feeding more than 8 times/d whereas larger fish (20-60 g) only need 2 feeds/d to achieve maximum growth.

- The investigations of cryopreservation of *A. ocellatum* may be a crucial step in an exhaustive study of wild isolates for genetic, pathogenic, morphological or behavioural differences. This in turn may have implications for the treatment of different isolates. Long-term cell culture may not be representative of the original isolates. It appears that currently the only option for storage with retention of viability in *A. ocellatum* is of a short-term duration (circa 7 weeks) and invoked by dropping the environmental temperature below 14º C but remaining above freezing.

- Detection of *A. ocellatum* by PCR-based diagnostic practices provides greater sensitivity and specificity than traditional histological methods. While previously developed ELISA techniques are also specific, PCR diagnosis is more rapid and does not rely on the host being infected to detect the presence of the parasite in the facility. Due to the speed and accuracy of the technique, PCR diagnosis is a very useful tool that can be used to time treatments, and help describe the dynamics of the parasite within a given facility. Additionally, use of PCR diagnostics to identify entry pathways of the parasite into a facility is a possibility.

- Biosecurity risk and potential of aerosols to disperse *A. ocellatum* into and within facilities has a profound effect on management of the disease. This finding explains infection dynamics where cross contamination with equipment was minimised yet infection still spread. Further, many other marine protozoan infections may transfer via this pathway, many of which are disease agents in aquaculture. The use of PCR diagnostics on air filters may further help to assess entry of *A. ocellatum* into facilities and identify where to block these pathways to infection.

- Treatment with chemotherapeutics has been used as the primary treatment for *A. ocellatum* infections for decades. While the chemicals used have changed over the years, most are still non-specific and are administered as bath treatments. Presently, chemotherapeutic treatment represents the best method of control for *A. ocellatum* infections. Many compounds are successful in removing trophonts from the host, however many of these fail to stop the parasite from continuing its life cycle. Hydrogen peroxide was found in the present study to affect all stages of the parasite and should be considered as a useful therapeutant for aquaculture. This is not only due to its cheap cost, but also its short half life and minimal tissue residue. Additionally, fresh water is a successful treatment for *A. ocellatum* infections. However, many fish are less tolerant of fluctuations in salinity and its use should only be undertaken with euryhaline fish. More chemicals may prove useful in treating *A. ocellatum* infections, although cost-effectiveness of hydrogen peroxide or freshwater may mean that they remain the chemotherapeutant of choice.

9. APPENDICES

9.1 Intellectual Property

All information brought into this project or developed during the project is public domain.

9.2 Staff

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