Intensive rearing techniques and feeding behaviour of larval and juvenile mulloway, *Argyrosomus japonicus*

Thesis submitted by
Debra Anne Ballagh BSc (Hons)

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For the degree of Doctor of Philosophy
School of Marine & Tropical Biology
James Cook University
Townsville
Australia

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The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A1102).

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Conferences

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Abstract

Mulloway, *Argyrosomus japonicus*, is a euryhaline sciaenid that is popular with seafood consumers and recreational fishers. The species is an emerging aquaculture candidate and culture techniques have been developed. But with industry production low and fingerling costs estimated to be as high as $2 each, there is an imperative to improve production technology and lower costs. This study aimed to improve mulloway hatchery efficiency through:

- the development of specific disinfection protocols for mulloway eggs to reduce the potential for transmission of pathogens on the surface of eggs.
- an improved understanding of embryonic development.
- optimization of light and feeding regimes to increase growth and survival of larvae and juveniles.
- a better understanding of the biological mechanisms underpinning feeding behaviour for larval and juvenile stages.

Disinfection of fish eggs with ozone is an important practice to reduce the incidence of infection by pathogens in the hatchery. However, ozone can reduce hatching success and can induce deformities in hatched larvae. In this study, the embryonic development of mulloway was described in greater detail than previously available to create a visual reference of egg stages and to assist in the development of a safe ozone disinfection protocol. The potential impact of ozone exposure is measured using a CT value, which is the concentration of ozone (mg l^{-1}) multiplied by the contact time (min). Initially, the combined effects of ozone CT (0, 0.1, 0.5, 1 or 5) and treatment temperature (19, 22 or 25ºC) on the hatching success of mulloway larvae were evaluated. The highest CT value that did not negatively

affect hatching was a CT of 1, and the most appropriate water temperature for disinfection was 22°C, as there were no apparent negative impacts of treating eggs at this temperature and mulloway eggs are currently incubated at 22°C at the Port Stephens Fisheries Institute (PSFI) hatchery. Embryos at four different stages of embryonic development (3, 8, 20 or 27 h post fertilisation; HPF) were then exposed to ozone (CT = 1) and no significant effect was detected on hatchability at any of the embryonic stages examined. Accordingly, it is suggested that eggs should be disinfected with ozone early in development to reduce negative impacts on development caused by nodavirus and other pathogens.

The photoperiod and light intensity requirements of early and advanced larvae were investigated to determine an appropriate light regime. Two experiments were conducted to investigate the effects of a range of photoperiods (24L:0D, 18L:6D, 12L:12D, 6L:18D or 0L:24D) and light intensities (1 or 10 μmol s^{-1} m^{-2}) on the growth, swim bladder (SB) inflation, food consumption and survival of early (2-8 dah [days after hatching]) and advanced (14-34 dah) mulloway larvae. The two light intensities tested had no effect on the parameters measured in early or advanced larvae and there were no significant interactions between photoperiod and light intensity. In early larvae, the optimal photoperiod was determined to be 12L:12D, which promoted growth, survival and SB inflation. Once SB inflation has been achieved, the photoperiod should be adjusted to 18L:6D to increase feeding time and promote growth. In advanced larvae, no differences in growth were observed at the end of the experiment; however, survival was significantly better in the 0L:24D photoperiod compared with all other photoperiods examined. It is possible that there are physiological effects of maintaining larvae in complete darkness, and until further research is completed, it is recommended that an 18L:6D

photoperiod is used. This protocol is expected to optimise growth and subsequently reduce the overall time that larvae remain in the hatchery.

The optimal weaning procedures for mulloway larvae were also investigated. Three experiments were conducted to compare weaning strategies using live feeds; rotifers (*Brachionus plicatilis*) and *Artemia*, and formulated pellet diets. Initially, the effects of feeding *Artemia* at different levels (0, 50 or 100% ration of *Artemia* fed from 18 dah [days after hatching]; based on current hatchery protocols) and a pellet diet from two larval ages (14 or 23 dah) were investigated. Results indicated that some *Artemia* should be in the diet of larvae to promote growth; however, it is possible to feed half the usual amount without reducing growth rates. Growth of larvae was not influenced by the time of pellet introduction. The size (total length [TL]) at which mulloway larvae selected *Artemia* equally or in preference to rotifers, and pellet (400 µm) equally or in preference to *Artemia*, respectively was then determined. The mean size (± SEM) at which mulloway larvae began selecting *Artemia* equally to rotifers was 5.2 ± 0.5 mm TL and selected pellets equally to *Artemia* at 10.6 ± 1.8 mm TL. This has led to the establishment of weaning protocols for larval mulloway that optimise larval growth while reducing feed costs by minimising the amount of *Artemia* used during production.

Next, the optimal photoperiod and feeding interval requirements for rearing juvenile mulloway were examined. A two factor experiment incorporating three photoperiods (12L:12D, 18L:6D or 24L:0D), and five feeding intervals (1, 3, 6, 12 or 24 h between feeds) determined the effects on somatic growth rate, feeding efficiency and survival of juvenile mulloway (2.7 ± 0.5 g). The growth indices used included fish weight, length, food conversion ratio (FCR), condition factor (CF), and the coefficient of variation of weight (CVwt). No interactions were found for

any parameters measured. The 12L:12D photoperiod produced significantly poorer survival than other photoperiods; however, feeding interval did not influence survival. It was determined that feeding can be optimised by rearing juvenile mulloway in an 18L:6D photoperiod and by feeding once every 12 h. The 24L:0D photoperiod reduced growth (weight and length) and increased the FCR, and the 12L:12D photoperiod reduced survival with the result that the fish in the 18L:6D photoperiod had optimal growth and survival parameters. The only feeding interval to affect growth (weight and length) was the 24 h feeding interval; there was no difference in growth detected between the 1, 3, 6, or 12 h feeding intervals. It was also determined that it would take 10 h for 50% of the feed consumed in one meal to be evacuated from the stomach. Given that there was no significant difference in growth and feeding efficiency in juvenile mulloway between the 1, 3, 6 and 12 h feeding intervals, it would be more efficient for producers to feed their fish no more than twice daily (or every 12 h). The increase in feeding efficiency associated with these feeding techniques is likely to increase production and reduce the time mulloway remain in the hatchery.

Finally, the sensory basis for feeding was investigated for larval and juvenile mulloway, including the contribution of vision, mechanoreception and chemoreception to the feeding ability of larval and juvenile mulloway. Firstly, an investigation into the ablation of the mechanosensory neuromasts using streptomycin sulphate (SS) determined that larvae exposed to SS at a concentration of 0.75 g l^{-1} for 3 h had a reduced startle response but did not exhibit abnormal swimming behaviour. The contribution of each sensory system to feeding was then examined for larval and juvenile mulloway between 3.5 and 53.3 mm. From 3.5 to 4.1 mm, mulloway larvae consumed significantly more live food items when fed in

light conditions compared with those fed in dark conditions, indicating that vision was the primary sense mode used for feeding. From 5.0 mm, the mechanosensory system appeared to play an increasingly important role in feeding and it was observed that the number of live food items consumed was significantly greater when vision and mechanoreception were available until larvae reached 7.5 mm. Evidence of an increase in the contribution of the olfactory organs to feeding was also observed from 8.7 mm, while fish were feeding on live feeds. From 10.6 mm, fish were fed pellets and while fish were observed feeding in both light and dark conditions, feeding success became greater in fish fed in the light providing evidence that the role of vision was again the primary sense mode. This change in the contribution of the sensory organs to feeding as development proceeds in larval and juvenile mulloway is discussed.

The protocols developed in this thesis have now been adopted in practice at the PSFI and have improved growth and survival rates. The average production costs of juvenile mulloway reared at the PSFI is now estimated to be $1.05 per fingerling, and this is expected to have increased the viability of mulloway as an aquaculture species. In particular, the reduction of the quantity of *Artemia* required to rear larval mulloway has reduced by 50%, which is a significant saving in production costs. In addition, the protocols developed for ozone disinfection of mulloway eggs has been adopted for other species such as Australian bass (*Macquaria novemaculeata*), yellowtail kingfish (*Seriola lalandi*) and southern blue-fin tuna (*Thunnus maccoyii*). This thesis has also highlighted new research priorities for mulloway cultivation and has formed the basis of rearing protocols for yellowtail kingfish and southern blue-fin tuna, which are now under investigation to determine optimal culture techniques.

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List of Abbreviations

A *Artemia*

ABARE Australian Bureau of Agricultural & Resource Economics

ANOVA Analysis of variance

B.W. Body weight

CF Condition factor

CRC Cooperative Research Centre

CT Concentration (mg l^{-1}) x Time (min) of ozone exposure

CVwt Coefficient of variation of weight

D Dark

DAH Days after hatching

DO Dissolved oxygen

FAO Food and Agriculture Organization

FCR Food conversion ratio

FRDC Fisheries Research and Development Corporation

HPF Hours post fertilisation

IPNV Infectious Pancreatic Necrosis Virus

L Light

LHRHa Luteinising Hormone Releasing Hormone analogue

NSW New South Wales

P Pellet

PSFI Port Stephens Fisheries Institute

R Rotifer

SB Swim bladder

SD Standard deviation

SEM Standard error of mean

SGR Specific growth rate

SJNNV Striped Jack Nervous Necrosis Virus

SNK Student Newman Keuls

SS Streptomycin sulphate

SW Saltwater

TL Total length

VER Viral encephalopathy and retinopathy

VNN Viral Nervous Necrosis

Chapter 1

General Introduction

1.1 Aquaculture

Aquaculture is one of the world's fastest growing food-producing sectors and accounts for 47% of the world's fish food supply (FAO, 2009). The products produced by aquaculture are varied and include marine and freshwater fish, molluscs, crustaceans, aquatic plants and other aquatic animals. In terms of aquaculture production, the largest category is freshwater fish, and aquaculture now accounts for 76% of global freshwater fish production (FAO, 2009). Although substantially less volume of marine fish compared with freshwater fish is produced from aquaculture, this category is increasing rapidly and is having a major impact on world fish markets (FAO, 2009). This in part can be attributed to declining wild marine fish stocks and the relatively high market value of marine fish, which has made this category increasingly appealing for aquaculture production. Marine finfish currently contribute to only 3% of the world's aquaculture production by volume, but make up 8% of the total value (FAO, 2009).

Aquaculture is also considered to be the fastest growing primary industry in Australia (NSW DPI, 2008), and in 2008 aquaculture production was worth $868 million (ABARE, 2009). The suite of Australian species that have been investigated for their suitability for aquaculture is comprehensive and includes finfish,

crustaceans, edible molluscs and pearl oysters (ABARE, 2009); however, finfish aquaculture dominates this sector making up 62% of Australian aquaculture production (O'Sullivan and Savage, 2009). Marine finfish are particularly important in Australia as the two most economically significant species produced in the country are marine; Atlantic salmon (*Salmo salar*) and southern bluefin tuna (*Thunnus maccoyii*) (ABARE, 2009).

The increasing demand for food produced by aquaculture, coupled with the potential high value returns of marine finfish, provide an economic incentive for research into species-specific aquaculture production methods. The expansive and relatively unprotected coastline of Australia presents unique challenges with regard to the selection of suitable aquaculture species. With the exception of salmonids, imported into Australia over 100 years ago, the species currently being researched are generally endemic and well suited to the environmental conditions, are fast growing and receive a good market price. However, a key limiting factor to aquaculture production in Australia and around the world is the availability of knowledge about species biology and the subsequent understanding of optimal culture conditions.

In Australia, mulloway (*Argyrosomus japonicus*) has been identified as a promising aquaculture species; it is distributed widely along Australia's coastline, has good growth rates and is a popular angling and food fish. In addition, wild mulloway stocks have been classified as overfished (Scandol et al., 2008) and there is a need to reduce our dependence on wild stocks. Recent production figures state that aquaculture production of mulloway in Australia is worth $4.9 million (O'Sullivan and Savage, 2009), and now further research is required to increase knowledge of the species' biology and improve the efficiency of production.

1.2 Aquaculture of mulloway

Mulloway is a euryhaline sciaenid that has been produced in small quantities in NSW since the early 1990s (Battaglene and Talbot, 1994). The species is regarded as a prized angling fish with good eating qualities and has been cultured for wild stock enhancement (Taylor et al., 2005) and for a developing aquaculture market (Battaglene and Talbot, 1994; Fielder et al., 1999). Relatively little is known of the species' biology; however, small scale production and research of mulloway has produced encouraging results (Battaglene and Talbot, 1994; Fielder and Bardsley, 1999). The species is distributed along Australia's southern coastline (Edgar, 2000), and is thought to spawn in coastal waters close to beaches. Eggs and larvae are dispersed in surrounding coastal waters, eventually migrating to the upper reaches of estuaries as juveniles (Gray and McDonall, 1993; Silberschneider and Gray, 2008). In Australia, the species is currently cultured in NSW and South Australia (O'Sullivan and Savage, 2009), and aquaculture programs for this species are also developing in South Africa (Battaglene and Talbot, 1994; Tom Hecht, personal communication).

As a highly desirable species, mulloway can fetch a good market price ($12 kg^{-1} wholesale, Sydney Fish Market, 2008); however, the industry is currently limited by high production costs. In 2006-07, cultured mulloway made up 1.6% of fish tonnage produced by Australian aquaculture, yet it represented only 1% of the market value (O'Sullivan and Savage, 2009). In NSW in 2004, the cost of producing a 70 mm mulloway fingerling was estimated at $2. The high cost was due, at least in part, to a high risk of batch failure that could be attributed to a limited understanding of the biological and physical requirements of the species.

A preliminary study on hatchery protocols for rearing mulloway was completed by Battaglene and Talbot (1994) who were the first to produce the species in a hatchery and to close the life-cycle. Battaglene and Talbot (1994) described initial larval rearing techniques and provided important information that has supported preliminary investigation of the potential for the industry. However, if mulloway production is to increase it must be economically viable and a significant reduction in labour, food and operating costs is needed as well as an increase in productivity. Cost-effective production of high quality fingerlings is fundamental to those goals.

Increasing our limited knowledge of the biological and physical requirements of larval and juvenile mulloway is essential for improving hatchery productivity, reducing production costs for fingerlings and removing this restriction to industry growth. Preliminary research into mulloway culture has focused on hormone induction and broodstock management (Battaglene and Talbot, 1994), parasite identification (Hayward et al., 2007), and investigations into larval rearing and salinity and potassium tolerances (Battaglene and Talbot, 1994; Fielder and Bardsley, 1999; Doroudi et al., 2006). Critical gaps in our current knowledge include suitable egg management procedures; light and feeding regimes that promote growth, ontogeny and survival; as well as the biological mechanisms that underpin these factors. Key research areas identified as critical for improvement in hatchery efficiency include:

- development of specific disinfection protocols for mulloway eggs to reduce the potential for transmission of pathogens on the surface of eggs,
- an improved understanding of embryonic development,

- a detailed understanding of the potential for increasing growth and increasing survival through modifying the light and feeding regime for different larval and juvenile stages, and
- a better understanding of the biological mechanisms underpinning feeding behaviour for larval and juvenile stages

1.2.1 Egg disinfection

Hatchery disinfection and hygiene protocols play an important role in the hatchery process as a reduction in the incidence of infection by pathogens can reduce the costs associated with disease management and mortality. Ozone is a popular disinfectant in hatcheries as it is highly effective in the inactivation of pathogens; however, it can have deleterious effects on the hatching success of some species (Hall et al., 1981; Grotmol and Totland, 2000; Buchan et al., 2006; Ben-Atia et al., 2007). Some of the factors that can influence the effect of ozone on fish eggs include the concentration of ozone and the treatment time, the size of the egg, the treatment temperature and the stage of embryonic development (Grotmol and Totland, 2000; Ben-Atia et al., 2007). While there are risks associated with ozone treatment, successful application of this disinfectant can reduce the incidence of disease causing pathogens and reduce hatchery costs.

A common pathogen of marine fish hatcheries is nodavirus, a neuropathogenic virus responsible for significant mortalities in aquaculture worldwide (Munday and Nakai, 1997). A nodavirus was identified at the Port Stephens Fisheries Institute (PSFI) hatchery just prior to the commencement of this study, and it became a priority to develop an egg disinfection protocol to reduce the

vertical transmission of this and other pathogens, while ensuring that development of the eggs was not affected. Many of the factors identified to influence the tolerance of fish eggs to ozone are species specific and therefore it was important to develop protocols specific to mulloway eggs.

1.2.2 Embryonic development

Studies into finfish embryonic development are relatively uncommon when compared to the vast array of aquaculture studies. Despite this, information on the embryonic development of each species is required to be able to identify abnormal development. Abnormal embryonic development has been used as an indicator of egg quality (Shields et al., 1997; Moran et al., 2007) and can be a predictor of larval deformities. An understanding of embryonic development can assist with studies into the embryology of the species, and may help to answer questions about the treatment effects of culture conditions and broodstock nutrition on the incidence of development anomalies (Martins et al., 2009). An understanding of embryonic development can also assist hatchery operators to identify non-viable batches of eggs before significant resources are allocated to them. The time and resources allocated to rearing sub-optimal animals can be costly and fish with undetected deformities can substantially increase the cost of production, which in part can result as deformed fish are often un-saleable.

1.2.3 Sensory development

The three major sensory systems employed by marine fish for detecting prey and evading predators are vision, mechanoreception and chemoreception. Typically, the sensory organs are undeveloped at hatching but development is rapid and ensures larvae have the features required for exogenous feeding (Blaxter, 1986; Blaxter, 1988; Cobcroft and Pankhurst, 2003; Mukai et al., 2008). The dominant sensory system of early larvae is usually vision, but as ontogeny proceeds, mechanoreception and chemoreception can play an increasingly important role in prey detection and predator evasion.

There are many key milestones in larval ontogeny that must be reached in order to achieve good growth rates and high survival, but swim bladder (SB) inflation, first exogenous feeding and weaning are milestones that are often bottlenecks in larviculture and can result in peaks in mortality (Battaglene and Talbot, 1990; Cahu and Zambonino Infante, 2001; Engrola et al., 2009). The limited sensory ability of newly hatched larvae means light is usually required for feeding (Blaxter, 1986); however, as development progresses and larvae begin to utilise other sensory systems, they may have an increasing ability to survive and grow in different environments (Pankhurst, 2008). Many factors can influence SB inflation and first feeding, including the light and feeding regimes applied and the level of sensory development achieved. As the feeding and light requirements of a species can change, the optimal culture conditions should be re-examined during each stage of hatchery production.

To examine the sensory basis for prey detection and feeding behaviour, fish fed in the light and dark can be compared for feeding ability to determine the

instance of visual and non-visual feeding (Pankhurst, 2008). Non-visual feeding can then be further investigated by pharmacologically ablating the superficial neuromasts of fish, thus blocking mechanoreception and leaving only chemoreception (and inner-ear mechanoreception) in dark conditions (Wersall and Flock, 1964; Matsuura et al., 1971, Jones and Janssen, 1992; Pankhurst, 2008).

The contribution of each sensory organ to feeding is unknown for larval and juvenile mulloway, and therefore this study aimed to determine changes in the availability of sensory organs and the corresponding changes in feeding behaviour through a series of feeding trials as development proceeded.

1.2.4 Photoperiod and light intensity

Photoperiod and light intensity have been studied in depth for many species (Sayer, 1998; Boeuf and Le Bail, 1999), as optimal light conditions are generally species specific and improvements in growth, survival and food conversion ratios can be obtained by manipulating the light regime (Blaxter, 1986). As discussed earlier (1.2.3), newly hatched larvae require light to feed; however, optimal light conditions can change as ontogeny proceeds and this can reflect ontogenetic changes in the sensory systems.

Larvae require a minimum light intensity to effectively detect and catch food, while light intensities that are too great can be stressful and cause mortality (Boeuf and Le Bail, 1999). Many marine larvae also require a period of darkness to properly inflate their SB (Battaglene and Talbot, 1990). The benefits of photoperiod manipulation to improve growth rates have also been well documented. Optimal photoperiod conditions are often a balance between increased feeding time (Sayer,

1998; Petit et al., 2003), and reducing the energy expenditure that can be associated with light (Fielder et al., 2002; Biswas and Takeuchi, 2003; Ginés et al. 2004).

1.2.5 Feeding requirements

Another key restraint to larviculture is the cost of feeding, in particular those costs associated with live feeds. Rotifers (*Brachionus plicatilis*) and *Artemia* nauplii contribute significantly to hatchery costs, and it has been estimated that *Artemia* can contribute up to 17% of hatchery costs for some finfish species, which is second only to the cost of labour (Candreva et al., 1996). The weaning process has been identified by many research groups as a bottleneck in the hatchery process and inadequate information regarding the feeding requirements of a species can restrict feeding opportunities and limit growth (Person Le Ruyet et al., 1993; Cahu and Zambonino Infante, 2001). Battaglene and Talbot (1994) first identified the need for feeding rates to be examined for rearing larval mulloway in order to ensure optimal growth rates were obtained.

As newly hatched larvae absorb yolk and begin feeding it is important to provide nutritionally rich foods that will promote growth and reduce energy expenditure. Typically, rotifers are offered to first feeding mulloway larvae until they are large enough to begin consuming *Artemia* nauplii, and at the PSFI this usually occurs when larvae reach 4 mm in length. Larvae are then maintained on *Artemia* until development allows for consumption and digestion of an inert microdiet, and the first introduction of pellets usually occurs when fish reach 8 mm in length. The stage of development for successful introduction of each new food source, along with the feeding rate of each food type is not properly understood for

mulloway and therefore feeding requirements, including the time of introduction of each new food type and the quantity of food provided, need to be examined in order to promote efficiency within the hatchery.

Another important feeding requirement for cultured fish is the feeding frequency or feeding interval. This is generally well studied for many finfish species as feeding interval can affect growth rates and can influence the total mass gained and specific growth rate of fish (Reddy and Leatherland, 2003). Changes in feeding interval can also influence the size variation of a cohort of fish. Wang et al. (1998) observed that more frequent feeding reduced the size variation of hybrid sunfish larvae. A significant variation of weight within a population can lead to hierarchical feeding patterns and this may further influence the growth performance of individuals (Sayer, 1998), and increase the incidence of cannibalism. A review of optimal feeding frequencies for a range of species completed by Zhou et al. (2003) highlighted the variability in feeding habits for different species and reported that differences can be the result of different feeding behaviour or stomach capacity.

There is a strong relationship between feeding interval and stomach capacity and this can be influenced by the gastric evacuation rate. How quickly appetite will return after feeding is strongly dependent on stomach fullness, and while food is being digested and evacuated from the stomach, fish become increasingly motivated to feed (Olsen and Balchen, 1992).

1.3 Purpose of this thesis

The principal objectives of the research described in this thesis were to determine optimal physical and biological conditions for rearing mulloway in hatcheries. 'Optimal' were defined as those conditions that contribute to maximum survival, growth and development.

The major objectives were:

- to develop ozone disinfection protocols for eggs
- to describe the embryonic development of the species
- to investigate the sensory basis of feeding during development of the species
- to examine optimal light requirements of larval and juvenile mulloway
- to examine optimal feeding requirements for larval and juvenile mulloway

The remainder of this thesis is presented as a series of related but self-contained chapters (Chapters 2 to 6) with a final discussion chapter (Chapter 7) that draws major conclusions from earlier chapters together and summarises the body of work. The references for each chapter are provided at the end of this thesis.

Chapter 1 is the General Introduction, and it outlines the status of mulloway culture in Australia and NSW. This chapter introduces the topics covered and outlines the objectives of the thesis.

Chapter 2 describes a series of trials that determined the tolerance of mulloway to ozone to develop an appropriate disinfection protocol which may reduce the transfer of pathogens from brood stock to eggs and larvae. The embryonic development of the species is described for reference in this thesis and

for future use as a reference guide by hatchery operators. This chapter has been published as a short communication in Aquaculture 318, 475-478.

Chapter 3 describes a series of trials that investigated the optimal light intensity and photoperiod conditions for rearing early larvae (less than 4 mm) and advanced larvae (greater than 4 mm), and determined the effects of different light regimes on SB inflation, growth, feeding and survival.

Chapter 4 describes a series of trials that investigated efficient weaning techniques for mulloway including a suitable ration of *Artemia* and an appropriate time to introduce pellets. This chapter also investigated preference selection of food types in advanced mulloway from rotifers to *Artemia* and then *Artemia* to a pellet microdiet. This chapter has been published online in Aquaculture Research 41, e493-e504.

Chapter 5 describes the optimal feeding interval and photoperiod conditions of juvenile mulloway and the gastric evacuation of the species. This research enabled an efficient feeding protocol to be developed for juvenile mulloway. This chapter has been published in Aquaculture 277, 52-57

Chapter 6 presents research into the sensory development of the species from early larvae to juvenile mulloway. The results were then used to determine some of the biological mechanisms underpinning the results of previous chapters.

Chapter 7 is the General Discussion and the results of the research are discussed with reference to the implications for mulloway aquaculture in Australia.

Chapter 2

Embryonic development of mulloway, *Argyrosomus japonicus*, and egg surface disinfection using ozone[1]

[1]Content from the following chapter is published as a short communication:

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2.1 Introduction

Mulloway (*Argyrosomus japonicus*) are thought to spawn in coastal waters close to beaches (Silberschneider and Gray, 2008) and the buoyant, fertilised eggs hatch after 28-30 h incubation at 23°C (Battaglene and Talbot, 1994). They are currently cultured in NSW at the PSFI hatchery along with yellowtail kingfish (*Seriola lalandii*), snapper (*Pagrus auratus*) and Australian bass (*Macquaria novemaculeata*), and like any aquaculture species are susceptible to pathogens such as bacterial contamination, parasites and viruses that can affect the viability of eggs and larvae (Kusuda et al., 1986; Muroga, 2001; Olafsen, 2001; Hayward et al., 2007). In 2004, a new strain of nodavirus was found in larvae of Australian bass cultured at the PSFI. The virus was identified as the Australian bass nodavirus (Peter Kirkland, pers. comm. Elizabeth Macarthur Agricultural Institute), and it was determined that management of viruses and other pathogens should include an egg surface disinfection regime.

Nodaviruses are a family of neuropathogenic viruses capable of causing disease of the nervous tissue. They are a major pathogen of a wide range of finfish

species and cause significant mortalities in aquaculture worldwide (Munday and Nakai, 1997). The disease caused by nodavirus has been named viral encephalopathy and retinopathy (VER) (Munday et al., 1992) and also viral nervous necrosis (VNN) (Yoshikoshi and Inoue, 1990); VNN will be the term used in this manuscript. The disease is characterized by vacuolation and necrosis of the central nervous system and leads to a range of symptoms that include hyper-inflated swim bladders and atypical swimming, such as whirling, rotating and hyperactivity (Munday and Nakai, 1997; Grotmol et al., 1999).

Virus particles can be transferred horizontally to larvae after hatching, and it has been suggested that the entry point of infection occurs through the intestinal epithelium (Grotmol et al., 1999). Hatchery techniques usually employed to control horizontal transfer of nodavirus in marine hatcheries include strict hygiene protocols, sterilization of influent water and exposure of culture surfaces to high temperatures and pH (Frerichs et al., 2000). The virus can also be transferred vertically, where nodavirus particles present in broodstock ovaries are expelled during spawning (Munday and Nakai, 1997). The point of infection for vertical transfer may be through the connective tissue covering the oocytes, and can be most effectively controlled using egg surface disinfection by submersion of fertilised eggs in iodine or more commonly, ozone baths (Arimoto et al., 1996; Grotmol and Totland, 2000; Azad et al., 2005).

Ozone (O_3) is used for water treatment and disinfection in aquaculture because of its strong oxidation properties. Ozone in aquaculture is usually generated using corona discharge, which converts oxygen (O_2) to ozone (O_3) on contact (Summerfelt and Hochheimer, 1997). For egg disinfection the ozone gas is injected into a water bath in which the fertilised eggs are then submerged. The strength of an

ozone bath is measured using a CT value, which is the concentration of ozone (mg l^{-1}) multiplied by the contact time (min). The oxidising properties of ozone are effective in inactivation of pathogens and some studies have reported successful inactivation of some viruses using an ozone CT of 0.1-0.25 (Liltved et al., 1995; Arimoto et al., 1996). However, ozone can also be harmful to the eggs themselves and therefore, tolerance of eggs to ozone must be assessed on a species by species basis (Liltved et al., 1995; Grotmol et al., 2003).

A reduction in hatching ability has been reported as a common side effect of some ozone treatments (Hall et al., 1981; Grotmol and Totland, 2000; Buchan et al., 2006) and could be due to alterations made to the egg surface, including hardening, or a reduced secretion of hatching enzyme (Grotmol and Totland, 2000). It has been suggested that the tolerance of fish eggs to ozone may be correlated to the diameter of the egg, and also the temperature of incubation. When the eggs of gilthead sea bream (*Sparus aurata*) were treated with ozone at 19ºC using CT values of 0.6 and 1.2, hatchability was not significantly affected; however, when the CT value was increased to 2.4 and 4.8 hatching success was significantly reduced (Ben-Atia et al., 2007). In contrast, haddock (*Melanogrammus aeglefinus*) eggs that were incubated at 6ºC were capable of tolerating ozone CT values of up to 30 without survival decreasing (Buchan et al., 2006).

Only one case of nodavirus has been reported in mulloway larvae and consequently little is known of the biology of the virus and potential impacts to hatchery production. However, nodavirus has been previously detected in the larvae of another sciaenid, red drum (*Sciaenops ocellatus*), and significant mortality was observed from the resulting VNN (Oh et al., 2002). Nodavirus is therefore likely to

cause similar problems for larval production of mulloway if left uncontrolled or unmanaged.

This study was designed to determine the range of ozone CT exposure that did not negatively impact mulloway egg development. The embryonic development of mulloway was described from fertilisation through to hatching to create a visual reference of egg stages for use in mulloway hatcheries and egg disinfection protocols. The first of two experiments was then designed to investigate the combined effects of ozone CT (0, 0.1, 0.5, 1 or 5) and treatment temperature (19, 22 or 25ºC) on the hatching success of mulloway larvae. The second experiment, investigated the effect of ozone on the hatching success of larval mulloway at four different stages of embryonic development (3, 8, 20 or 27 h post fertilisation; HPF). Hatching success for both experiments was measured by the percentage of larvae hatched, the time and duration of hatching and deformities at hatching.

2.2 Materials and methods

2.2.1 Mulloway embryonic development sequence

Mulloway broodstock were induced to spawn using Luteinising Hormone Releasing Hormone analogue (LHRHa) (50 μg kg^{-1}). After fertilisation, a sample of eggs was transferred immediately to the laboratory where digital photographs were taken using a Motic Image Analyser (Extech Equipment Pty. Ltd., Boronia, Victoria, Australia). The remaining eggs were harvested from the tank and transferred to a holding bath maintained at 22ºC. Photographs were taken of eggs from the holding bath at 45, 60, 75, 90 and 120 min after fertilisation and then every hour until hatching. The development of mulloway embryos at 22ºC was then documented using the photographs.

2.2.2 Experiment 1: The interactive effects of ozone CT and temperature on mulloway hatching success

A factorial experiment was designed to determine the effect of ozone CT (CT = 0, 0.1, 0.5, 1 or 5) in combination with three temperatures (19 ± 0.1, 22 ± 0.2 or 25 ± 0.2ºC). Fertilised mulloway eggs were obtained from a commercial mulloway hatchery (Clear Water Mulloway, Millers Forest, NSW, Australia). The eggs were divided into 45 groups, each with 120 eggs, and held in small-size, soft mesh aquarium nets to aid in transfer. Ozone was generated using an Ozotech ozone generator (Model - OZ1BTUS, California, USA) and was bubbled using a ceramic air stone into sea water held in 10 l buckets. The concentration of ozone was

measured three times using a photometer (Model – ozone 1000, Palintest, UK) to ensure an accurate reading was obtained and the concentration of ozone remained stable. The concentration of ozone was adjusted to correspond with that of the desired CT value, therefore standardizing the treatment time for each replicate to 1 min. Each group of eggs was then treated individually with their respective ozone CT value and temperature at the gastrula stage (8-10 HPF) of development. Eggs were then transferred to 10 l hatching vessels, which were suspended in a water bath at the desired treatment temperature. The facilities available limited our ability to replicate hatching temperature; however, the temperature in each experiment vessel was measured hourly to ensure consistency. The 10 l treatment vessels were moderately aerated to maintain egg buoyancy for the duration of the experiment. The eggs were then observed and the hatching milestones: time to start of hatching, 50% hatched and the completion of hatching, were recorded. Larvae and un-hatched eggs were then harvested and examined using a dissection microscope (M5 – 89734, Wild Heerbrugg, Switzerland) to provide total percentage of hatched larvae and larval deformities.

2.2.3 Experiment 2: The effect of ozone treatment on hatching success for mulloway eggs treated at four stages of embryonic development

The effect of ozone on mulloway eggs treated at four embryonic development stages (3 HPF, cleavage; 8 HPF, gastrulation (early neurulation); 20 HPF, late development; and 27 HPF, larvae preparing to hatch) was investigated. Based on the results of Experiment 1, an ozone CT value of 1 was used, as this was the highest CT that did not negatively affect hatching.

Mulloway broodstock at the PSFI were induced to spawn using temperature cues (Partridge et al., 2003), and fertilized eggs were immediately collected and transferred to a holding tank which was maintained at 22 ± 0.2ºC. Replicates of 100 eggs were counted into small–size, soft mesh nets approximately 15 min before the allocated treatment time. Each sampling time consisted of four ozone treated replicates and four control replicates. The control replicates were handled as for ozone treatments but were treated with an ozone CT of 0. Ozone was generated and measured as described for Experiment 1. Eggs were treated for 1 min (1 or 0 mg l^{-1} ozone) and were then transferred to 100 l incubation tanks and maintained at 22 ± 0.3ºC in dark conditions with moderate aeration to maintain egg buoyancy. Eggs were monitored using torch light for the start of hatching, 50% hatched and the completion of hatching. Larvae and un-hatched eggs were then harvested and examined using a dissection microscope (M5 – 89734, Wild Heerbrugg, Switzerland) to provide total percentage of hatched larvae and larval deformities.

2.2.4 Statistical analyses

Statistical analyses were conducted using Statgraphics Version 4.1 (STSC, USA). In Experiment 1, a two-factor analysis of variance (ANOVA) was used to determine the interactive effects of CT value and temperature on the percentage of hatched larvae. As the interactions between CT value and temperature were significant, data were compared using one-factor ANOVA separately for each temperature and each CT. As less than 50% of eggs in the 19ºC and CT = 5 treatment hatched, data for those treatments were not examined for hatching time (HPF 50% hatched) and duration of hatching. The data for Experiment 1 (percent

hatched) was not normally distributed, and transformation failed to normalise the data. However, we are confident that ANOVA is robust enough to cope with these data (Underwood, 1981), and to reduce the risk of a Type 1 error, significance levels were adjusted to $\alpha = 0.01$.

In Experiment 2, means were compared using a two-factor ANOVA ($\alpha = 0.05$) for the percentage of larvae hatched, hours (HPF) until hatching (50% of larvae) and the duration of hatching (hours from initial hatch to final hatch). Where statistical differences were found in analyses ($P<0.05$), the means were separated by the Student – Newman – Keuls test (SNK).

2.3 Results

2.3.1 Embryonic development sequence of mulloway

Fertilised eggs (Fig. 1a) were positively buoyant, spherical in shape (839 ± 15 μm), transparent and contained one, sometimes two, oil globules. The first cell division (denoting the onset of cleavage) took place approximately 45 min post-fertilisation at the animal pole. The next three successive cleavages occurred every 15 min, but began to slow after the fourth division with the result that a clearly defined blastodermal cap (the blastodisc) was visible overlying the yolk mass, 4 HPF (Fig. 1b). Between 4 and 6 HPF, the blastodisc extended radially to eventually encompass the yolk (Fig. 1c) with only a small opening to the perivitelline space, the blastopore, remaining. Onset of gastrulation was apparent 7-8 HPF with the formation of a thickening cellular mass, the embryonic shield (Fig. 1d). Development of a discreet embryonic axis (denoting the onset of neurulation) was evident by 11 HPF (Fig. 1e) and eyes were visible at 13 HPF (Fig. 1f). Stellate melanophores emerged around the head at 16 HPF, and had spread along the entire body by 21 HPF. The embryo had begun to take on the appearance of a larvae by 20 HPF (Fig. 1g) and had begun to increase in length and deplete the yolk stores. The heart, which develops below the neural tube during gastrulation, began to beat at 25 HPF (Fig. 1h) and the embryo began to move. Embryonic development was completed and hatching began to occur at 28 HPF (Fig. 1i), in which 2.3 mm larvae emerged with a 0.8 mm yolk sac, non pigmented eyes and a non-patent digestive tract.

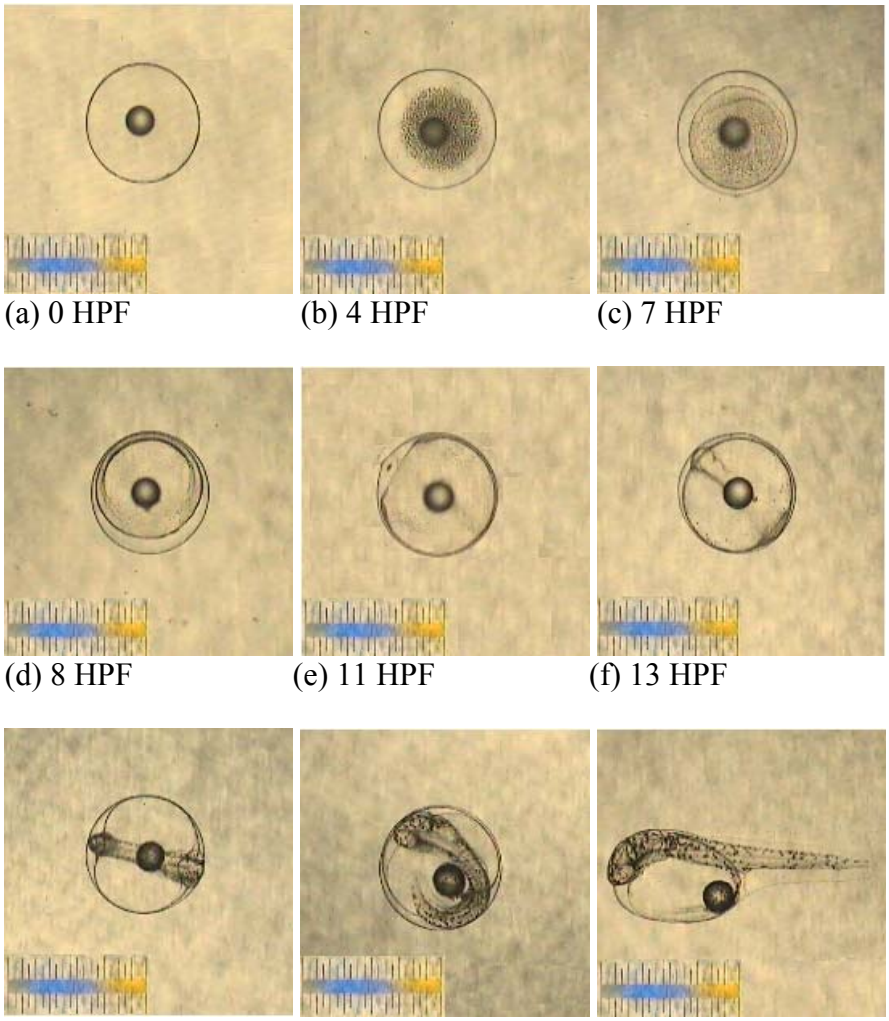

Figure 1: Embryonic development sequence of mulloway eggs incubated at 22ºC. The measurement scale is equal to 1 mm. (a) Fertilised mulloway egg 0 h post fertilisation (HPF); (b) Early blastula stage was reached 4 HPF; (c) The embryonic shield appeared, indicating the onset of gastrulation, at 7 HPF; (d) Early gastrulation, in which the embryo was entering the neurula stage of development. This development stage was studied in Experiment 2; (e) The embryo had reached the neurula stage at 11 HPF, and the neural tube had appeared; (f) Muscle, skeletal structures and organs had begun to develop at 13 HPF; (g) The embryo, 20 HPF, now appearing to take the form of a larval fish, had begun to grow longer and was starting to deplete yolk stores; (h) The heart began beating and the larvae began to move at 25 HPF; (i) A 2.3 mm larvae hatched with a 0.8 mm yolk sac, non pigmented eyes and a disconnected digestive tract at 28 HPF.

2.3.2 Experiment 1: The interactive effects of ozone CT and temperature on mulloway hatching success

All groups of eggs treated with an ozone CT of 5 had a significantly poorer hatching percentage compared with eggs treated with the lower ozone CT values (Table 1). This was exacerbated at the coolest temperature (19ºC), and produced the poorest hatching percentage observed (3.7 ± 1.6%; mean ± SEM). No significant difference in hatching percentage was found between the eggs treated with an ozone CT of 0, 0.1, 0.5 or 1 (Table 1).

Eggs treated and incubated at 25ºC hatched (50% of larvae) significantly faster than eggs maintained at 19 or 22ºC (Table 1). Eggs maintained at 22ºC and treated with a CT of 1 hatched (50% of larvae) significantly faster than eggs treated with CT values of 0.1 or 5 at 22ºC (Table 1). No difference in the hatching time (50% of larvae) was observed for any CT value at 25ºC. No significant difference in hatching time existed within the 19ºC treatments, except for those eggs treated with a CT of 5 where only 3.7% of eggs hatched. This treatment therefore did not generate a value for the time taken for 50% of larvae to hatch.

Significant differences existed for the duration of hatching time (i.e. from the start of hatching to the end of hatching) between temperatures and CT values (Table 1). The eggs treated with a CT value of 5 typically had a significantly longer hatching duration than other CT values. No significant difference existed in hatching duration between the 0, 0.1, 0.5 or 1 CT values within the 19ºC treatment. The duration of hatching at 22ºC occurred most slowly for eggs treated with a CT value of 5. Similarly, eggs at 25ºC hatched more slowly when treated with a CT value of 5 than those treated with CT values of 0, 0.1 or 0.5. The duration of

hatching time varied significantly for eggs treated with a CT value of 1 at 25ºC compared with those treated at 19 and 22ºC.

Table 1: Values (mean ± SEM; $n = 3$) of the percentage of fish hatched, the time until 50% of larvae had hatched (hours post fertilisation; HPF) and the duration of hatching for mulloway eggs treated with ozone in combinations of temperature and ozone CT. (Experiment 1) [1]

Temperature ºC	CT	Percent hatched [2]	HPF to 50% hatched [3]	Duration of hatching (h) [3]
19	0	97.8 ± 2.2^{a}	33.4 ± 0.0^{y}	9.2 ± 1.1^{y}
	0.1	99.0 ± 0.5^{a}	34.2 ± 0.8^{y}	8.0 ± 1.1^{y}
	0.5	98.1 ± 1.9^{a}	33.7 ± 0.4^{y}	6.9 ± 1.4^{xy}
	1	99.0 ± 0.6^{a}	33.6 ± 0.0^{y}	8.6 ± 1.3^{y}
	5	$3.7 \pm 1.6^{b\,y}$	---	---
22	0	99.3 ± 0.4^{a}	$32.3 \pm 0.8^{ab\,y}$	$7.0 \pm 0.1^{b\,y}$
	0.1	99.0 ± 1.0^{a}	$34.1 \pm 0.2^{b\,y}$	$4.5 \pm 0.8^{a\,x}$
	0.5	100.0 ± 0.0^{a}	$32.8 \pm 1.0^{ab\,y}$	$7.1 \pm 0.1^{b\,y}$
	1	98.9 ± 0.1^{a}	$30.6 \pm 1.0^{a\,x}$	$7.1 \pm 0.2^{b\,y}$
	5	$74.8 \pm 12.6^{b\,x}$	$34.6 \pm 0.5^{b\,y}$	$9.8 \pm 1.6^{c\,x}$
25	0	99.2 ± 0.4^{a}	28.7 ± 0.2^{x}	$3.6 \pm 0.7^{a\,x}$
	0.1	100.0 ± 0.0^{a}	28.9 ± 0.1^{x}	$4.0 \pm 0.4^{a\,x}$
	0.5	99.5 ± 0.3^{a}	29.5 ± 0.1^{x}	$3.6 \pm 0.9^{a\,x}$
	1	99.7 ± 0.3^{a}	29.1 ± 0.3^{x}	$4.4 \pm 0.1^{ab\,x}$
	5	$64.6 \pm 15.3^{b\,x}$	29.8 ± 0.7^{x}	$6.5 \pm 1.1^{b\,x}$

[1] Values with the same letter or no letter in the superscript within each temperature (between CT values; a, b, c) and within each CT value (between temperatures; x, y) are not significantly different ($P>0.01$).

[2] Two-factor ANOVA revealed significant effects of temperature, CT, and the interaction between temperature and CT ($P<0.01$). Results of one-factor ANOVA, SNK, presented.

[3] One-factor ANOVA, SNK

2.3.3 Experiment 2: The effect of ozone treatment on hatching success for mulloway eggs treated at four stages of embryonic development

No significant differences were observed between the percentage of hatched larvae when eggs were treated with ozone at any of the embryonic development stages (3, 8, 20, 27 HPF) or the CT values (CT = 0 and 1) (Table 2). No significant differences existed in the time taken for 50% of larvae to hatch or the duration of hatching for any of the embryonic development stages or the CT values (Table 2). Therefore, no significant differences in hatching success occurred for mulloway eggs treated with a CT value of 1, compared with a CT value of 0 at any of the developmental stages measured.

Table 2: Values (mean ± SEM; n = 4) for the percentage of fish hatched, the time until 50% of larvae had hatched (hours post fertilisation; HPF) and the duration of hatching (h) for mulloway eggs treated with ozone. (Experiment 2) [1]

Treatment	HPF	Percent hatched (mean ± SEM)	HPF to 50% hatched	Duration of hatching (h)
CT = 1	3	100 ± 0.0	26.1 ± 0.2	2.0 ± 0.4
	8	100 ± 0.0	27.1 ± 0.2	1.8 ± 0.3
	20	95 ± 5.0	27.9 ± 0.3	1.4 ± 0.4
	27	100 ± 0.0	27.6 ± 0.1	2.1 ± 0.6
CT = 0	3	97.5 ± 2.5	27.2 ± 0.5	1.9 ± 0.2
	8	97.5 ± 2.5	27 ± 0.3	1.9 ± 0.3
	20	85 ± 12.0	28.1 ± 0.3	1.8 ± 0.3
	27	97.5 ± 2.5	27.9 ± 0.1	1.6 ± 0.3

[1] There were no significant differences (P>0.05, two-factor ANOVA, SNK) observed between any of the values.

2.4 Discussion

This study has determined that mulloway larvae incubated at 22ºC began to hatch from 28 HPF. Cleavage began approximately 45 minutes post-hatching and, as is characteristic of teleost fishes in general, was meroblastic (Bone et al., 1995), and confined to the animal pole of the egg, such that cleavage was 'partial' and did not involve the relatively large yolk mass. Successive cleavages occured every 15 min but began to slow after the fourth division. This change in the rate of early cleavage is similar to that of yellowtail kingfish (*S. lalandi*), another temperate marine species, for which the rate of cleavage slows at the 32 cell stage (Moran et al., 2007). Once the blastoderm had extended to envelope the yolk, the mulloway eggs developed quickly; and the embryonic shield was apparent by 7 HPF. The latter arises from the thickening of the germ ring during gastrulation, when the cells rearrange to form three pluripotent germ layers the ectoderm, endoderm and mesoderm. These later give rise to all tissues, organs and the neural tube (Kunz, 2004; Gilbert, 2006). First, the embryonic shield elongates to form the dorsal-ventral axis of the future embryo (Kunz, 2004; Gilbert, 2006). Soon thereafter, in 'neurulation', the development of the neural tube ensues, giving rise to the central and peripheral nervous systems, associated sensory organs, and the neural chord (Gilbert, 2006); all ectodermal in origin.

Stellate melanophores appeared on the head of mulloway embryos around 16 HPF, and by 21 HPF had gradually spread along the entire body. However, pigmentation of the eyes did not occur during embryonic development, but began 1-2 days after hatching. The embryo began to increase in length and take the form of a larval fish by 20 HPF. The increase in the length of the embryo, accompanied by

depletion in the yolk volume, is common in the latter stages of development (Battle, 1944). The heart develops during gastrulation and forms in a cavity between the germ layers called the coelom (Gilbert, 2006). The heart began to beat 25 HPF and coincided with the first movements of the embryo. Embryo movements later play a role in hatching, facilitating rupture of the egg envelope which is weakened by a hatching enzyme, secreted from glands on the head of the embryo (Kunz, 2004).

The trials that followed the embryonic development study have determined that ozone can be applied to mulloway eggs and therefore may be suitable for disinfection purposes. In Experiment 1, the highest concentration of ozone used to treat mulloway eggs without negatively impacting on hatching success was a CT value of 1 (a CT value of 5 significantly reduced hatching success). This is similar to results of a study into the tolerance of gilthead sea bream (*S. aurata*) eggs to ozone, where it was determined that eggs treated with an ozone CT value greater than 1.2 had significantly reduced hatching success (Ben-Atia et al., 2007). These authors suggested the reduction in hatching may have been the result of hardening of the egg chorion by high ozone concentrations which inhibited the ability of the larvae to break through the egg surface. In our trial, the negative effect on hatch rate of a high ozone CT was maximal at the lowest temperature (19ºC), where only 3.7% of larvae hatched. Ben-Atia et al. (2007) suggested that ozone tolerance is species dependent and not temperature dependent. However, for mulloway it appears that low temperatures increase the negative effects of ozone on hatch rate.

In this trial, mulloway eggs hatched more quickly and the duration of hatching was shorter when treated at 25ºC than at the other temperatures. This is typical of teleost eggs, where hatching time is dependent on temperature (Kinne, 1963). Mulloway larvae produced at the PSFI hatchery are currently incubated and

cultured at 22ºC and while hatching in this trial occurred more quickly at 25ºC, there is no apparent benefit to hatching by treating mulloway eggs at the higher temperature and therefore it is recommended that the eggs continue to be treated at the standard temperature of 22ºC. Eggs maintained at 22ºC and exposed to a CT value of 0 in Experiment 1 took longer to hatch than eggs maintained at 22ºC in the embryonic development trial. Photoperiod can influence the incubation period in some species (Duncan et al., 2008), therefore, the ambient photoperiod used in Experiment 1 may have increased the incubation period of mulloway eggs.

While it is important to determine the tolerance of the eggs to ozone, it is also necessary to determine the level at which the pathogen is inactivated by ozone. Nothing is reported on the effective CT value required to inactivate the Australian bass nodavirus, therefore it may be prudent to treat mulloway eggs with the maximum ozone CT value the species can tolerate in order to minimise the risk of nodavirus infection. The striped jack nervous necrosis virus (SJNNV) has been well studied and it was established that an ozone CT value of 0.25 is required to inactivate SJNNV (Arimoto et al., 1996). The inactivation of infectious pancreatic necrosis virus (IPNV) using ozone was also studied and it was found that exposure of the IPNV to ozone for 60 s at 0.1-0.2 mg l^{-1} (CT = 0.1-0.2) successfully inactivated the virus (Liltved et al., 1995). Therefore, as a CT value of 1 did not reduce hatching success, but is likely to be effective as a disinfectant, it is recommended that mulloway eggs should be disinfected using an ozone CT value of 1 in water of 22ºC. It is important to note that this study has not determined the upper limit of the tolerance of mulloway eggs to ozone and further studies may determine a higher maximum tolerance level of mulloway eggs to ozone.

The ozonation protocol developed in Experiment 1 was then tested on mulloway embryos at four stages of development (3, 8, 20 or 27 HPF), to determine if mulloway embryos were more susceptible to the effects of ozone at different stages of development. As described previously, at 3 HPF, eggs were undergoing cleavage; at 8 HPF, gastrulation had begun, the embryonic shield had developed and the neurual tube was forming (eggs in Experiment 1 were also treated at this stage); at 20 HPF the embryo was in the final stages of development and had visible eyes, pigment and skeletal structures; and by 27 HPF the embryo was nearing hatching and the presence of hatching enzyme was likely (Kunz, 2004).

No differences were observed between the hatching successes of embryos treated at any of the developmental stages and as the intention of the ozone treatment is to disinfect the eggs of viruses and bacteria, it would be sensible to treat eggs early in their development. Grotmol et al. (1999) found that after inoculation with nodavirus, the incubation period of Atlantic halibut (*Hippoglossus hippoglossus*) eggs significantly reduced with increasing dose rate. It is therefore advisable to treat eggs with ozone soon after fertilisation to reduce the chance of interference to the hatching process by pathogens.

In summary, mulloway eggs incubated at 22ºC begin to hatch approximately 28 HPF. Ozone disinfection up to a CT value of 1 did not reduce hatching success, while a CT of 5 produced poor hatching results. To maintain standard egg development the eggs should be treated and incubated at 22ºC, which is the current protocol employed at the PSFI hatchery. Ozone toxicity (CT = 1) does not change with embryonic development and therefore it is advisable to disinfect mulloway eggs early in development to reduce adverse effects associated with viruses and bacteria on the incubation and hatching of fish eggs. The susceptibility of the

Australian bass nodavirus and other pathogens to ozone still needs to be determined to ensure the protocols listed here are appropriate for disinfection. Also, the effects of ozone treatment on the post-hatching development of larvae need to be considered in future experiments.

Chapter 3

Optimal photoperiod and light intensity for feeding and ontogeny of larval mulloway, *Argyrosomus japonicus*

3.1 Introduction

Some of the most widely investigated parameters for improving larval rearing protocols are photoperiod and light intensity requirements. Optimal photoperiod and light intensity can improve growth rates by increasing the available feeding time of visually mediated feeders and by improving the ability of larvae to detect prey (Sayer, 1998; Boeuf and Le Bail, 1999). The primary sensory mode for feeding of many species of larvae is vision, and for most species the eyes are pigmented and considered functional by first feeding (Blaxter, 1986; Pankhurst, 2008). Marine fish generally rely on a mosaic of retinal cone photoreceptor cells at first feeding, which allow for prey detection in daylight conditions (Takashima and Hibiya, 1995; Campbell, 1996; Kunz, 2007). Larvae later develop retinal rod photoreceptor cells, which are stimulated under lower light intensities and thus allow the larvae to adapt vision to light and dark environmental conditions (Blaxter, 1986; Takashima and Hibiya, 1995). As such, daylight provides for acute visual resolution and image formation by cones whereas rod vision sacrifices acuity for increased sensitivity in dim light.

The receptivity of fish to light is different for each species and can change with increasing ontogeny (Boeuf and Le Bail, 1999). Most larvae require a

minimum light intensity to properly detect and catch food, while light intensities that are too great can be stressful and cause mortality (Boeuf and Le Bail, 1999). For many species, optimal light intensity requirements are similar to those found in natural environmental conditions. Summer flounder (*Paralichthys dentatus*) larvae were found to perform better under low light conditions, consistent with their spawning grounds in offshore shelf waters at depths of up to 200 m (Watanabe and Feeley, 2003), while the growth and survival of black sea bass (*Centropristis striata*) improved with increasing light intensities and authors reported this to be consistent with shallow, near-shore locations where the larvae are found in nature (Copeland and Watanabe, 2006).

In contrast to light intensity, improved growth and development of larvae can be achieved by providing an artificial photoperiod. Many trials have determined that an artificially long day length stimulates growth in marine species (Boeuf and Le Bail, 1999). In first feeding snapper (*Pagrus auratus*) larvae, increasing photoperiod was found to promote growth and development (Fielder et al., 2002). Trotter et al. (2003) also observed improved growth, survival and swim bladder (SB) inflation of striped trumpeter (*Latris lineate*) under a long day length (18-24 h of light).

SB inflation is often investigated in conjunction with photoperiod as inflation usually occurs in darkness and around the time of first feeding. An extended dark period has been observed to promote SB inflation in many species of marine larvae and therefore it is necessary to consider this milestone when examining optimal photoperiod regimes (Battaglene and Talbot, 1990). A successful photoperiod regime for marine larvae is one that provides adequate

darkness for SB inflation while maintaining optimal light hours for growth and survival.

The current light management protocols for intensive clear water culture of mulloway (*Argyrosomus japonicus*) at the PSFI hatchery include: complete darkness until first feeding; a 12L:12D photoperiod from first feeding at 2 days after hatching (dah) until 10 dah; followed by an 18L:6D photoperiod. Light intensities are usually set at 1-2 μmol s^{-1} m^{-2}. Two experiments were designed to examine the light requirements of mulloway larvae. The first experiment investigated the effects of photoperiod and light intensity on the growth (length and weight), SB inflation and survival of early larval mulloway (2-8 dah), and the second experiment investigated the effects of photoperiod and light intensity on the growth (length and weight), food consumption and survival of advanced larval mulloway (14-34 dah).

3.2 Materials and methods

3.2.1 Fish and facilities

Fertilised mulloway eggs were sourced from a local mulloway hatchery (Clear Water Mulloway, Millers Forest, NSW). Eggs were treated with an ozone CT value of 0.2 at the PSFI to disinfect the eggs of pathogens and were then transferred to a 2000 l clear water flow-through (1.5 l min^{-1}) larviculture tank. The tank was maintained in darkness until 1 dah, when the water level in the tank was lowered to concentrate the larvae, and fish for Experiment 1 were removed. The tank was then refilled and the photoperiod was set at 12L:12D and the light intensity was set at 1 μmol s^{-1} m^{-2}. Larvae were maintained on nutritionally enriched (Algamac 3050, Aquafauna Bio-Marine Inc., California, USA) rotifers (*Brachionus plicatilis*) at 10 ml^{-1}. Larvae for Experiment 2 were removed in a similar manner 13 dah.

3.2.2 Water quality analyses

Water quality was measured using a water quality meter (Horiba U-10, Japan) and the mean and standard deviation calculated. The mean water quality parameters for both trials were: pH (8.2 ± 0.8), dissolved oxygen (8.1 ± 0.5 mg l^{-1}), temperature (20.1 ± 0.8ºC), and salinity (31.5 ± 2.1‰). Water quality was consistent across all tanks for the duration of each experiment.

3.2.3 Experiment 1: Light intensity and photoperiod requirements of early larval mulloway

Groups of 800 larvae (1 dah); (mean total length [TL] = 2.4 mm), were transferred to each of 27, 100 l flow-through (200 ml min^{-1}) experiment tanks. The tanks were maintained in darkness until the following day (2 dah), when the experiment began. The experimental treatments included two light intensities (1 or 10 μmol s^{-1} m^{-2}) and five photoperiods (24L:0D, 18L:6D, 12L:12D; 6L:18D or 0L:24D). Black plastic sheeting surrounded each tank to exclude light leakage between adjacent tanks and from within the experiment room and daily operations were carried out using torchlight. Lights were set to turn on immediately at 0830 h, and larvae were fed nutritionally enriched rotifers (10 ml^{-1}) at 0900 and 1500 h each day. Water quality parameters were taken from 8 randomly selected tanks each day and each tank was siphoned daily to remove wastes. Larvae (n = 20) were sampled every two days for length (mm), dry weight (mg), SB inflation and the presence or absence of food in their stomach. The experiment ended on day 8 (after hatching), and the total survival was assessed.

3.2.4 Experiment 2: Light intensity and photoperiod requirements of advanced larval mulloway

Groups of 800 larvae (13 dah) (mean TL, 4.5 mm) were transferred to each of 27, 100 l flow-through (2 l min^{-1}) experiment tanks. The experiment treatments included two light intensities (1 or 10 μmol s^{-1} m^{-2}) and five photoperiods (24L:0D, 18L:6D, 12L:12D, 6L:18D or 0L:24D). As in Experiment 1, black plastic sheeting

surrounded each tank to exclude light leakage and daily operations were performed using torchlight. Lights were set to turn on immediately at 0830, and larvae were fed at 0900 and 1500 h each day. Larvae were fed nutritionally enriched rotifers (10 ml^{-1}) for the first three days of the experiment and were then weaned on to nutritionally enriched *Artemia* (1 ml^{-1}). As in Experiment 1, water quality parameters were taken from 8 randomly selected tanks each day and each tank was siphoned to remove wastes. Larvae (n = 20) were sampled every four days for length (mm), dry weight (mg) and the presence or absence of food. The experiment ended on day 34 (after hatching), and the total survival was assessed.

3.2.5 Statistical analyses

Data were assessed for homogeneity of variance using Cochran's test (Underwood, 1997). When variances were heterogeneous, data were log transformed to establish homogeneity. The experiments were designed for two-factor ANOVA after the exclusion of the 0L:24D photoperiod treatment, in which larvae were maintained in 24 h of darkness and did not include light intensity as a factor. Therefore, all treatments that included light were analysed using two-factor ANOVA (α = 0.05) to determine the effects of photoperiod and light intensity on length, weight, SB inflation, food consumption and survival. All treatments, including the 0L:24D photoperiod, were then analysed using one-factor ANOVA to determine differences between fish held in 24 h of darkness and fish held in other photoperiods. Where statistical differences were found, (P<0.05), the means were separated using the Student-Newman-Keuls (SNK) test. Statistical analyses were conducted using Statgraphics Version 4.1 (STSC, USA).

3.3 Results

3.3.1 Experiment 1: Light intensity and photoperiod requirements of early mulloway larvae

Photoperiod had a significant (P<0.05) effect on the growth, development, feeding and survival of early larvae; however, there were no effects of light intensity on any of the parameters measured and there were no interactions observed between photoperiod and light intensity (Tables 3-6).

The weight of larvae increased in all treatments throughout the experiment; however, no significant difference was observed between the weights at any of the sample periods (Table 3). No larvae in the 0L:24D treatment were alive at the 8 dah sample period, therefore no weight value could be recorded.

Table 3: Weights (mg; means ± SEM) of mulloway larvae exposed to different photoperiods and light intensities at each sample period (4, 6 and 8 days after hatching [dah]). (Experiment 1)[1]

Photoperiod (h)	Light intensity (μmol s^{-1} m^{-2})	4 dah	6 dah	8 dah
24L:0D	1	0.035 ± 0.005	0.058 ± 0.008	0.068 ± 0.012
	10	0.044 ± 0.004	0.061 ± 0.001	0.074 ± 0.007
18L:6D	1	0.040 ± 0.001	0.043 ± 0.002	0.080 ± 0.012
	10	0.027 ± 0.014	0.049 ± 0.008	0.069 ± 0.007
12:L12D	1	0.035 ± 0.012	0.047 ± 0.011	0.061 ± 0.006
	10	0.035 ± 0.003	0.048 ± 0.001	0.056 ± 0.004
6L:18D	1	0.030 ± 0.003	0.047 ± 0.006	0.064 ± 0.009
	10	0.022 ± 0.003	0.043 ± 0.007	0.056 ± 0.000
0L:24D	0	0.029 ± 0.009	0.039 ± 0.005	-

[1] Weight was not significantly affected (P>0.05; two-factor ANOVA - treatments that contained light; one-factor ANOVA - all treatments) by photoperiod, light intensity or the interaction between photoperiod and light intensity.

Significant differences were observed between fish lengths in different photoperiods for each sample period (Table 4). Larvae in the 6L:18D and 0L:24D photoperiods were significantly shorter than all other photoperiods for each sample period. Larvae sampled at 6 and 8 dah were significantly longer in the 18L:6D photoperiod compared with those in the 12L:12D photoperiod. No significant difference in length was observed between larvae in the 24L:0D photoperiod and the 18L:6D and 12L:12D photoperiods. No larvae in the 0L:24D treatment were alive at the 8 dah sample period, therefore no length value could be recorded.

Table 4: Lengths (mm; means ± SEM) of mulloway larvae exposed to each photoperiod and light intensity at each sample period (4, 6 and 8 days after hatching [dah]). (Experiment 1)[1]

Photoperiod (h)	Light intensity (μmol s^{-1} m^{-2})	4 dah	6 dah	8 dah
24L:0D	1	$2.8 \pm 0.0^{b*}$	$3.2 \pm 0.0^{b*}$	3.6 ± 0.1^{bc}
	10	$2.8 \pm 0.0^{b*}$	$3.3 \pm 0.0^{b*}$	3.7 ± 0.0^{bc}
18L:6D	1	$2.8 \pm 0.0^{b*}$	$3.4 \pm 0.1^{c*}$	3.8 ± 0.1^{c}
	10	$2.8 \pm 0.0^{b*}$	$3.4 \pm 0.1^{c*}$	3.7 ± 0.0^{c}
12L:12D	1	$2.8 \pm 0.1^{b*}$	$3.2 \pm 0.1^{b*}$	3.6 ± 0.0^{b}
	10	$2.8 \pm 0.0^{b*}$	$3.2 \pm 0.0^{b*}$	3.6 ± 0.1^{b}
6L:18D	1	2.6 ± 0.0^{a}	2.6 ± 0.1^{a}	3.0 ± 0.0^{a}
	10	2.6 ± 0.1^{a}	2.8 ± 0.0^{a}	3.0 ± 0.0^{a}
0D:24D	0	2.6 ± 0.1	2.8 ± 0.0	-

[1] Mean lengths at each sampling period with the same letter in the superscript between photoperiods are not significantly different ($P>0.05$; two-factor ANOVA - treatments that contained light); there were no significant differences in lengths of fish between light intensities, and no significant interactions existed between photoperiod and light intensity.
* denotes significant difference from 0L:24D ($P<0.05$; one-factor ANOVA - all treatments).

The proportion of fish feeding was significantly affected by photoperiod (Table 5). At the first sample period (4 dah) significantly fewer larvae in the 6L:18D and 0L:24D photoperiods were consuming food than in the other photoperiods. At the second sample period (6 dah) the number of larvae consuming food had increased in the 6L:18D photoperiod and there was no significant difference in food consumption between this photoperiod and the other light photoperiods; however, there were still significantly fewer larvae feeding in the dark photoperiod (0L:24D).

Table 5: The percentage of fish feeding (means ± SEM) when exposed to each photoperiod and light intensity at each sample period (4, 6 and 8 days after hatching [dah]). (Experiment 1)[1]

Photoperiod (h)	Light intensity (μmol s^{-1} m^{-2})	4 dah	6 dah	8 dah
24L:0D	1	94.0 ± 6.0^{b} *	78.0 ± 1.3 *	100.0 ± 0.0
	10	88.0 ± 11.7^{b} *	98.3 ± 0.2 *	100.0 ± 0.0
18L:6D	1	86.7 ± 10.0^{b} *	96.0 ± 0.4 *	96.7 ± 0.3
	10	97.7 ± 2.3^{b} *	100.0 ± 0.0 *	100.0 ± 0.0
12:L12D	1	90.3 ± 2.3^{b} *	75.0 ± 1.0 *	93.3 ± 0.1
	10	95.7 ± 2.2^{b} *	89.3 ± 1.1 *	92.7 ± 0.4
6L:18D	1	2.3 ± 2.3^{a}	69.0 ± 1.3 *	61.7 ± 1.2
	10	3.0 ± 2.4^{a}	71.0 ± 0.4 *	81.7 ± 0.3
0L:24D	0	6.0 ± 3.1	12.0 ± 0.3	-

[1] The percentage of fish feeding at each sampling period with the same letter in the superscript between photoperiods are not significantly different ($P>0.05$; two-factor ANOVA - treatments that contained light); there were no significant differences in the percentage of fish feeding between light intensities, and no significant interactions existed between photoperiod and light intensity.
* denotes significant difference from 0L:24D ($P<0.05$; one-factor ANOVA - all treatments).

Survival was significantly reduced in the 6L:18D photoperiod compared with larvae in photoperiods with 12 to 24 h of light (Table 6). Survival in the 0L:24D photoperiod was significantly lower than in all other photoperiods and no larvae remained at the completion of the experiment.

SB inflation was significantly improved in larvae held in the 6L:18D photoperiod compared with the 0L:24D, 18L:6D and 24L:0D photoperiods at the first sample period, 4 dah (Table 6). No significant differences occurred in SB inflation between larvae in the 12L:12D photoperiod and the other photoperiods. Larvae in all photoperiods had inflated their SB by 6 dah.

Table 6: The percentage survival and SB inflation (means ± SEM) of mulloway larvae exposed to each photoperiod and light intensity. (Experiment 1)[1]

Photoperiod (h)	**Light intensity (μmol s^{-1} m^{-2})**	**% Survival[2]**	**% SB Inflation[3]**
24L:0D	1	$15.4 \pm 3.5^{b*}$	82.0 ± 3.2^{a}
	10	$20.0 \pm 11.5^{b*}$	89.0 ± 0.6^{a}
18L:6D	1	$29.2 \pm 6.1^{b*}$	78.3 ± 5.8^{a}
	10	$33.8 \pm 2.6^{b*}$	93.3 ± 4.1^{a}
12:L12D	1	$20.5 \pm 5.3^{b*}$	94.0 ± 3.5^{ab}
	10	$16.1 \pm 3.3^{b*}$	89.3 ± 3.6^{ab}
6L:18D	1	$4.9 \pm 1.6^{a*}$	$98.0 \pm 2.0^{b*}$
	10	$6.8 \pm 1.3^{a*}$	$100 \pm 0.0^{b*}$
0L:24D	0	0.0 ± 0.0	88.3 ± 3.3

[1] The percentage survival and SB inflation with the same letter in the superscript between photoperiods are not significantly different ($P>0.05$; two-factor ANOVA - treatments that contained light); there were no significant differences in the percentage survival and SB inflation between light intensities, and no significant interactions existed between photoperiod and light intensity.
[*] denotes significant difference from 0L:24D ($P<0.05$; one-factor ANOVA - all treatments).
[2] Percentage survival was measured at the end of the experiment.
[3] Percentage SB (swim bladder) inflation was measured at 4 dah (days after hatching).

3.3.2 Experiment 2: Light intensity and photoperiod requirements of advanced mulloway larvae

As in Experiment 1, photoperiod had a significant effect on the growth, feeding and survival of advanced larvae; however, there were no effects of light intensity on any of the parameters measured and there were no interactions observed between photoperiod and light intensity.

The weights of larvae were not significantly different at the completion of the experiment. The only difference observed in fish weight occurred at 22 dah, at which time the larvae in the 18L:6D photoperiod were significantly heavier than those in the 6L:18D and 0L:24D photoperiods (Table 7).

Table 7: Weights (mg; means ± SEM) of mulloway larvae exposed to each photoperiod and light intensity at each sample period (18, 22, 26, 30 and 34 days after hatching [dah]). (Experiment 2)[1]

Photoperiod (h)	Light intensity (μmol s^{-1} m^{-2})	18 dah	22 dah	26 dah	30 dah	34 dah
24L:0D	1	0.58±0.30	0.70±0.05^{ab}	1.60±0.15	3.22±0.15	5.25±0.01
	10	0.51±0.03	0.75±0.02^{ab}	1.55±0.29	3.44±0.50	6.60±0.69
18L:6D	1	0.50±0.15	0.68±0.02^{b}	1.31±0.05	2.28±0.23	4.54±0.08
	10	0.43±0.06	0.77±0.04^{b} *	1.62±0.35	3.26±0.77	5.46±1.00
12L:12D	1	0.36±0.00	0.63±0.06^{ab}	1.31±0.02	2.43±0.11	4.97±0.47
	10	0.45±0.10	0.54±0.05^{ab}	1.47±0.10	2.34±0.04	3.87±0.01
6L:18D	1	0.26±0.00	0.53±0.01^{a}	1.20±0.05	2.76±0.12	4.84±0.24
	10	0.24±0.03	0.49±0.02^{a}	1.01±0.06	1.72±0.03	3.47±0.01
0L:24D	0	0.54±0.23	0.47±0.02	1.02±0.14	1.87±0.12	3.51±0.55

[1] Mean weights at the 22 dah sampling period with the same letter in the superscript between photoperiods are not significantly different (P>0.05; two-factor ANOVA - treatments that contained light). Weight was not significantly affected by photoperiod (except at the 22 dah sampling period), light intensity or the interaction between photoperiod and light intensity.
* denotes significant difference from 0L:24D (P<0.05; one-factor ANOVA - all treatments)
The mean weight of fish at commencement of the experiment (13 dah) was 0.15 mg.

Larvae lengths were also not significantly different at the end of the experiment. However, significant differences did exist for the first three sample periods (18, 22 and 26 dah) (Table 8), when larvae held in the long light photoperiods (18L:6D and 24L:0D) were significantly longer than those in the long dark photoperiods (0L:24D and 6L:18D).

Table 8: Lengths (mm; means ± SEM) of mulloway larvae exposed to each photoperiod and light intensity at each sample period (18, 22, 26, 30 and 34 days after hatching [dah]). (Experiment 2)[1]

Photoperiod (h)	Light intensity (μmol s^{-1} m^{-2})	18 dah	22 dah	26 dah	30 dah	34 dah
24L:0D	1	$5.7 \pm 0.2^{b*}$	$7.7 \pm 0.2^{bc*}$	$9.9 \pm 0.4^{b*}$	12.2 ± 0.3	14.2 ± 0.0
	10	$5.7 \pm 0.0^{b*}$	$7.6 \pm 0.3^{bc*}$	$9.6 \pm 0.2^{b*}$	12.3 ± 0.3	15.1 ± 0.4
18L:6D	1	$5.6 \pm 0.1^{b*}$	$7.7 \pm 0.1^{c*}$	9.1 ± 0.1^{ab}	11.4 ± 0.1	13.8 ± 0.2
	10	$5.5 \pm 0.1^{b*}$	$7.8 \pm 0.1^{c*}$	9.7 ± 0.4^{ab}	12.2 ± 0.9	14.5 ± 0.5
12L:12D	1	5.2 ± 0.1^{a}	$7.4 \pm 0.2^{b*}$	9.1 ± 0.1^{ab}	11.4 ± 0.0	14.5 ± 0.2
	10	5.1 ± 0.0^{a}	$7.1 \pm 0.2^{b*}$	9.2 ± 0.1^{ab}	11.4 ± 0.0	13.3 ± 0.0
6L:18D	1	5.3 ± 0.1^{a}	6.8 ± 0.1^{a}	8.9 ± 0.1^{a}	11.3 ± 0.3	14.1 ± 0.4
	10	5.2 ± 0.1^{a}	6.7 ± 0.2^{a}	8.4 ± 0.2^{a}	10.6 ± 0.3	11.6 ± 1.0
0L:24D	0	5.0 ± 0.1	6.5 ± 0.1	8.2 ± 0.4	10.5 ± 0.4	12.8 ± 0.4

[1] Mean lengths at each sampling period with the same letter in the superscript between photoperiods are not significantly different ($P>0.05$; two-factor ANOVA - treatments that contained light); there were no significant differences in lengths of fish between light intensities, and no significant interactions existed between photoperiod and light intensity.
* denotes significant difference from 0L:24D ($P<0.05$; one-factor ANOVA - all treatments).
The mean length of fish at commencement of the experiment (13 dah) was 4.5 mm.

The percentage of larvae consuming food was high throughout the experiment and was not affected by photoperiod or light intensity. The percentages (mean ± SEM) of fish feeding ranged from 90.0 ± 7.6 to 100 ± 0.0 %. All fish were observed feeding from 26 dah.

Survival was significantly improved in the 0L:24D photoperiod compared with survival in all other photoperiods (Table 9).

Table 9: The percentage survival (means ± SEM) of mulloway larvae exposed to each photoperiod and light intensity at completion of the experiment. (Experiment 2) [1]

Photoperiod (h)	**Light intensity (μmol s^{-1} m^{-2})**	**% Survival**
24L:0D	1	0.7 ± 0.7*
	10	9.7 ± 3.0*
18L:6D	1	18.6 ± 4.6*
	10	23.3 ± 9.9*
12L:12D	1	14.3 ± 9.7*
	10	1.5 ± 1.5*
6L:18D	1	21.3 ± 2.5*
	10	2.8 ± 2.6*
0L:24D	0	54.5 ± 12.9

[1] There were no significant differences in the percentage survival between photoperiods, light intensities or the interaction between photoperiod and light intensity (P>0.05; two-factor ANOVA - treatments that contained light).
* denotes significant difference from 0L:24D (P<0.05; one-factor ANOVA - all treatments)

3.4 Discussion

This study determined that length, survival, food consumption and SB inflation of early larval mulloway were influenced by photoperiod; however, there were no observed effects of the light intensities tested. Larvae in the 0L:24D and 6L:18D photoperiods were significantly shorter than fish held in longer light periods, which is similar to many studies that have reported poor growth in photoperiods with long dark periods and attributed this to the lack of feeding time available to larvae that rely on vision for feeding (Cobcroft and Pankhurst, 2003; Yoseda et al., 2008). No significant differences were observed between weights of larvae; however, as the mean dry weights of larvae were as low as 0.022 mg, it is likely that unavoidable error in the weighing process may have influenced these results.

Feeding rate was low in larvae maintained in the 0L:24D and 6L:18D photoperiods at the first sample period (4 dah); however, a small percentage of larvae did consume food. The incidence of food consumption in larvae fed during darkness has been identified before and Huse (1994) suggested the cause may be accidental ingestion through osmoregulatory drinking. The percentage of larvae consuming food in the 6L:18D photoperiod increased considerably by the second sample period (6 dah), and significantly more larvae were consuming food in the light photoperiods than in the 0L:24D photoperiod. The continued lack of food ingested by larvae in the 0L:24D photoperiod resulted in poor survival and by the third sample period (8 dah), no larvae had survived. Similar results have been reported with marine larval species suffering mortality through inadequate food consumption in dark conditions. A study into Atlantic cod (*Gadus morhua*) found

reduced survival in short light photoperiods and authors attributed this to a shorter feeding period and less chance of encountering prey (Puvanendran and Brown, 2002). Similarly, food consumption and survival in vundu catfish (*Heterobranchus longifilis*) larvae were reduced in long dark periods compared with long light periods and the authors suggested this was the result of restricted visual environment (Nwosu and Holzlöhner, 2000). The reduced growth and survival in mulloway larvae held in long dark photoperiods provides evidence that early larval mulloway are predominantly visual feeders and require light to detect prey.

SB inflation was also influenced by photoperiod, and at the first sample period (4 dah), a significantly greater percentage of larvae had inflated their SB in the 6L:18D photoperiod compared with larvae in the 0L:24D, 18L:6D and 24L:0D photoperiods. Improved SB inflation in photoperiods with long dark periods is not uncommon for Australian marine larval species. SB inflation in striped trumpeter (*Latris lineate*) (Trotter et al. 2003) and sand whiting (*Sillago ciliata*) (Battaglene et al. 1994) has been reported to occur after the onset of darkness, and it has been suggested that this may be an adaptation for predator avoidance (Martin-Robichaud and Peterson, 1998). SB inflation in mulloway larvae was achieved in all treatments by the second sample period (6 dah); however, as Battaglene and Talbot (1994) discussed, poor SB inflation can reduce growth and survival in mulloway larvae, and therefore efficient SB inflation, around the time of first feeding, should be promoted by providing an adequate dark phase.

The optimal photoperiod for early mulloway larvae should include a long dark phase to ensure SB inflation, in conjunction with an adequate light phase to enhance feeding time and improve survival. As fish in the 12L:12D photoperiod did not perform poorly in any of the parameters measured but had adequate darkness for

SB inflation and enough light to allow for feeding, it is a suitable photoperiod for early larvae. However, as growth (length) was greater in the long light photoperiods it is recommended that the photoperiod be adjusted to 18L:6D once SB inflation has been completed.

The effects of photoperiod and light intensity on advanced mulloway larvae were different to those observed for the early larvae trial. Growth (weight and length) in the long dark photoperiods (0L:24D and 6L:18D) was not significantly different to those of the long light photoperiods at the completion of the experiment; however, differences were observed in early samples. As larvae developed and increased in length, their apparent ability to perform well in long dark periods improved. Larvae held in the longer dark periods had significantly poorer growth up until, and including, the second sample period (22 dah); however, by the third sample period (26 dah) no differences existed between the lengths or weights of larvae in any of the photoperiods. Marine fish predominantly rely on vision for prey detection; however, mechanoreception and chemoreception play an increasingly important role in feeding as larval development proceeds (Blaxter, 1986; Pankhurst, 2008). As the suite of sensory organs develops, the feeding opportunities available to larvae increase (Cobcroft and Pankhurst, 2003). The improved function of the sensory organs may have increased the feeding ability of larvae in the dark phase, and may explain the improved growth rates in fish from the 0L:24D and 6L:18D photoperiods at the later sampling periods.

In addition to good growth rates, the survival of larvae maintained in the 0L:24D photoperiod was significantly better than in all other photoperiods. The improved feeding ability of advanced larvae in dark conditions compared with early larvae, is likely to have contributed to this increase in survival percentage but it

does not explain why survival was better than the other photoperiods. Cannibalism was reported by Battaglene and Talbot (1994) to be a significant contributor to mortality of larval mulloway. However, larval mulloway that are kept in the dark when food is unavailable or in short supply display reduced cannibalism and agonistic behaviour (Battaglene, 1996). Mulloway have been observed in larval rearing tanks to consume other larvae tail first. This has also been recorded for walleye (*Sander vitreus*), and it was reported that the prey larvae are swallowed tail first because the large head size of the prey makes it difficult to consume (Summerfelt, 2005). The execution of tail first cannibalism is likely to require vision and this may explain the increased mortality of fish exposed to light periods.

In addition to the role light plays in feeding and survival of larval fish, pigmentation and digestive enzyme activity can also be affected by light (Boeuf and Le Bail, 1999; Shan et al., 2008). Doolan et al. (2007) described the difficulty of marketing snapper (*Pagrus auratus*) with pigmentation anomalies, while Shan et al. (2008) described changes in digestive enzyme activity of miiuy croaker (Miichthys miiuy) larvae exposed to differnct photoperiod regimes. It is unclear what effect 24 h of darkness will have on the pigmentation and enzymatic activity of mulloway and therefore it is recommended that the photoperiod should include some light until the potential consequences of 24 h of light are investigated.

In summary, early larval mulloway require an adequate dark phase for successful SB inflation but growth and survival are improved in longer light photoperiods. Therefore, as early larvae in the 12L:12D photoperiod displayed successful SB inflation, good growth rates and good survival, it is suggested that larvae are maintained in a 12L:12D photoperiod from first feeding until SB inflation is complete. The light regime should then be changed to a 18L:6D photoperiod to

maximise feeding time and growth rates. It was then determined that advanced mulloway larvae are able to feed in light and dark conditions; however, as it was determined that 18 h of light was the most suitable photoperiod for growth in early larvae and it has been reported that 18 h of light is also appropriate for rearing juvenile mulloway (Ballagh et al., 2008), it is concluded that advanced larvae should also be reared in 18 h of light.

Chapter 4

Weaning requirements of larval mulloway,

Argyrosomus japonicus[1]

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4.1 Introduction

Hatchery production of mulloway (*Argyrosomus japonicus*) and other finfish species in Australia is limited in part by high production costs (Ballagh et al., 2008; Fielder et al., 2008b). Some of the most significant costs are attributed to feeding, particularly those associated with live feeds, such as rotifers and *Artemia*, required to rear early larvae. It is well documented that the cost and quality of *Artemia* can fluctuate over time as the supply is dependent on both worldwide aquaculture demand and weather patterns affecting the primary harvest areas (Sorgeloos et al., 2001; Callan et al., 2003). In addition to the unstable nature of *Artemia* supply, the percentage of hatchery feed costs attributed to *Artemia* is substantial and it is therefore desirable to find alternative feeding strategies for rearing marine fish larvae.

While there is a strong argument to reduce reliance on *Artemia*, some risks are associated with early weaning and reduced *Artemia* use. Person Le Ruyet et al. (1993) reported that up to 80% of *Artemia* costs could be saved by weaning sea bass (*Dicentrachus labrax*) 15 days earlier than the usual protocol; however, the risks of

doing so included a decrease in larval weight gain, increased larval size variation and skeletal abnormalities. Recent larval weaning studies have endeavoured to reduce feed costs and to overcome problems associated with decreasing the use of *Artemia*; however, many studies have suggested that some *Artemia* should be included in the weaning process to improve growth and survival (Callan et al., 2003; Curnow et al., 2006; Fletcher et al., 2007).

The weaning success of any finfish species from live feeds onto a formulated microdiet is partly dependent on the composition of the diet and the ability of the larvae to select and digest a non-live food (Person Le Ruyet et al., 1993; Kolkovski, 2001; Shaw et al., 2003). Stomach development and the production of digestive enzymes are regarded as indicators for the transition from live feeds to microdiets (Watanabe and Kiron, 1994; Cahu and Zambonino Infante, 2001; Chen et al., 2006); however, the rate of ontogeny varies between species (Kolkovski, 2001). It is considered that the composition of microdiets, including binders and proteins, make digestion of the pellets difficult (Lindner et al., 1995; Partridge and Southgate, 1999). In addition, amino acids are freely available in live feeds and support larval digestion in marine fish larvae (Rønnestad et al., 1999). The reduced ability of early larvae to digest protein in particular has been investigated in detail with various studies reporting that microdiets containing pre-digested and soluble protein are more easily digested by marine larvae (Kvåle et al., 2002; Tonheim et al., 2007).

Shifts in food type preference have been measured against larval age (Hung et al., 2002; Shaw et al., 2003), weight (Olsen et al., 2000), and length (Mayer and Wahl, 1997), but the underlying factor that links these parameters is larval ontogeny. Sensory and visual development (Jones and Janssen, 1992; Pankhurst,

2008), locomotive ability (Blaxter, 1986) and mouth gape (Fernández-Diaz et al., 1994) all play a role in prey detection, capture and consumption. Additionally, the prey taxa, size and abundance can influence larval preference (Pryor and Epifanio, 1993; Mayer and Wahl, 1997).

Weaning success and feeding efficiency can be improved by introducing new food sources at appropriate stages of larval development. At the PSFI mulloway hatchery, approximately 250,000 larvae are typically stocked into either a 2,000 l intensive clear water tank or a 10,000 l green water tank (with resident algae). The density of live prey is increased as larval size increases. Larval development is measured against length rather than age and current weaning protocols use large strain rotifers (*Brachionus plicatilis*) from first feeding (2 days after hatching [dah], 2.5 mm TL) at a rate of 4 ml^{-1} for a 10,000 l tank and up to 20 ml^{-1} for a 2000 l tank. When larvae attain a mean length of 4-5 mm, rotifers are supplemented with enriched *Artemia*, which are initially introduced at a low rate (0.2-1 ml^{-1}). The *Artemia* density is then increased to accommodate the increased demand as larvae develop. A 400 μm (diameter) weaning diet is introduced when fish attain length of approximately 8 mm, and the concentration of *Artemia* is gradually reduced until larvae are considered to be weaned (when all sampled fish are consuming mostly pellet).

Three experiments were conducted to examine if the efficiency of mulloway weaning practices can be improved by decreasing the amount of *Artemia* required during the weaning process and determining the optimal time of *Artemia* introduction to reduce the costs of production. The first experiment aimed to investigate the effects of a range of live feed and pellet combinations on growth and weaning success of larval mulloway. The other two experiments were short-

duration feeding trials that aimed to investigate the transition by larval mulloway from rotifers to *Artemia* and then from *Artemia* to a pellet microdiet. The outcomes of the *Artemia* and pellet microdiet transition trial were then confirmed in a pilot commercial-scale production run.

4.2 Materials and methods

4.2.1 Fish and facilities

Captive mulloway broodstock held at the PSFI, NSW, Australia were induced to spawn using temperature cues after exposure to a truncated photo-therm regime (described in Partridge et al., 2003). Fertilised eggs were collected on four different occasions, quantified and then treated with ozone (CT = 0.2) to disinfect eggs of pathogens, before being transferred to a 2000 l flow-through (1.5 l min^{-1}) clear water larval rearing tank for both Experiments 1 and 2, and an 8000 l flow through (5 l min^{-1}) clear water larval rearing tank for Experiment 3 and the confirmation trial. The rearing tanks were all maintained in clear water so that resident microalgae did not affect feeding preferences.

4.2.2 Water quality analyses

Water quality (mean ± SD; pH, dissolved oxygen [DO], temperature and salinity) parameters were measured daily in each experiment tank using a water quality meter (Horiba U-10, Japan). A rapid test kit (E. Merck, Model 1.08024, Germany) was used to measure total ammonium (NH_4^+ mg l^{-1}) (Table 10). Water quality variables were consistent across all treatment tanks for the duration of the experiments. Water quality in Experiments 2 and 3 was similar to that found in the holding tanks.

Table 10: Water quality parameters (mean ± SD) measured daily in each experiment.

Experiment	pH	DO mg l^{-1}	Temperature °C	Salinity g l^{-1}	Total Ammonium mg l^{-1}
Experiment 1	8.2 ± 0.1	6.7 ± 0.3	23.3 ± 0.8	35.3 ± 0.1	0.1 ± 0.0
Experiment 2	8.1 ± 0.1	7.5 ± 1.6	20.1 ± 1.4	32.0 ± 0.4	0.1 ± 0.1
Experiment 3	8.1 ± 0.2	7.6 ± 0.2	22.5 ± 0.8	30.4 ± 0.8	0.4 ± 0.1
Confirmation experiment	7.8 ± 0.1	10.1 ± 1.0	21.3 ± 0.3	35.7 ± 0.1	0.2 ± 0.1

4.2.3 Experiment 1: Effects of different feed combinations on weaning success and growth of mulloway larvae

Experiment 1 examined the effects of feeding six different combinations of live feeds and a 3/5 Proton pellet microdiet (INVE, Dendermonde, Belgium) on the weaning success and growth of larval mulloway. The 3/5 Proton diet was selected as it produced comparable results to two other commercially available diets for growth and survival in snapper (*Pagrus auratus*), another temperate marine fish (Fielder et al., 2008a). Mulloway larvae stocked at 125 fish l^{-1} were fed enriched (Algamac 3050, Aquafauna Bio-Marine Inc., California, USA) rotifers at 10 ml^{-1} in a 2000 l holding tank until 13 dah (mean TL 4.9 ± 0.1 mm), when 800 larvae were stocked into each of 30, flow through (200 ml min^{-1}) experiment tanks (n = 5 replicate tanks/treatment; tank walls were black and conical floors were white) containing 100 l of 10 μm filtered estuarine water. The experiment treatments (Table 11) commenced on the following day (14 dah) and continued until larvae in all treatments were considered to be successfully weaned from live feeds to the pellet diet. Each experiment tank was siphoned daily to remove excess food and waste and the internal overflow screens were cleaned. Lights were set to turn on and

off immediately without a dimming effect at 0800 and 2000 h, respectively (8 μmol s^{-1} m^{-2} in the light phase; LI-COR, model Li-1776, USA). Enriched rotifers (maintained at 10 ml^{-1}) were fed to larvae in all treatments twice daily (0900 and 1500 h) throughout the experiment (Table 11).

Table 11: Feed treatments applied to fish throughout the feeding trial (n = 5). (Experiment 1) [1]

Treatment	Rotifers	*Artemia* [2]	Pellet diet
1	14-29 dah	18-27 dah [a]	23-29 dah
2	14-29 dah	18-27 dah [b]	23-29 dah
3	14-29 dah	Nil	23-29 dah
4	14-29 dah	18-27 dah [a]	14-29 dah
5	14-29 dah	18-27 dah [b]	14-29 dah
6	14-29 dah	Nil	14-29 dah

[1] dah - days after hatching
[2] a = 100% ration, b = 50% ration

Nutritionally enriched *Artemia* were fed twice daily to four of the six treatments (Treatments 1, 2, 4 and 5) from 18-27 dah and were maintained at either a standard PSFI ration (100%) or half the standard ration (50%). The standard ration began at 0.4 ml^{-1} per feed at 18 dah (5.0 ± 0.1 mm, mean larvae length) and was doubled each day until 21 dah. The ration was then halved each day until 27 dah, which was the last time *Artemia* were fed to larvae. The residual concentration of live feeds was determined for each tank before the addition of each feed. The pellet microdiet was broadcast 4-6 times daily from either 14 dah (Treatments 4, 5 and 6) or 23 dah (Treatments 1, 2 and 3) until completion of the experiment (29 dah). The quantity of pellet broadcast at the start of the experiment was 0.2 g day^{-1} and increased daily (approximately 17%) to ensure the increase in feed demand was met.

Twenty larvae were sampled from each tank every four days and euthanased using a lethal dose of ethyl-*p*-aminobenzoate (100 mg l^{-1}; Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia). Larvae were examined immediately using a dissection microscope (M5 – 89734, Wild Heerbrugg, Switzerland) for TL (mm) from the top of the snout to the end of the tail, and for food type consumed. The 20 fish from each tank were then pooled and dried (105ºC, 16 h) to obtain the mean dry weight (mg) of fish. Survival was calculated for each treatment replicate at the end of the experiment and excluded the fish removed for sampling [Percent survival = (Final no. of fish / 720) * 100].

4.2.4 Experiment 2: Transition of mulloway larvae from rotifers to Artemia

Experiment 2 was a short-duration feeding experiment that examined the transition of mulloway larvae from enriched rotifers to enriched *Artemia* as larval length increased. The mean TL of larvae at the start of the experiment was 4.1 mm, which was smaller than the mean TL of fish at the start of Experiment 1 and allowed for a comparison of results. Larvae for the experiment were sourced each experiment day from a 2000 l tank (described for Experiment 1), and were maintained on rotifers at a density of 10 ml^{-1}. For three days leading up to and during the experiment, larvae in the holding tank were given one feed of *Artemia* each day at a density of 0.1 ml^{-1} so they were not naive to the new food source at commencement of the experiment protocols. The tank water was exchanged at a rate of 1.5 l min^{-1}, which ensured clearance of live feeds from the tank during the night and also ensured the digestive tract of the larvae was cleared. Twenty fish from the holding tank were sampled daily until the mean TL reached 4.1 mm. On

each experiment morning thereafter, and before the addition of food in the holding tank, the sample of larvae (n = 20) was examined to confirm that no residual food remained in the digestive tract from the previous day. Then, 100 larvae were transferred every experiment day to each of 15 randomly positioned blue experiment vessels containing 10 l of 10 μm filtered and lightly aerated static estuarine water (ambient light conditions, similar to the light conditions of the larviculture tank). Larvae were acclimated for 30 min before they were fed rotifers at 2 ml^{-1} (Treatment 1), *Artemia* at 2 ml^{-1} (Treatment 2) or both rotifers and *Artemia* at 1 ml^{-1} each (Treatment 3). Larvae were given 1 h to feed (methods described by Shaw et al., 2003) and were then euthanased using a lethal dose of ethyl-*p*-aminobenzoate (100 mg l^{-1}) and immediately preserved in 10% neutral buffered formalin for later dissection and stomach content assessment. A sub-sample of 20 fish from each replicate was examined using a dissection microscope and the stomach contents were teased apart to determine food type and quantity consumed by individual larvae.

4.2.5 Experiment 3: Transition of mulloway larvae from Artemia to a pellet microdiet

Experiment 3 was a short-duration feeding experiment that examined the transition of mulloway larvae from enriched *Artemia* to a pellet microdiet (Proton 3/5, INVE) as larval length increased. The mean TL of larvae at the start of the experiment was 8.3 ± 0.3 mm (mean ± SD), which was smaller than the mean TL of fish observed to be feeding on pellets in Experiment 1 and allowed for a comparison of results. Larvae for the experiment were sourced each day from an 8000 l flow-

through holding tank, where they were maintained on *Artemia* at a density of 1 ml^{-1}. For three days leading up to and during the experiment, larvae in the holding tank were broadcast-fed approximately 8 g of a 300-500 μm pellet (3/5 Proton) once each day so they were not naive to the new food source. Once the mean TL of mulloway reached 8.3 ± 0.3 mm, 100 larvae were transferred each experiment day to each of 15 randomly positioned blue experiment vessels containing 10 l of 10 μm filtered, lightly aerated static estuarine water, similar to those described in Experiment 2 (ambient light conditions, similar to that of the holding tank). Fish were transferred to the experiment vessels before feeding each experiment day and clearance of food from the guts of larvae was confirmed prior to transfer, as described in Experiment 2. Larvae were acclimated for 30 min before being offered either *Artemia* at 2 ml^{-1} (Treatment 1), a 300-500 μm pellet broadcast in 15 min intervals (Treatment 2) or both *Artemia* at 1 ml^{-1} and pellet broadcast in 15 min intervals (Treatment 3). The number of pellets provided was equivalent to the number of *Artemia* provided, which was determined by measuring the weight of a known number of pellets and then determining the required weight of pellets for the feeding trial. Larvae were allowed to feed for 1 h and were then euthanased using a lethal dose of ethyl-*p*-aminobenzoate and immediately preserved in 10% formalin for later dissection and stomach content assessment. As in Experiment 2, a sub-sample of 20 fish from each replicate was examined using a dissection microscope, and the food type and quantity consumed by individual larvae was recorded.

The optimal feeding outcomes of Experiment 3 were then confirmed in a pilot commercial scale production run at the PSFI. The mean TL of larvae selected for the start of this experiment was 9.8 ± 1.1 mm (mean ± SD) as this was smaller than the mean TL of fish that were feeding on pellets in Experiment 1 and

Experiment 3 and enabled a comparison of results. Mulloway larvae were housed in two larviculture tanks containing 8000 l of 10 μm filtered estuarine water and maintained on enriched *Artemia* at 1 ml^{-1}. Once the average length of fish within the tanks was 9.8 ± 1.1 mm the larvae were offered a 300-500 μm pellet diet (3/5 Proton) in addition to the 1 ml^{-1} ration of *Artemia*. The ration of *Artemia* was reduced over the following three days to 0.8 ml^{-1}, 0.6 ml^{-1} and 0.3 ml^{-1}, respectively. The larvae were then maintained on *Artemia* at 0.3 ml^{-1} until all fish were observed to be feeding on pellets (7 days from the initial addition of pellet). Pellets were distributed to each tank using an automatic belt feeder and through regular manual broadcasting. Twenty larvae were sampled daily from each tank and the number of pellets and *Artemia* in the stomachs of individual larvae and TL were recorded. The TL was recorded for every fish, which differed from the initial feed selection experiments where the mean TL of fish in the holding tank was recorded each day.

4.2.6 Statistical analyses

Statistical analyses were conducted using Statgraphics Version 4.1 (STSC, USA). In Experiment 1, data were analysed for homogeneity of variance using Cochran's test. The experiment was designed for two-factor ANOVA to determine the effects of the *Artemia* ration size and the time of pellet introduction on weight, TL and survival. Where statistical differences ($P<0.05$) were found, the means were separated using the Student-Newman-Keuls test (SNK). Where significant interactions were found, one-factor ANOVA and the SNK test were used to establish where the significant differences existed.

In Experiments 2 and 3, data in Treatment 3 were analysed for the mean number of food items consumed. Significant differences (P<0.05) were determined using a two sample paired *t*-test. Chesson's selectivity index (α) (Chesson, 1978) was also used to compare feeding preferences of mulloway larvae for each prey type.

$$\alpha_i = \frac{r_i / n_i}{\sum_{i=1}^{m} r_i / n_i}, \qquad i = 1,\ldots m$$

Where r_i is the number of items of prey type i in the larvae diet, n_i is the number of items of prey type i in the environment and m is the total number of prey types. Only larvae that had selected one or more prey items were used in the calculation of the selection index, to exclude non-feeding fish from the analysis.

Significant differences in selection (α) were determined using a *t*-test to compare α to neutral selection (α = 0.5) for each length with the equation (Chesson, 1983)

$$t = \frac{\alpha_i - 0.5}{\sqrt{s^2 / k}}$$

Where α_i is the sample mean and s^2 is the sample variance of the k estimators of α_i. Alpha levels of P<0.01 were considered significant for this analysis to reduce the risk of a type I error.

The percentage of fish feeding on each prey type was recorded for all treatments in Experiments 2 and 3 to determine feeding ability of larval mulloway. The percentage of fish feeding in Treatments 1 and 2 were compared using a two sample *t*-test, while in Treatment 3 (offered two food types) a two sample paired *t*-test was used to compare the percentages of fish that were feeding. Those fish in Treatment 3 that were not feeding were excluded from the analysis.

4.3 Results

4.3.1 Experiment 1: Effects of different feed combinations on weaning success and growth of mulloway larvae

No interactions existed ($P>0.05$) between the two factors, time of pellet introduction (14 or 23 dah) and *Artemia* ration size (0, 50 or 100% ration), for larval weight or TL at all sampling times. Also, no effects of the two levels of pellet introduction time were observed for larval weight or TL. Fish that were not fed *Artemia* performed poorly compared to fish in treatments that were fed *Artemia*. At completion of the experiment, significant differences ($P<0.05$) existed between the mean weight or TL of fish in treatments that were not fed *Artemia*, (i.e. Treatments 3 and 6) compared with those that were fed *Artemia* (Fig. 2, Fig. 3, Table 12) at completion of the experiment. However, no significant differences in mean weight or TL were found between treatments fed a half ration (Treatments 2 and 5) and a full ration (Treatments 1 and 4) of *Artemia* (Fig. 2, Fig. 3, Table 12). Fish in treatments that were not fed *Artemia* began consuming pellets earlier than fish that were fed *Artemia*. At 21 dah, two replicates from Treatment 6 (not fed *Artemia*) contained fish that were feeding on minimal amounts of pellet. The average TL (mean ± SEM) of fish in this treatment was 7.2 ± 0.1 mm (Fig. 3). At 25 dah, the mean TL of fish in Treatments 3 and 6 (not fed *Artemia*) was 8.8 ± 0.3 mm and 9.6 ± 0.3 mm, respectively, and all replicates contained fish that fed on pellets. At 29 dah, the mean TL of fish ranged from 10.0 ± 0.3 mm (Treatment 3) to 14.5 ± 0.5 mm (Treatment 4), and all fish in all treatments fed on pellets.

A significant interaction (P<0.05) occurred between time of pellet introduction (14 or 23 dah) and *Artemia* ration size (0, 50 or 100% ration) for survival (Table 12). This was caused by significantly poorer survival in fish fed a 50% ration of *Artemia* and offered pellets from 23 dah than fish fed a 50% ration of *Artemia* and offered pellets from 14 dah. There were no significant differences in the survival of fish between the other feeding regimes (0 and 100% rations).

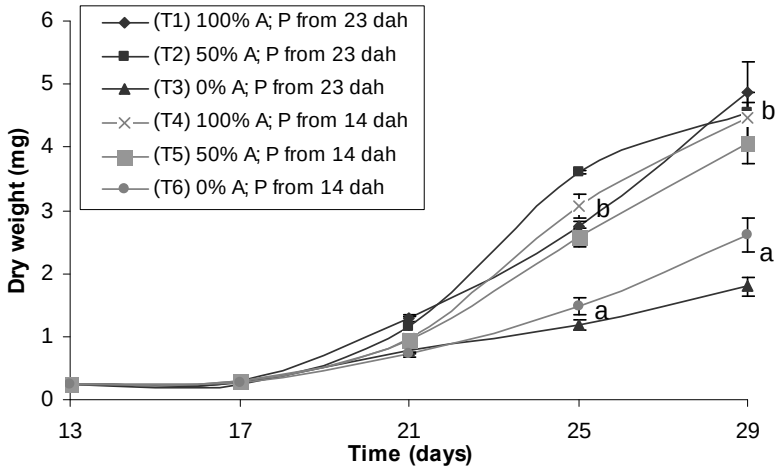

Figure 2: Dry weights (mean ± SEM; n = 5) of fish fed six different feeding regimes (Table 2) throughout the trial (Experiment 1). Different letters indicate significant differences (P<0.05; two-factor ANOVA, SNK). T = Treatment, A = *Artemia* and P = Pellets.

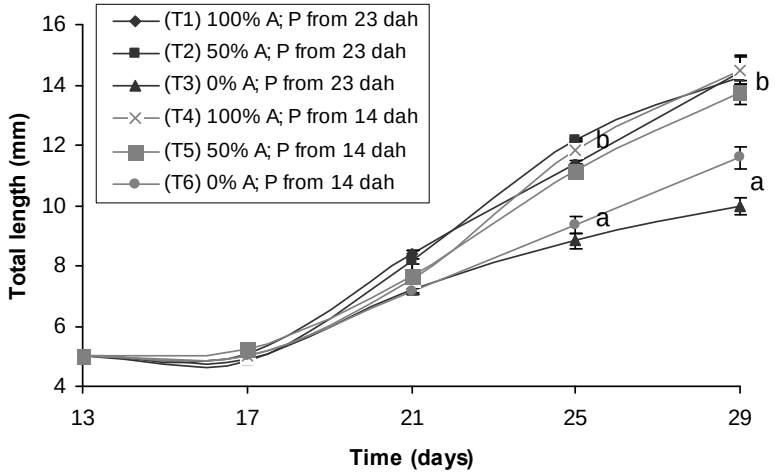

Figure 3: Total length (mean ± SEM; n = 5) of fish fed six different feeding regimes (Table 2) throughout the trial (Experiment 1). Different letters indicate significant differences (P<0.05; two-factor ANOVA, SNK). T = Treatment, A = *Artemia* and P = Pellets.

Table 12: Mean (± SEM) final dry weight, total length and survival of fish in each feeding treatment at the end of the experiment. (Experiment 1) [1, 2, 3]

Treatment	*Artemia* (18 dah)	Pellet diet	Weight (mg)	Length (mm)	Survival (%)
1	100%	23 dah	4.9 ± 0.5^{b}	14.4 ± 0.6^{b}	5.5 ± 3.0
2	50%	23 dah	4.5 ± 0.2^{b}	14.2 ± 0.2^{b}	1.0 ± 0.6^{x}
3	0%	23 dah	1.8 ± 0.1^{a}	10.0 ± 0.3^{a}	3.0 ± 1.5
4	100%	14 dah	4.5 ± 0.4^{b}	14.5 ± 0.5^{b}	3.7 ± 1.2
5	50%	14 dah	4.1 ± 0.3^{b}	13.7 ± 0.4^{b}	13.1 ± 4.1^{y}
6	0%	14 dah	2.6 ± 0.3^{a}	11.6 ± 0.4^{a}	7.4 ± 3.0

[1] dah - days after hatching
[2] A full ration (100%) of *Artemia* began at 0.4 ml^{-1} $feed^{-1}$ at 18 dah and was doubled each day until 21 dah. The ration was then halved each day until 27 dah, which was the last feed of *Artemia*. The 50% ration was half of the full ration.
[3] Different letters in superscript within the same column indicate significant differences ($P<0.05$; two-factor ANOVA, SNK). a and b indicates effects of *Artemia* ration; x and y indicates effects of pellet introduction. Where significant interactions ($P<0.05$) occurred, superscripts are presented in italics.

4.3.2 Experiment 2: Transition of mulloway larvae from rotifers to Artemia

The percentage of feeding larvae in Treatment 1, offered only rotifers, increased over the duration of the trial from 21.0 ± 0.9% (mean TL 4.1 ± 0.3 mm) to 88.0 ± 1.0% (5.4 ± 0.5 mm) (Table 13). The percentage of larvae feeding in Treatment 2, offered only *Artemia*, also increased throughout the experiment from 11.0 ± 0.7% (mean TL 4.1 ± 0.3 mm) to 81.0 ± 0.9% (5.5 ± 0.4 mm) (Table 13). Larvae in Treatment 3 were offered both rotifers and *Artemia* and as the mean TL of larvae increased the acceptance of *Artemia* in preference to rotifers increased. A significantly greater percentage of larvae consumed rotifers in preference to *Artemia* until the mean larval TL reached 5.1 ± 0.1 mm, at which time there was no significant difference observed between the percentage of fish consuming rotifers or *Artemia*. Once the mean TL reached 5.5 ± 0.1 mm, a significantly greater percentage of fish consumed *Artemia* in preference to rotifers (Table 13).

Table 13: The percentage (mean ± SEM; $n = 5$) of fish feeding on each food type, in each treatment, on experiment days. (Experiment 2) [1, 2, 3]

Length (mm)	4.1 ± 0.3	4.4 ± 0.4	4.9 ± 0.4	5.1 ± 0.5	5.2 ± 0.5	5.4 ± 0.5	5.5 ± 0.4
Treatment 1 – R	21 ± 0.8^{b}	71 ± 1.7	76 ± 1.1^{b}	88 ± 0.9^{b}	81 ± 0.9^{b}	88 ± 1.0^{b}	86 ± 0.4
Treatment 2 – A	11 ± 0.7^{a}	67 ± 1.2	39 ± 1.1^{a}	57 ± 0.7^{a}	59 ± 1.2^{a}	56 ± 0.6^{a}	81 ± 0.9
Treatment 3 – R	91 ± 4.0^{y}	75 ± 4.7^{y}	91 ± 3.7^{y}	63 ± 7.8	55 ± 7.6	53 ± 3.4	24 ± 1.7^{x}
Treatment 3 – A	14 ± 5.9^{x}	40 ± 7.0^{x}	38 ± 6.7^{x}	54 ± 8.3	63 ± 7.4	64 ± 2.7	85 ± 1.5^{y}

[1] Lengths (mean ± SEM, $n = 20$ replicate fish) are those of fish in the holding tank on a given experiment day.
[2] Fish in Treatment 1 were offered only rotifers (R), fish in Treatment 2 were offered only *Artemia* (A), and fish in Treatment 3 were offered both R and A. Treatment 3 is represented in the 3rd and 4th rows as two food types were available.
[3] The mean percentages of fish feeding in Treatments 1 and 2 were compared using a two sample t-test. The mean percentages of fish that were feeding (excluding non-feeding fish) on each prey type in Treatment 3 were compared using a two sample paired t-test. Different superscripts at the same length indicate significant differences ($P<0.05$) between Treatments 1 and 2 (a and b), and between prey types within Treatment 3 (x and y).

The mean number of rotifers and *Artemia* consumed by mulloway larvae was also dependant on fish length (Fig. 4). Larvae in Treatment 3 consumed significantly more rotifers than *Artemia* until the mean larval TL reached 5.1 ± 0.1 mm, at which time there was no significant difference observed between the mean number of rotifers and *Artemia* consumed. From 5.2 ± 0.1 mm, larvae began to consume significantly more *Artemia* than rotifers. The number (mean ± SEM) of *Artemia* consumed by larvae in Treatment 3 increased steadily from 0.1 ± 0.03 *Artemia* h^{-1} for larvae of 4.1 ± 0.07 mm, to 13.7 ± 0.31 *Artemia* h^{-1} for larvae of 5.5 ± 0.1 mm.

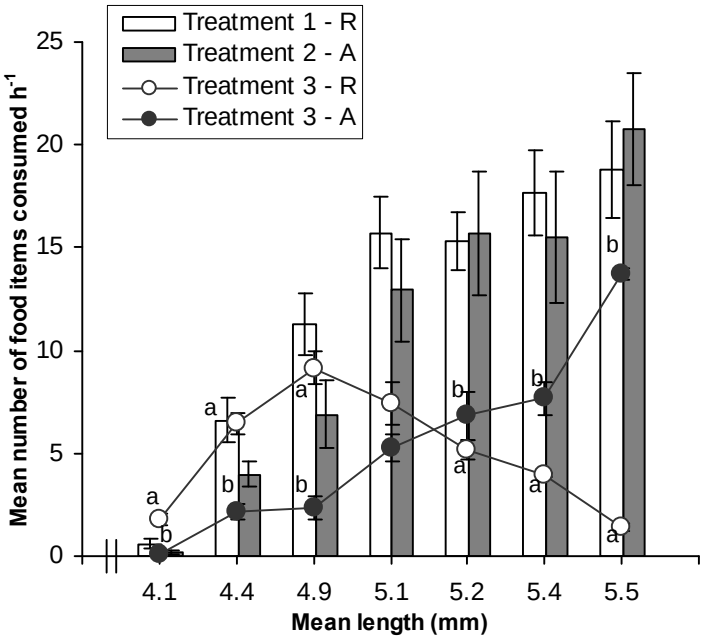

Figure 4: The number of food items (mean ± SEM; n = 5) consumed by fish in 1 h, for each treatment, on each experiment day (Experiment 2). Lengths (means; n = 20) are those of fish in the holding tank on a given experiment day. Treatment 1 (only offered rotifers) and Treatment 2 (only offered *Artemia*) are represented by the bar graph. Treatment 3 (offered both rotifers and *Artemia*) is represented by the line graph, with each food type presented as a separate line. Different letters between Treatment 3 values at the same length indicate significant differences (P<0.05; two sample paired t-test).

Chesson's selectivity index (α) showed significant (P<0.01) selection of rotifers for fish of 4.1 ± 0.3 and 4.9 ± 0.4 mm TL (Fig. 5). From 5.1 ± 0.5 to 5.4 ± 0.4 mm TL, there was not a significant difference in selection between either rotifers or *Artemia* and neutral selection (α = 0.5). Larvae of 5.5 ± 0.4 mm TL showed significant (P<0.01) selection for *Artemia* (Fig. 5).

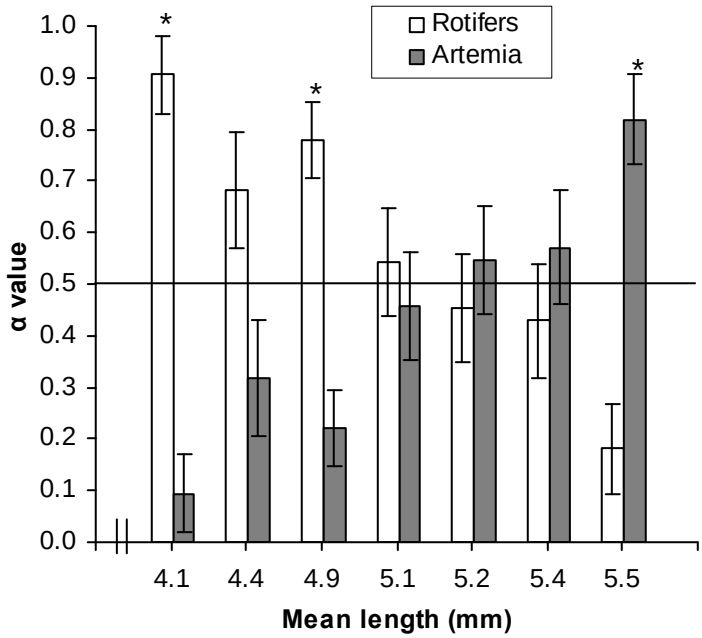

Figure 5: Chesson's selectivity index (α; mean ± SEM; n = 5) for fish fed a mixed diet containing 50% rotifers and 50% *Artemia* (Experiment 2). Fish that did not consume either prey were excluded from the analyses. Mean lengths displaying the '*' symbol indicate a significant difference from neutral selection (P<0.01; two sample paired t-test).

4.3.3 Experiment 3: Transition of mulloway larvae from Artemia to a pellet microdiet

The percentage of feeding larvae in Treatment 1, offered only *Artemia*, ranged from 95.0 ± 0.5% (mean TL 8.3 ± 0.3) to 100.0 ± 0.0% (11.6 ± 0.2 mm) (Table 14). The percentage of larvae feeding in Treatment 2, offered only pellets, increased throughout the experiment from 25.0 ± 0.3% (mean TL of 8.3 ± 0.3) to

96.0 ± 0.5% (12.5 ± 0.3 mm) (Table 14). Larvae in Treatment 3 were offered both *Artemia* and pellets and as the mean TL increased the acceptance of the pellets in preference to *Artemia* increased. A significantly greater percentage of larvae consumed *Artemia* in preference to pellets until the mean TL reached 10.6 ± 0.4 mm, at which time there was no significant difference observed between the percentages of fish consuming *Artemia* or pellets. Once the mean TL reached 11.6 ± 0.2 mm, a significantly greater percentage of fish consumed pellets in preference to *Artemia*. However, this significant difference was not evident for fish with a mean TL of 12.5 ± 0.3 mm.

Table 14: The percentage (mean ± SEM; $n = 5$) of fish feeding on each food type, in each treatment, on experiment days. (Experiment 3) [1, 2, 3]

Length (mm)	8.3 ± 0.3	8.8 ± 0.4	10.0 ± 0.3	10.6 ± 0.4	11.6 ± 0.2	12.0 ± 0.4	12.5 ± 0.3
Treatment 1 – A	95 ± 0.5^{b}	96 ± 0.4^{b}	96 ± 0.4^{b}	98 ± 0.2^{b}	100 ± 0.0^{b}	98 ± 0.2^{b}	97 ± 0.4
Treatment 2 – P	25 ± 0.3^{a}	55 ± 0.7^{a}	70 ± 0.7^{a}	77 ± 1.7^{a}	86 ± 0.9^{a}	81 ± 1.0^{a}	96 ± 0.5
Treatment 3 – A	97 ± 1.2^{x}	98 ± 1.2^{x}	83 ± 2.7^{x}	73 ± 4.6	53 ± 5.1^{x}	78 ± 2.0	78 ± 4.6
Treatment 3 – P	8 ± 2.0^{y}	26 ± 3.3^{y}	44 ± 8.4^{y}	79 ± 2.8	81 ± 3.3^{y}	87 ± 2.0	81 ± 4.0

[1] Lengths (mean ± SEM, $n = 20$ replicate fish) are those of fish in the holding tank on a given experiment day.
[2] Fish in Treatment 1 were offered only *Artemia* (A), fish in Treatment 2 were offered only pellets (P), and fish in Treatment 3 were offered both A and P. Treatment 3 is presented in the 3rd and 4th rows as two food types were available.
[3] The mean percentages of fish feeding in Treatments 1 and 2 were compared using a two sample t-test. The mean percentages of fish that were feeding in Treatment 3 (excluding non-feeding fish) were compared using a two sample paired t-test. Different superscripts at the same length indicate significant differences ($P<0.05$) between Treatments 1 and 2 (a and b), and within Treatment 3 (x and y).

The mean number of *Artemia* and pellets consumed by mulloway larvae was also dependent on fish length (Fig. 6). Larvae in Treatment 3 consumed significantly more *Artemia* than pellets until the mean larval TL reached 10.6 ± 0.4 mm, at which time there was no significant difference observed between the mean number of *Artemia* and pellets consumed. From a mean TL of 11.6 ± 0.2 mm, larvae began to consume significantly more pellets than *Artemia* (Fig. 6). The number (mean ± SEM) of pellets consumed by larvae in Treatment 3 increased steadily from 1.0 ± 0.2 pellets h^{-1} for larvae of 8.3 ± 0.3 mm mean TL, to 13.5 ± 0.6 pellets h^{-1} for larvae of 11.6 ± 0.2 mm mean TL. The mean number of pellets consumed did not increase for larvae of 12.0 ± 0.4 and 12.5 ± 0.3 mm mean TL; however, they did continue to consume more pellets than *Artemia* (Fig. 6).

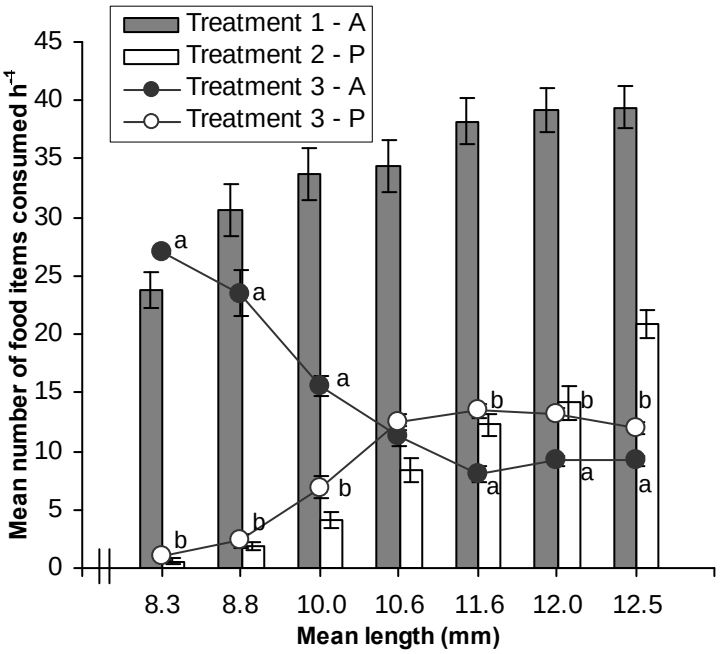

Figure 6: The number of food items (mean ± SEM; n = 5) consumed by fish in 1 h for each treatment, on experiment days (Experiment 3). Lengths (means; n = 20) are those of fish in the holding tank on a given experiment day. Treatment 1 (only offered *Artemia*) and Treatment 2 (only offered pellets) are represented by the bar graph. Treatment 3 (offered both *Artemia* and pellets) is represented by the line graph, with each prey type presented as a separate line. Different letters between Treatment 3 values at the same length indicate significant differences (P<0.05; two sample paired t-test).

Chesson's selectivity index (α) showed significant (P<0.01) selection of *Artemia* for fish of 8.3 ± 0.3 and 8.8 ± 0.4 mm mean TL (Fig. 7). Larvae of 10.0 ± 0.3 and 10.6 ± 0.4 mm mean TL were observed to have no significant difference in selection between *Artemia* or pellets and neutral selection (α = 0.5). Once the mean TL of larvae reached 11.6 ± 0.2 mm, significant selection for pellets was observed (Fig. 7). However, this significant difference was not evident for fish with a mean TL of 12.5 ± 0.3 mm.

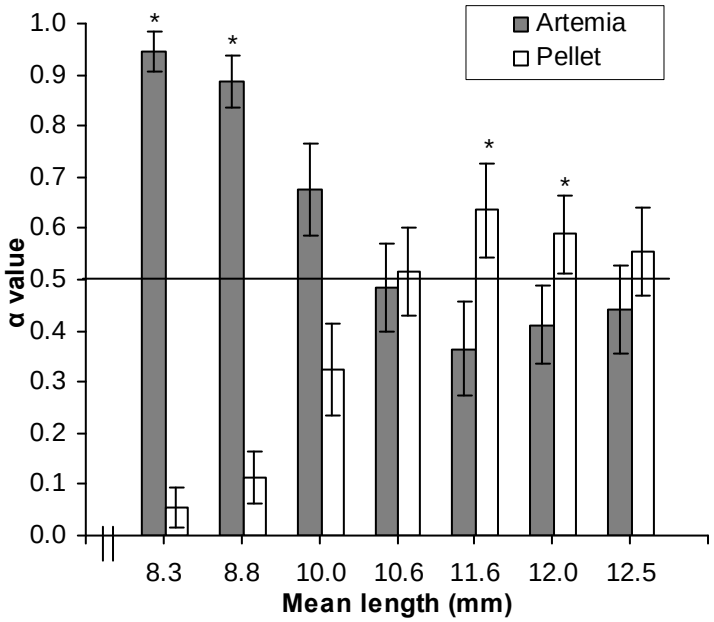

Figure 7: Chesson's selectivity index (α; mean ± SEM; n = 5) for fish fed a mixed diet containing 50% *Artemia* and 50% pellet microdiet (Experiment 3). Fish that did not consume either prey were excluded from the analyses. Mean lengths displaying the '*' symbol indicate a significant difference from neutral selection (P<0.01; two sample paired t-test).

In the confirmation of these results (Experiment 3), larvae were observed to consume significantly more pellets than *Artemia* once the mean TL of fish was between 10.0 and 11.0 mm. There was no significant difference observed between the mean numbers of food items consumed for fish of 8.0 ± 0.5 to 10.0 ± 0.5 mm TL. The percentage of fish that consumed pellets increased linearly ($r = 0.936$) from 47% in fish with a mean TL of 8.0 ± 0.5 mm to 100% in fish with a mean TL of 12.0 ± 0.5 mm. As expected, the percentage of fish consuming *Artemia* decreased consistently ($r = 0.518$) and ranged from 89.0% in fish with a mean TL of 10.0 ± 0.5 mm to 13.0% in fish with a mean TL of 15.0 ± 0.5 mm.

4.4 Discussion

This study has determined that mulloway larvae can be weaned directly from rotifers to the pellet microdiet tested without the use of *Artemia* (Experiment 1); however, this significantly reduces growth. Fish that were fed *Artemia* in combination with rotifers and pellets also weaned successfully but displayed better growth rates. These results suggest the weaning process can be achieved more cost effectively by reducing the amount of *Artemia* currently used at the PSFI by half and is consistent with other studies which reported similar results when larvae were weaned onto pellet microdiets without the use of *Artemia*. Both Callan et al. (2003) and Fletcher et al. (2007) determined that it was possible to co-feed Atlantic cod (*Gadus morhua*) larvae a microdiet along with a reduced amount of *Artemia* without compromising growth and survival; however, if *Artemia* was excluded from the diet, growth and survival were reduced. Curnow et al. (2006) also found that the weaning process for barramundi (*Lates calcarifer*) should include some *Artemia* (5% of previous industry ration) in conjunction with a pellet microdiet in order to stimulate feeding until the stomach is fully developed. The importance of including *Artemia* in the weaning process is apparent; however, the early introduction of pellets has also been reported to improve weaning success in marine larvae. Alves Jr. et al. (2006) determined that fat snook (*Centropomus parallelus*) could be weaned onto a pellet microdiet earlier if a suitable co-feeding period was applied; while Brown et al. (1997) discussed the importance of a co-feeding period to improve survival in Atlantic wolffish (*Anarhichas lupus*) larvae.

Some of the benefits of incorporating live feeds in the weaning process include improved larval digestion by stimulating enzyme secretion from the

pancreas and activating zymogens in the gut to increase overall enzymatic activity (Pedersen and Hjelmeland, 1988; Person Le Ruyet et al., 1993). The reduced growth rates observed when mulloway larvae were not offered *Artemia* in conjunction with pellets, may indicate that larval digestive enzymes were inadequate to digest and assimilate nutrients from this pellet diet. In addition, all fish (Experiment 1) not offered *Artemia* (Treatments 3 and 6) fed on pellets at mean lengths of 8.8 and 9.6 mm, respectively. These lengths are larger than those determined appropriate to introduce *Artemia*, but smaller than was suggested to introduce pellets in feed selection Experiments 2 and 3, respectively. Again, this indicates that larvae in these treatments were ready to consume a larger food source, but digestive capacity at this time was inadequate to effectively digest the pellet. There is evidence to suggest that co-feeding early larvae both live feeds and inert diets can enhance weaning success, growth and survival, by pre-conditioning the larvae to accept the manufactured diets. Cañavate and Fernández-Díaz (1999) determined that weaning success in sole (*Solea senegalensis*) was improved by co-feeding before metamorphosis rather than after, and discussed the difficulty in encouraging advanced larvae to accept an inert diet once they were accustomed to *Artemia*.

In Experiment 1, it is interesting that fish in all treatments fed on pellets once the minimum mean total length of 10.0 mm was attained. This is similar to the outcomes of feed selection Experiment 3, in which fish selected pellets equally to *Artemia* by 10.6 mm, and suggests this length is suitable for the introduction of pellets to mulloway larvae. Furthermore, a similar study by Curnow et al. (2006) found that a reduction in live feed use in barramundi (*L. calcarifer*) could be facilitated by supplying a Gemma Micro diet (Skretting) but not with the Proton

diet. It may be useful to investigate weaning success in mulloway using a range of pellet microdiets to determine if further reductions in *Artemia* reliance are possible.

The reduced survival of fish fed pellets late (23 dah) rather than earlier (14 dah) when they were also offered a 50% ration of *Artemia* was likely to have been the result of natural mortality that frequently occurs in larval experiments. This reduction in survival is not likely to be the result of treatment effects, but rather from handling stress.

In Experiment 2, the transition of food preference from rotifers to *Artemia* occurred once the mean size of mulloway larvae was 5.2 mm, at which time larvae selected *Artemia* equally to rotifers. Fish that were only fed *Artemia* consumed them at a greater rate than fish offered both rotifers and *Artemia*, which indicates that larvae were capable of feeding on *Artemia* but were preferentially selecting rotifers until they reached 5.2 mm in length. Additionally, the percentage of fish selecting *Artemia* increased steadily and was similar to the percentage of fish consuming rotifers once larvae reached 5.2 mm. The selection of smaller prey types up until larvae reached 5.2 mm can be explained on the basis of energy spent and profits gained. Mayer and Wahl (1997) discussed prey preference of larval walleye (*Stizostedion vitreum*) in terms of profit as the fish selected strongly for prey types that improved growth and survival. Selective feeding has been described as an evolutionary tendency to maximise energy intake and must be an adaptive feature of larval fish to optimise energy consumption (Greene, 1986; Schoener, 1987). Mulloway larvae may have selected *Artemia* once swimming ability and sensory development had improved.

In Experiment 3, the transition of food preference from *Artemia* to the Proton pellet diet occurred once the size of mulloway was 10.6 mm. The fish that

were only fed pellets consumed them at a much greater rate than fish offered a choice between *Artemia* and pellets, indicating that larvae were capable of consuming pellets but were preferentially selecting *Artemia* until reaching a mean length of 10.6 mm. Similarly, Chesson's selectivity index indicated that the transition from *Artemia* to pellets occurred between 10.0 and 10.6 mm, after which selection of pellets was significantly greater than neutral selection. In addition, the percentage of fish selecting pellets increased with fish length and was similar to the percentage of fish consuming *Artemia* once larvae reached 10.6 mm. Olsen et al. (2000) found that less digestible prey types were not selected by Atlantic halibut (*Hippoglossus hippoglossus*), and it is possible that smaller mulloway larvae were not selecting pellets as they were more difficult to digest. Once mulloway larvae reached 10.6 mm, digestive capacity may have been sufficient to enable them to benefit from the more energy rich pellets. Inclusion of pellets for fish of this length may reduce resistance to weaning arising from fish becoming accustomed to *Artemia*, as discussed by Cañavate and Fernández-Díaz (1999), while allowing larvae to benefit from the less digestible pellets.

The results of the confirmation pilot commercial scale trial were similar to that of Experiment 3. It was demonstrated that mulloway larvae began to select pellets in preference to *Artemia* once mean total larval length was between 10.0 and 11.0 mm. The initial experiment measured food preference against the average length of fish in the holding tank on a given day, while the confirmation trial measured food preference against individual larvae lengths. As the results of the two experiments were similar, it suggests that the methodology used in the initial experiment was sufficient to rely on the results obtained using average lengths each day and not individual lengths. The results of the three experiments examining the

transition from *Artemia* to pellets were similar. In Experiments 1 and 3, and in the confirmation trial, larvae total lengths at the time of pellet selection were 10.0, 10.6 and 10.0-11.0 mm, respectively. The similarity in results strongly supports the conclusion that 10.0-11.0 mm is an appropriate length to begin weaning larval mulloway from *Artemia* to the pellet microdiet.

These feeding studies have shown that the process for weaning larval mulloway onto a Proton microdiet needs to include some *Artemia* in order for the transition to be successful without compromising growth. The weaning process should begin with rotifers until the mean size of larvae reaches approximately 5.0 mm in length, after which, *Artemia* should be maintained until the mean fish total length is between 10.0 and 11.0 mm. The microdiet can then be introduced and the amount of *Artemia* gradually reduced over the following days, until all fish are considered to be successfully weaned. These protocols are expected to maintain growth rates while reducing costs by minimising the amount of *Artemia* required for weaning and optimising the time of introduction for each new food source.

Chapter 5

Photoperiod and feeding interval requirements of juvenile mulloway, *Argyrosomus japonicus*[1]

[1]Content from his chapter has been published as:

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5.1 Introduction

As with many new aquaculture species in Australia, mulloway (*Argyrosomus japonicus*) production is currently restricted by high costs. In order to improve production efficiency in hatcheries it is important to optimize the conditions in which the fish are reared. These conditions can include the physical culture environment (temperature, salinity, light intensity and photoperiod) (Black, 1998) and general nutritional parameters such as diet composition, ration and feeding frequency (Jobling, 1998).

The benefits of photoperiod manipulation to improve growth rates have been well documented. Boeuf and Le Bail (1999) suggested that day length or photoperiod may indirectly modify growth by increasing food intake and/or muscle mass by exercise. Red sea bream (*Pagrus major*) and largemouth bass (*Micropterus salmoides*) have displayed a higher weight gain and specific growth rate in longer photoperiods (Petit et al., 2003; Biswas et al., 2005), which have been primarily attributed to an increase in the length of feeding time available and a corresponding

increase in the food consumed (Sayer, 1998; Petit et al., 2003). Alternatively, by increasing the length of feeding time, energy expenditure may increase with the result that some species have reduced growth rates (Fielder et al., 2002; Biswas and Takeuchi, 2003). This was evident in the work conducted by Ginés et al. (2004), who found that the growth rate of immature gilthead sea bream (*Sparus aurata*) was reduced when held under continuous light even though food consumption was greater than other photoperiods.

Manipulation of photoperiod can also affect feed conversion ratios or efficiencies and the condition factor of fish (Boeuf and Le Bail, 1999; Biswas et al., 2005), and can be largely species specific, demonstrating the importance of investigating the effect photoperiod has on growth and feeding efficiency of each new aquaculture species. Better food conversion efficiencies were achieved in *M. salmoides* under continuous light when compared to a 12L:12D photoperiod (Petit et al., 2003), and the growth of *P. major* was enhanced by improved food conversion efficiencies in longer photoperiods (Biswas et al., 2005). In contrast, Biswas and Takeuchi (2003) found longer photoperiods resulted in reduced condition in Nile Tilapia (*Oreochromis niloticus* L.), while Barlow et al. (1995) reported no difference in condition of juvenile barramundi (*Lates calcarifer*) when reared in 12 or 24 h of light.

Feeding frequency also affects growth rates and can influence the total mass gained and specific growth rates (SGR) of juvenile fish (Reddy and Leatherland, 2003). The optimal feeding frequency may be directly related to feeding efficiency as appropriately spaced feeds may better allow food to be ingested, digested and assimilated (Biswas and Takeuchi, 2003). A review of optimal feeding frequencies for a range of species completed by Zhou et al. (2003) highlights the variability in

feeding habits for different species and reported that differences can be the result of different feeding behavior or stomach capacity. In addition, the most efficient feeding interval is also likely to be influenced by the gastric evacuation rate of the species and how quickly appetite will return after feeding (Smith, 1989). Appetite is strongly dependent on stomach fullness, and while food is being digested and evacuated from the stomach, fish become increasingly motivated to feed (Olsen and Balchen, 1992).

A change in feeding interval can affect food consumption, food conversion ratios (FCR) and the coefficient of variation for weight (CVwt) (Dwyer et al., 2002). Wang et al. (1998) observed that more frequent feeding reduced the size variation of hybrid sunfish larvae. A significant variation of weight within a population can lead to hierarchical feeding patterns and thus may further influence the growth performance of individuals having different social status (Sayer, 1998). Cannibalism within a cohort of fish is also facilitated by size variation (Kestemont et al., 2003), with the result that mortality rate within the population will increase.

The combined effects of photoperiod and feeding interval have been investigated on species such as Nile tilapia (*O. niloticus*) and snapper (*Pagrus auratus*) with optimal combinations resulting in significantly improved growth rates (Biswas and Takeuchi, 2003; Tucker et al., 2006). Initially this study investigated the interactive effects of a range of feeding intervals and photoperiods on the somatic growth rate and feeding efficiency of juvenile mulloway as assessed by change in body weight and length, FCR, CF, CVwt and survival. We then assessed the rate of gastric evacuation after one feed as measured by change in stomach fullness as a percentage of body weight over time.

5.2 Materials and methods

5.2.1 Fish and facilities

Juvenile mulloway, 2.7 ± 0.5 g (mean ± SEM), reared at the PSFI were stocked into 60 l (700 x 360 x 238 mm) clear acrylic experiment tanks. Fish used in Experiments 1 and 2 were from different cohorts of fish. A clear perspex lid covered each tank and the light intensity (18W fluorescent tubes, Davis) at the air-water interface was 1-2 μmol s^{-1} m^{-2} during the light phase (LI-COR, model Li-1776, USA). Lights were set on a timer to turn on and off immediately. Tank effluent water was collected in a 1000 l sump, pumped through 25 μm cartridge filters into a trickle biofilter and then recirculated to tanks at a flow rate of 0.5 l min^{-1}. Approximately 150% of the total system seawater was exchanged daily.

For Experiment 1, banks of tanks were divided into three photoperiod treatment bays; each bay separated with black plastic sheeting to exclude light leakage between adjacent photoperiod treatments.

5.2.2 Water quality analyses

Water quality (mean ± SD) was measured daily using a water quality meter (Horiba U-10, Japan): pH (7.77 ± 0.14), dissolved oxygen (5.99 ± 0.22 mg l^{-1}), temperature (21.9 ± 1.8°C), and salinity (31.2 ± 1.8‰). Total ammonium (NH_4^+) (0.31 ± 0.13 mg l^{-1}) was measured each day using a rapid test kit (E. Merck, Model 1.08024, Germany). Water quality parameters were consistent across all treatment tanks for the duration of the experiments.

5.2.3 Experiment 1: The effects of photoperiod and feeding interval on juvenile mulloway

A two-factor experiment was conducted over 30 days at the PSFI to determine the interactive effects of photoperiod and feeding interval on the growth and feeding efficiency of juvenile mulloway.

Five feeding intervals were investigated (one feed every 1, 3, 6, 12 or 24 h) across three photoperiods (24L:0D, 18L:6D or 12L:12D). Each treatment combination was replicated four times with each replicate tank containing 20 fish. Individual treatment combinations are expressed as photoperiod and feeding interval (light:dark, hours between feeds e.g. 12L:12D; 12 h represents 12L:12D photoperiod; 12 h feeding interval).

All fish were fed 10% of their body weight daily (Ridley Aqua-feed, Narangba, Australia; Native Fish Starter: crude protein - 52%; crude fat - 12%; ash - 13%; fibre - 3%), which exceeded the total intake, to ensure feed availability did not limit growth. Each day the total food for individual tanks was divided evenly across the number of feeds assigned to that tank. Uneaten feed was removed by siphon daily and was pooled for each tank in 10 day batches to be dried (16 h at 105°C) and weighed. The total biomass (g) of fish in each tank was measured every 10 days and feed weights were adjusted accordingly. Weights of feed delivered per feeding episode and weights of uneaten feed were used to calculate feed intake as a % of body weight/feeding episode. Fish were given 1 h prophylactic formalin treatments (200 ppm) on the day of each weight check to reduce the risk of infection by the dinoflagellate ectoparasite *Amyloodinium ocellatum*. Fish in all treatments were fed a reduced ration of 1% body weight in one feed on these occasions as the feeding

schedule was interrupted.

For the 12L:12D and 18L:6D photoperiod treatments, lights were set to turn on at 0730 h and the first feed addition for all photoperiods was at 0800 h. The 12L:12D, 12 h treatment did not enable enough hours of light to complete the two assigned feeds during the light phase. Therefore, the second feed was provided in the dark phase. Fish in the 12L:12D, 24 h treatment were fed just once during the light phase and a subsequent comparison of weight gain between this and the 12L:12D, 12 h treatment, may provide supporting evidence as to whether the fish fed in darkness.

Upon completion of the experiment the final percent survival within each tank and weight and length measurements of all fish were obtained. Feeding indices used to assess performance were: feed conversion ratio (FCR) = feed (dry weight)/ fish weight gain (wet weight) (Hardy, 1989); condition factor (CF) = 100 * ($weight_{(g)}$ / $length_{(cm)}^{b}$), where b is estimated by the length-weight relationship equation ($W = aL^{b}$) of the sampled populations (King, 1995); coefficient of variation of weight (CV_w) = (s / mean) x 100, where s is the standard deviation.

5.2.4 Experiment 2: Gastric evacuation of juvenile mulloway

A gastric evacuation experiment was conducted to determine the time taken for juvenile mulloway to evacuate an entire feed when fed to satiation. Fifty seven experiment tanks were each stocked with 15 juvenile mulloway raised from a separate cohort of fish and were acclimated to a feed regime of one feed at 0900 h for three consecutive days. Three randomly selected tanks were euthanased using a lethal dose of ethyl-p-amino-benzoate (100 mg l^{-1}) at 0900 h on the day of the trial

and were examined to ensure the fish stomachs were empty. The remaining treatment tanks were fed to satiation at 0900 h and fish from three randomly selected tanks were euthanased every 2 h for 36 h. Euthanased fish were immediately frozen for later dissection and analysis of stomach contents.

A sub-sample of five frozen fish from each replicate tank was thawed and the stomach from the cardiac to pyloric sphincter was dissected from each fish. The content of each stomach was then removed and the remaining whole fish and stomach content were dried at 105°C for 16 h. The dry weights of the fish and its stomach content were obtained and these data were used to determine the stomach fullness as a percentage of total body weight = (dry wt [g] of stomach contents / dry wt [fish – stomach contents]) * 100.

5.2.5 Statistical analyses

Data in Experiment 1 were assessed for homogeneity of variance using Cochran's test (Underwood, 1997). The experiment was designed for analysis by two-factor ANOVA to determine the effects of feeding frequency and photoperiod, and their interaction, on growth, FCR, CF, CVwt and survival. Where significant differences were found, the means were compared by the Student - Newman - Keuls test (SNK). Statistical analyses were conducted using Statgraphics Version 4.1 (STSC, USA).

5.3 Results

5.3.1 Experiment 1: The effects of photoperiod and feeding interavl on juvenile mulloway

There were no significant interactions ($P>0.05$) between photoperiod and feeding interval for all variables measured, therefore, photoperiod and feeding interval outcomes were discussed separately. The length and weight of fish increased in all experimental treatments regardless of feeding interval or photoperiod.

Photoperiod had a significant effect ($P<0.05$) on the weight of fish, with fish in the 12L:12D (7.4 ± 0.2 g) and 18L:6D (7.5 ± 0.2 g) photoperiods heavier after the 30 day trial than fish in the 24L:0D (6.6 ± 0.2 g) photoperiod (Fig. 8, Table 15). Photoperiod also influenced fish length (mean ± SEM; $n = 20$), with fish cultured in the 12L:12D photoperiod longest (8.7 ± 0.2 cm), followed by the 18L:6D photoperiod (8.4 ± 0.1 cm), and the 24L:0D photoperiod (7.9 ± 0.1 cm) (Table 15).

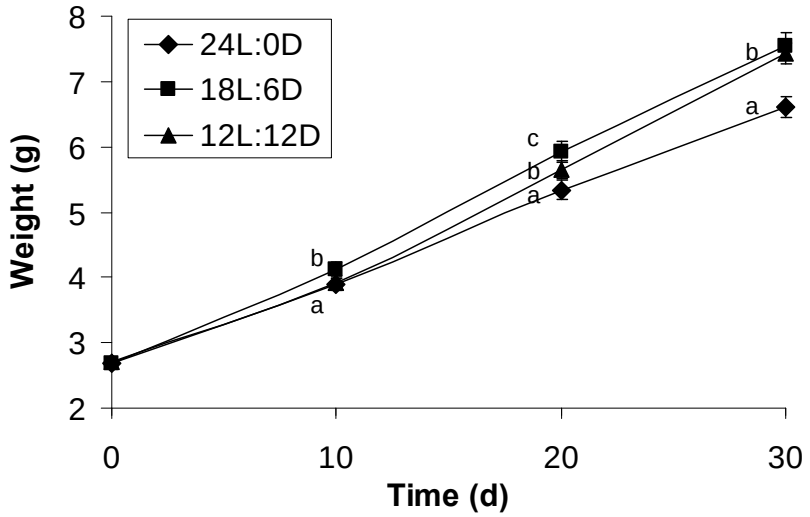

Figure 8: Mean ± SEM (n = 12) wet weights of juvenile mulloway held under three different photoperiods over the 30 day trial. Photoperiods were 12L:12D, 18L:6D; 24L:12D. Feeding interval data is pooled. Mean ± SEM values with different letters at the same time period indicate significant differences (P<0.05; two-factor ANOVA, SNK).

Table 15: Weight, length (total length), feed conversion ratio (FCR), condition factor (CF) and coefficient of variation for weight (CVwt) of mulloway from varying photoperiods and feeding intervals after 30 days. (Experiment 1) [1, 2, 3]

Main effect	Level	Weight (g)	Length (cm)	FCR	CF	CVwt
Photoperiod (h)	12	7.4 ± 0.3 ^{b}	8.7 ± 0.2 ^{c}	1.1 ± 0.1 ^{a}	5.8 ± 0.1 ^{a}	0.2 ± 0.0
	18	7.5 ± 0.3 ^{b}	8.4 ± 0.1 ^{b}	1.2 ± 0.0 ^{b}	6.4 ± 0.1 ^{b}	0.2 ± 0.0
	24	6.6 ± 0.3 ^{a}	7.9 ± 0.1 ^{a}	1.5 ± 0.1 ^{c}	6.4 ± 0.1 ^{b}	0.2 ± 0.0
Feed interval (h between feeds)	1	7.4 ± 0.3 ^{y}	8.4 ± 0.3 ^{y}	1.2 ± 0.0	6.2 ± 0.1	0.2 ± 0.0
	3	7.6 ± 0.5 ^{y}	8.5 ± 0.3 ^{y}	1.3 ± 0.1	6.3 ± 0.1	0.2 ± 0.0
	6	7.6 ± 0.2 ^{y}	8.5 ± 0.2 ^{y}	1.3 ± 0.1	6.3 ± 0.1	0.2 ± 0.0
	12	7.3 ± 0.3 ^{y}	8.4 ± 0.2 ^{y}	1.3 ± 0.1	6.1 ± 0.1	0.2 ± 0.0
	24	6.1 ± 0.3 ^{x}	7.8 ± 0.3 ^{x}	1.3 ± 0.0	6.1 ± 0.1	0.2 ± 0.0

[1] Within each factor and column, values (level means ± SEM; n = 20 for photoperiod, n = 12 for feeding interval) with the same letter in the superscript are not significantly different (P>0.05; two-way ANOVA, SNK)

[2] There were no significant interactions (P>0.05) between photoperiod and feeding interval for any of the growth indices.

[3] Feed conversion ratio (FCR) = Feed (dry weight)/ fish weight gain (wet weight) (Hardy, 1989); Condition factor (CF) = $100*(\text{weight}_{(g)}/\text{length}_{(cm)}^{b})$, where b is estimated by the length-weight relationship equation ($W = aL^{b}$) of the sampled populations (King, 1995); Coefficient of Variation of weight (CV_w) = (s/mean) x 100, where s is the standard deviation.

The FCR of fish was significantly affected ($P<0.05$) by photoperiod. Fish exposed to the 24L:0D photoperiod displayed a higher FCR than those reared in the 18L:6D photoperiod, while the 12L:12D photoperiod had the lowest FCR. The CF of mulloway was also affected by photoperiod with the CF of fish reared in 12L:12D lower than those reared in 18L:6D and 24L:0D (Table 15). The lower CF for the 12L:12D photoperiod was a result of significantly longer but not heavier individuals in comparison with the 18L:6D and 24L:0D photoperiods as previously described. Photoperiod did not affect CVwt ($P>0.05$) (Table 15).

Survival of fish held in 12L:12D (93.3 ± 1.6%) was significantly lower ($P<0.05$) than for fish held in 18L:6D (98.3 ± 0.5%) and 24L:0D (97.5 ± 1.5%). No significant difference ($P>0.05$) in survival of fish was found between the feeding intervals.

Feeding interval had a significant effect on the weight and length of fish (Fig. 9; Table 15). Mulloway cultured with a 24 h feeding interval were significantly ($P<0.05$) lighter and shorter than fish in all other feeding intervals (Table 15). The percentage body weight consumed per feed increased linearly (R^2 = 0.9992) from the 1h feeding interval to the 12 h feeding interval (Fig. 10). When the 24 h feeding interval is included this linear trend is not as strong (R^2 = 0.9838) as these fish consumed an average percentage body weight of more than twice that of fish held in the 12 h feeding interval (Fig. 10). However, the total weight of the feed consumed for this 24 h feeding interval was less than other feeding intervals as a result of smaller fish weights (Table 15).

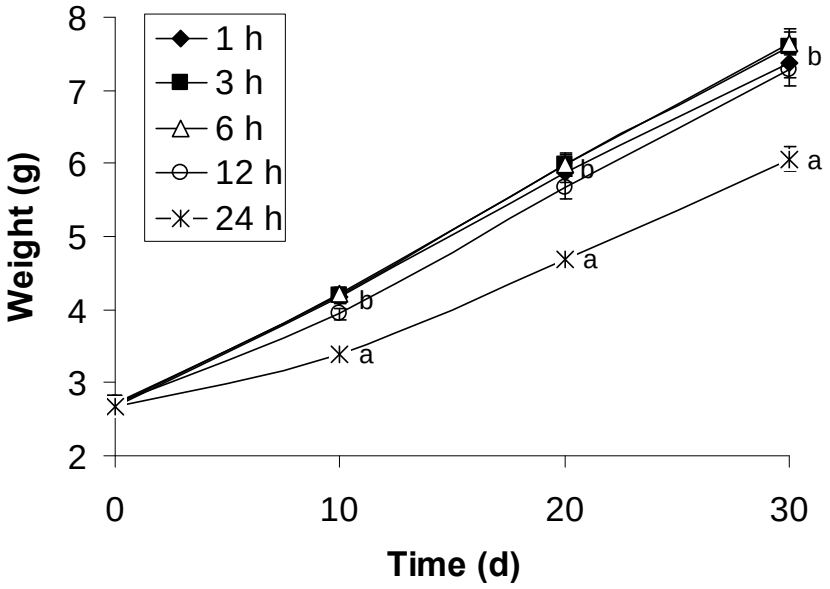

Figure 9: Mean ± SEM (n = 12) wet weight of juvenile mulloway reared on different feeding intervals over the 30 day trial. Durations between feeds were 1, 3, 6, 12 and 24 h. Photoperiod data is pooled. Mean ± SEM values with different letters at the same time period indicate significant differences (P<0.05; two-factor ANOVA, SNK).

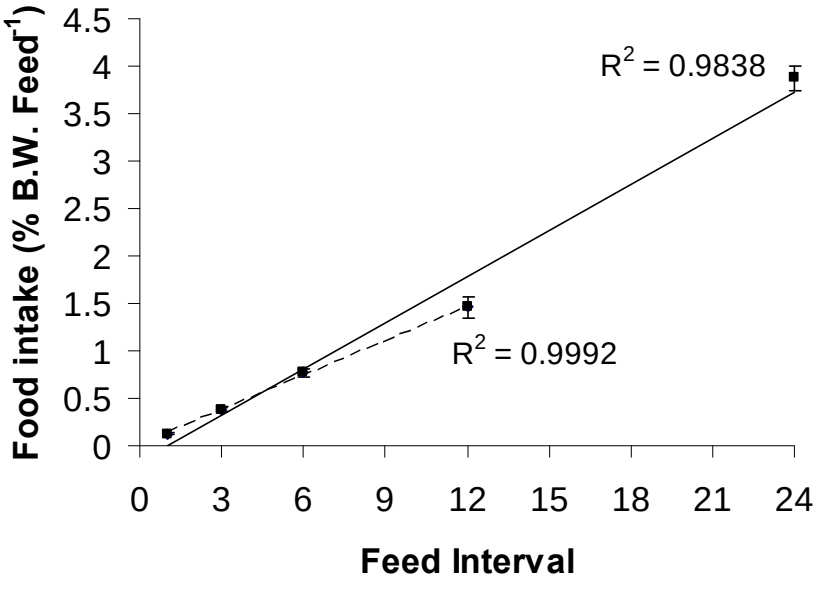

Figure 10: Mean ± SEM (n = 12) percentage of body weight consumed per feed for different feeding intervals over the 30 day trial. Feeding intervals were 1, 3, 6, 12 and 24 h between feeds. Photoperiod data is pooled. Trend lines: —— 1 to 24 h feeding intervals, – – – 1 to12 h feeding intervals.

The FCR, CF and CVwt were not significantly affected (P>0.05) by feeding interval over the 30 day experiment (Table 15); however, the FCR did vary significantly for each ten day weight period. From day 0 to day 10 the FCR for the 12 h feeding interval was higher than the 1, 3 and 6 h feeding intervals and the FCR for the 24h feeding interval was higher than all other feeding intervals. From day 11 to day 20 there was no difference in FCR between the feeding intervals but from day 21 to day 30 the FCR successively increased as feeding interval was decreased. The final result of this change in FCR is that no difference was found between feeding intervals for the average FCR over 30 days.

The fish in the 12L:12D, 12 h treatment were fed once under light and once in darkness. The mean weights (mean ± SEM; n = 4) of the fish in this treatment (7.8 ± 0.4 g) were higher than that of the treatment 12L:12D, 24 h (6.3 ± 0.2 g), which were only fed once in the light.

5.3.2 Experiment 2: Gastric evacuation of juvenile mulloway

The amount of food remaining in the stomach of juvenile mulloway after a single feed to satiation decreased steadily over a 20 h period (Fig. 11). At 10 h after feeding, 50% of the food consumed had been evacuated from the stomach. The linear relationship between 2 and 20 h after feeding (R^2 = 0.9477), can be expressed by stomach fullness (% body weight) = -0.3417 x (time after feeding) + 6.8101.

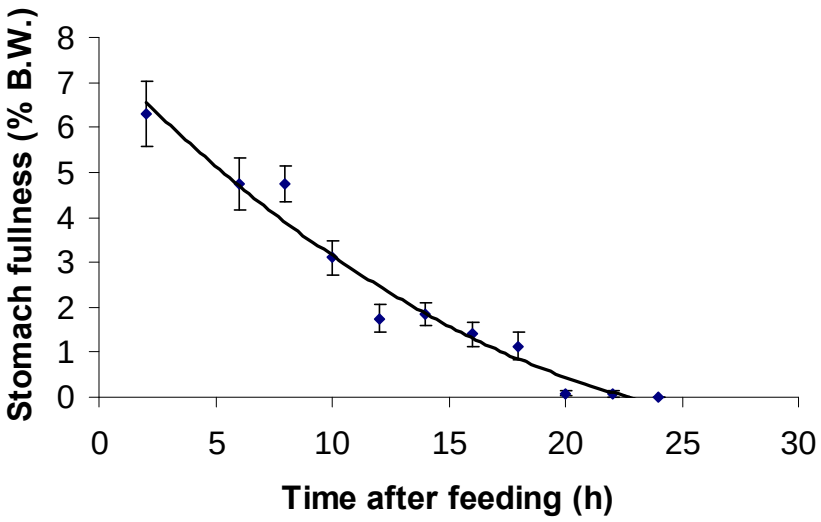

Figure 11: Change in stomach fullness assessed as the mean ± SEM (n = 15) weight of the stomach content as a percentage of fish body weight (g) of juvenile mulloway over time (post-prandial hours) after being fed once to satiation. All weights used in calculations are dry weights.

5.4 Discussion

This study has established that there are no interactive effects of photoperiod and feeding interval on juvenile mulloway, which differs from a previous study on juvenile snapper (*P. auratus*), another temperate marine species (Tucker et al., 2006).

Experiment 1 determined that photoperiod can have a significant effect on the survival and growth of juvenile mulloway. Under a 24L:0D photoperiod, significantly poorer growth (weight, length and FCR) of mulloway was obtained compared with that of fish reared under 12L:12D and 18L:6D photoperiods. This is consistent with previous research that demonstrated optimising photoperiod regimes is necessary to obtain high growth rates (Boeuf and Le Bail, 1999). Biswas and Takeuchi (2003) found that a 24L:0D photoperiod is less effective in obtaining high growth rates (in terms of Specific Growth Rate - SGR) for Nile tilapia (*O. niloticus*) when compared to 3L:3D, 6L:6D and 12L:12D photoperiods. These authors attributed the respective SGR values to the balance between energy intake and energy expenditure at the different photoperiod treatments. A continuous light regime is likely to require greater energy expenditure associated with increased swimming activity than shorter light regimes and may have contributed to significantly higher FCR and reduced growth rates for mulloway. In contrast to this, a continuous photoperiod has been associated with improved growth rates for some species as the length of feeding time available to fish is increased (Sayer, 1998). Tripple and Neil (2003) found an increase in the weight of juvenile haddock (*Melanogrammus aeglefinus*) reared under continuous light for 24 weeks compared to fish reared under natural photoperiods. Simensen et al. (2000) also found

improved SGR in Atlantic halibut (*Hippoglossus hippoglossus*) when reared under continuous light compared to fish reared under shorter photoperiods. As the results of photoperiod trials on fish growth have been inconsistent between species, it is apparent that photoperiod requirements are species specific and growth rates may partly depend on the species ability to reduce energy expenditure in long light phases.

The FCRs for juvenile mulloway were observed to increase with day length. The 12L:12D photoperiod produced the lowest FCR, while the 24L:0D photoperiod had the highest FCR. This suggests that juvenile mulloway are better able to convert energy into somatic growth during shorter photoperiods. In contrast to this, Rad et al. (2006) found that continuous light improved the FCR of *O. niloticus*, again indicating that growth performance under long photoperiods is species specific. Mulloway held in the long light phase also consumed less food per day than the shorter photoperiods, even though there was more light available for feeding. This is likely to have been influenced by the poorer FCRs, which contributed to smaller fish unable to consume as much as the larger fish from the other photoperiods.

In addition to the poorer FCRs observed in juvenile mulloway during long light phases, it appears that fish length is inversely related to day length. Melatonin levels are also inversely related to day length (Randall et al., 1995), and melatonin along with the pineal gland, have previously been reported to influence skeletal growth in fish (Fjelldal et al., 2004). It is well documented that melatonin levels are higher in fish during darkness (Randall et al., 1995; Porter et al., 1996; Bayarri et al., 2003) and this may have contributed to longer fish lengths in the treatments that had longer dark phases. The differences found in fish length also contributed to lower CF in the 12L:12D photoperiod, as these fish were longer but not heavier than

fish in the 18L:6D photoperiod. CF is said to be an indicator of the well being of fish (Bolger and Connolly, 1989) and although the fish were longer and displayed improved FCRs, the reduced CF may have contributed to the increased mortality rate observed in the 12L:12D photoperiod.

In the 12L:12D; 12 h treatment fish were fed once in the light and once in darkness. The weights of these fish were heavier than that of fish fed only once in the light (12L:12D; 24 h), providing evidence that fish may have been feeding in the dark by non visual means. This is similar to results of Harpaz et al. (2005), who found that juvenile barramundi (*L. calcarifer*), consumed food supplied in the light or dark. Sayer (1998) described how the incidence of nocturnal feeding can increase in winter when the photophase is shorter as there is less opportunity for daylight feeding. It may be useful to study this aspect of mulloway culture further to determine if additional gains in growth performance indices and survival could be obtained by night feeding in conjunction with daytime feeding under a range of short and long photoperiods.

Feeding interval also has a significant effect on the growth and feeding efficiency of juvenile mulloway. While there was no significant difference between the 1, 3, 6 and 12 h feeding intervals, the 24 h interval resulted in poorer growth. This is similar to results of Dwyer et al. (2002) who found that feeding yellowtail flounder (*Limanda ferruginea*) two or four daily meals produced better growth than fish fed once daily, attributing this to reduced food consumption in low feeding frequencies. The fish in the 24 h feeding interval did consume less feed than other feeding intervals but this was confounded by the poor FCRs in the first 10 days, which led to slow growth and smaller fish. Over time the FCRs improved and the percent body weight consumed per feed actually became greater than that of the

other feeding intervals. While the results of this study indicate that one feed per day is not a suitable feeding regime for juvenile mulloway, it is possible that fish may adapt to the regime over time and growth rates may improve.

Gastric evacuation rate and meal size are major factors affecting the post-prandial return of appetite and subsequent growth, with different species returning to feed after varying amounts of stomach evacuation (Smith, 1989). Species such as rainbow trout (*Oncorhynchus mykiss*) and squawfish (*Ptychocheilus oregonensis*) only feed once their stomach is near to empty, while juvenile pink salmon can return to feeding after as little as 15% of their stomach contents have been evacuated (Smith, 1989). Consequently, the difference in gastric evacuation and voluntary return to feeding between species greatly influences the optimal feeding regime.

The study on gastric evacuation of juvenile mulloway has shown that it can take up to 10 h before 50% of the food consumed in a single meal was evacuated from the stomach. The linear relationship between feed interval (h) and food intake (% B.W. Feed $^{-1}$) suggests that juvenile mulloway will continue to eat throughout the day but will eat larger amounts as the feeding frequency decreases, with the result that total food consumption does not increase appreciably with feeding frequency.

In conclusion, growth rates and efficient feeding can be optimised by rearing juvenile mulloway in an 18L:6D photoperiod and by feeding once every 12 h. The 24L:0D photoperiod produced poor growth (weight, length and FCR) compared to the 18L:6D and 12L:12D photoperiods and the 12L:12D photoperiod had reduced survival compared with the other photoperiods with the result that the fish in the 18L:6D photoperiod had optimal growth and survival parameters. The only feeding

interval to have an effect on growth (weight and length) was the 24 h feeding interval, with no difference in growth detected between the 1 h through to the 12 h feeding intervals. It was also determined that it would take 10 h for 50% of the feed consumed in one meal to be evacuated from the stomach. Given that there was no significant difference in growth and feeding efficiency in juvenile mulloway between the 1, 3, 6 and 12h feeding intervals, we suggest that it would be more efficient for producers to feed their fish no more than twice daily (or every 12 h).

Chapter 6

Investigation of the sensory basis of feeding of larval and juvenile mulloway, *Argyrosomus japonicus*

6.1 Introduction

Marine finfish larvae typically hatch very early in development with undeveloped sense organs (Blaxter, 1986; Cobcroft and Pankhurst, 2003; Mukai et al., 2008). After hatching, development is rapid to ensure larvae have the morphological features required to undertake exogenous feeding. There are three major sensory systems employed by fish for detecting prey and evading predators and they are vision, mechanoreception and chemoreception (Blaxter, 1986). However, for many species of larvae, vision, a sense dependent upon light for photoreceptor stimulation, is the primary sensory mode used for feeding and the eyes are generally pigmented and considered functional by first-feeding (Blaxter, 1988).

As larval ontogeny proceeds, mechanoreception and chemoreception can play an increasingly important role in feeding. In most fish species, the mechanosensory lateral line (rather than the inner ear; also a mechanosensory organ) is implicated in prey detection. A simple mechanical displacement organ called the neuromast is the sensory unit common to mechanosensory organs in fish. Larvae typically hatch with a few superficial neuromasts situated on the skin around the head and along the trunk (Jones and Janssen, 1992; Cobcroft and Pankhurst, 2003). These proliferate after hatching and increase in size, eventually becoming

enclosed in a bony canal but retaining continuity with the hydrodynamic environment around the fish via canal pores. Neuromasts of the mechanosensory lateral line detect small-scale hydrodynamic disturbances in the water immediately adjacent to the fish and those created by appendage beating of prey organisms can sometimes allow larvae to feed in dark environments (Pankhurst, 2008).

Chemosensory function in fishes is variously provided by the olfactory organs (paired nares on the head, equivalent to the nose), taste buds situated in and around the mouth and palette, and isolated 'taste' buds located superficially on the skin (Hara, 1971). As with other sensory organ systems small body size places constraints upon the size and complexity of chemosensory organs in early development of fish (Pankhurst and Butler, 1996; Cobcroft and Pankhurst, 2003) but this is overcome with ensuing growth and development, providing an increasing potential for chemical detection of prey.

To examine the sensory basis for prey detection and feeding behaviour, fish fed in the light and dark can be compared for feeding ability to determine the instance of visual and non-visual feeding (Pankhurst, 2008). Non-visual feeding can then be further investigated using the aminoglycoside antibiotic, streptomycin sulphate (SS), to pharmacologically ablate the neuromasts of the mechanosensory lateral line of fish, leaving only chemoreception for detection of live prey in dark conditions (Wersall and Flock, 1964; Matsuura et al., 1971, Jones and Janssen, 1992; Pankhurst, 2008).

The feeding behaviour of larval mulloway (*Argyrosomus japonicus*) has been studied before and it was determined that larvae are visual feeders from first-feeding but later develop the ability to feed in the dark (Chapter 3). Mulloway are a species of sciaenid and are typically thought to spawn in nearshore coastal waters

where eggs and larvae are dispersed in surrounding estuarine and coastal waters and are then recruited to the upper reaches of estuaries as juveniles (Silberschneider and Gray, 2008). Species that inhabit complex environments, such as estuaries, often rely on a combination of sensory modalities to utilise habitats and to locate and capture prey (Poling and Fuiman, 1999).

This study aimed to determine changes in the sensory organs available to larval and juvenile mulloway for feeding as development proceeded.

6.2 Materials and methods

6.2.1 Fish and facilities

Mulloway broodstock held at the PSFI were induced to spawn using temperature cues in September 2007 (methods described in Partridge et al., 2003). Fertilised eggs were collected, treated with ozone (Concentration [mg l^{-1}] x Time [min] = 1) to remove pathogens and transferred to an 8000 l clear-water holding tank. The temperature in the holding tank was maintained at 20-22ºC, and larvae were fed rotifers (5 ml^{-1}) from 2.5 mm (first feeding) until 5.5 mm, and then *Artemia* (0.5 ml^{-1}) until they were weaned onto a 400 μm pellet (from 10.5 mm). The tank water was exchanged at a rate of 5 l min^{-1}, which ensured clearance of live feeds from the tank during the night and no residual food remained in the digestive tract of fish from the previous day. Fish were maintained in ambient light.

6.2.2 Ablation of the lateral line in mulloway using streptomycin sulphate (SS)

The first experiment examined the efficacy of four concentrations (0.0, 0.5, 0.75 or 1.0 g l^{-1}) of streptomycin sulphate (SS) to ablate canal and superficial neuromasts of the mechanoreceptive lateral line of larval mulloway, leaving neuromasts of the inner ear intact (Wersall and Flock, 1964; Matsuura et al., 1971). Twenty larvae (3.5 mm) were gently transferred in a fine mesh dip-net from the 8000 l holding tank to each of nine, one l experiment vessels. Larvae were treated in their respective SS solution (n = 3) for 3 h and were then transferred individually to a Petri dish of sterilised estuarine water. In order to remove visual stimuli from

larvae, all white light was excluded from the room and larvae were tested under red lights. Larvae were approached with a blunt probe in order to produce a hydrodynamic disturbance close to the larvae and the presence or absence of a 'startle' (rapid evasive) response was recorded for each fish treated in an SS solution, as well as control fish that were exposed only to seawater. An appropriate dose for neuromast ablation determined here was used in subsequent feeding trials.

6.2.3 The contribution of the sensory organs to feeding

A series of short-duration trials made up the second experiment and were completed at regular size/age intervals of fish to examine the contribution of sensory organs to feeding.

6.2.3.1 Larval stages consuming live feeds

On the basis of the trial above, mulloway between 3.5 mm (early larvae) and 10 mm TL (advanced larvae) were either treated with 0.075 mg l^{-1} SS for 3 h in order to ablate the neuromasts of the mechanosensory lateral line, or a seawater (SW) 'control' treatment in which the mechanosensory lateral line remained intact. Fish were then allowed to feed in either the light or dark to separate visual and non-visual senses, thus forming four treatment groups:

- SS Dark (Chemoreception intact)
- SS Light (Vision and Chemoreception intact)
- SW Dark (Chemoreception and Mechanoreception intact)
- SW Light (all senses available)

On the morning of each trial, 20 larvae were sampled from the holding tank and examined under a dissection microscope for TL and to confirm that no residual food remained in the digestive tract from the previous day. Then, 30 larvae were transferred to each of 16 vessels made from a 100 mm length of 80 mm PVC pipe to which a piece of 1 mm nylon mesh was fitted to one end. The vessels were designed to contain larvae but allow movement of treatment solution. Half of the 16 vessels were suspended in a single tub containing a solution of SS (0.75 mg l^{-1}) (SS treatment), and the remaining 8 vessels were suspended in a bath of sterilised seawater (SW treatment); both solutions were lightly aerated. Fish remained in either the SS treatment or the SW treatment bath for 3 h and then the 30 fish from each vessel were transferred to a corresponding 10 l blue static water feeding vessel and placed in an individual experiment enclosure that was surrounded by black plastic sheeting to exclude light leakage. All sterilised seawater used in the trial had similar water quality parameters to the holding tank.

Fish were allowed to acclimate to experiment vessels for 1-2.5 h and were then fed a food type appropriate to their size, which started with rotifers (*Brachionus plicatilis*) until fish reached a mean TL of 5.5 mm, followed by *Artemia* until the mean TL reached 10.6 mm. Fish in each experiment replicate were fed in either light (7.4 μmol s^{-1} m^{-2}), which was similar to the holding tank, or dark (0 μmol s^{-1} m^{-2}) conditions for 1 h, after which, fish were euthanased with 100 ppm Aqui-S® and 20 of the 30 fish were randomly selected and placed on a cold slide, secured with a cold cover slip and examined immediately under a dissection microscope for the number of fish that had consumed food in 1 h and the number of food items in each individual stomach. When it was difficult to determine the number of food items in an individual stomach, the cold cover slip was removed

and the stomach and its content was teased apart. Feeding treatments were staggered at 30 min intervals to enable fish in each treatment to be harvested, euthanased and then examined immediately for evidence of feeding.

SS treated larvae were periodically checked throughout the feeding trial for a 'startle' response when approached with a blunt probe under red light conditions to ensure the concentration of SS remained adequate for mechanosensory lateral line ablation.

6.2.3.2 Fish consuming pellet diets

Once fish attained a mean TL of 10.6 mm they were weaned onto inert pellet diets which upon delivery either floated on the water surface or sank to the tank bottom and were unlikely to provide a sustained stimulus for mechanosensory lateral line stimulation. Accordingly, feeding of these fish was assessed without SS treatment under dark and light conditions, thus forming two treatment groups:

- Dark (non-visual senses available)
- Light (all senses available)

Feeding trials were conducted as for live feeds trials with the exception that fish were offered an appropriately sized pellet diet (3/5 Proton pellet microdiet, [INVE, Dendermonde, Belgium]; 5/8 Proton pellet diet; Ridley 1 mm crumble [Ridley Aqua-feed, Narangba, Australia; Native fish starter]; Ridley 2 mm pellet). In addition, fish >10.6 mm could not be examined under the microscope and therefore individual fish were dissected to remove the stomach and assess its content.

6.2.4 Statistical analyses

Data were assessed for homogeneity of variance using Cochran's test. When variances were heterogeneous, data were log transformed to establish homogeneity. One-factor ANOVA was then used to examine treatment effects on the number of larvae feeding and on the number of food items consumed. Where statistical differences were found ($P<0.05$), means were compared using the Student-Newman-Keuls test.

6.3 Results

6.3.1 Ablation of the lateral line in mulloway using streptomycin sulphate

All larvae in the control treatment (0 g l^{-1} SS) elicited a 'startle' response when approached with a blunt probe under red light conditions. Fifteen percent of larvae treated with SS at 0.5 g l^{-1} elicited a 'startle' response to the blunt probe, and the reactions of the fish were obvious and immediate. Ten percent of larvae treated with SS at 0.75 g l^{-1} reacted to the blunt probe; however, reactions were delayed and not immediate. Larvae treated with 1 g l^{-1} SS displayed no reaction to a blunt probe; however, larvae also appeared disoriented and did not react to a probe when lightly touched on the side flanks. Therefore 0.75 g l^{-1} SS appeared to be the most suitable for ablating the lateral line in mulloway larvae as any response when approached by a blunt probe was not immediate but larvae appeared to still behave normally.

6.3.2 The contribution of the sensory organs to feeding

6.3.2.1 Rotifer feeding stage

Fish from 3.5 ± 0.1 to 5.0 ± 0.1 mm TL (mean ± SEM) consumed significantly more (P<0.05) food items (rotifers) when fed in light conditions (SS Light & SW Light treatments), and vision was available, compared to those in dark conditions (SS Dark & SW Dark treatments) when non-visual senses were potentially available (Fig. 12). There was no significant difference in the mean number of prey consumed in the light when mechanoreception function was

unavailable (SS Light) compared to the SW Light treatment when all senses were available. Nor was there a significant difference in the number of prey consumed by larvae of these size classes when only chemoreception, or chemoreception and mechanoreception combined, were available (SS Dark & SW Dark treatments, respectively).

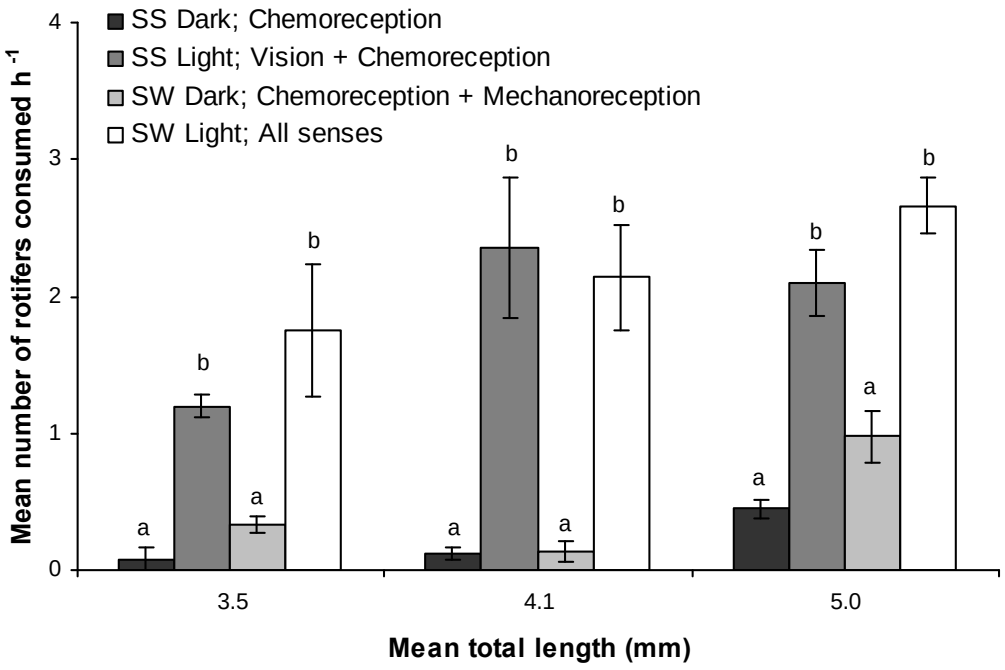

Figure 12: The number (mean ± SEM) of rotifers consumed by fish in 1 h. Fish were treated with streptomycin sulphate (SS) or seawater (SW) and fed in the dark or light. Different letters within a sample period indicate significant differences (P<0.05; one-factor ANOVA, SNK).

The proportion of fish from 3.5 ± 0.1 to 4.1 ± 0.1 mm TL feeding on rotifers was significantly greater (P<0.05) in the light treatment compared to dark treatments (Fig. 13). In fish of 3.5 mm TL the proportion of fish feeding was significantly higher in the SW Light treatment compared to the SS Light treatment; however, when fish reached 4.1 mm there was no significant difference in the proportion of fish feeding in these two treatments. The proportion of fish feeding in the dark when chemoreception alone, or chemoreception and mechanoreception

combined was available, was not significantly different in fish of these sizes. This relationship changed when fish attained a mean TL of 5.0 mm; a higher proportion of fish fed in the dark when both chemoreception and mechanoreception were available (SW Dark treatment) compared to when chemoreception alone was available (SS Dark treatment). Further to this, although significantly more fish fed when all senses were available (SS Light treatment) compared to chemoreception alone, there was no significant difference in the number of fish feeding in the SS Light and SW Dark treatments.

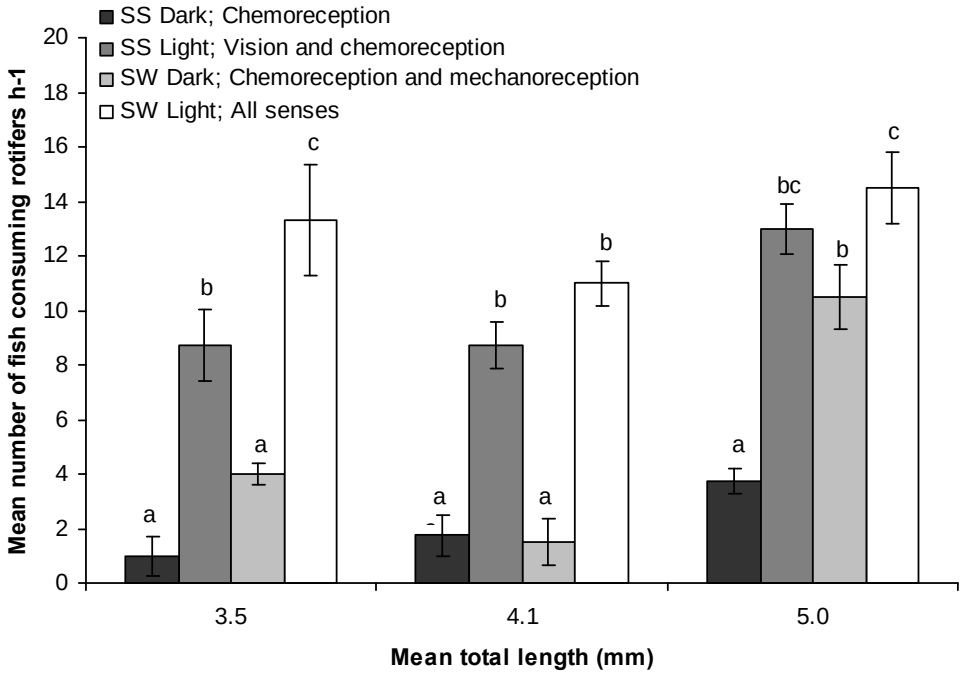

Figure 13: Number of fish (mean ± SEM) consuming rotifers in 1 h. Fish were treated with streptomycin sulphate (SS) or seawater (SW) and fed in the dark or light. Different letters within a sample period indicate significant differences (P<0.05; one-factor ANOVA, SNK).

6.3.2.2 Artemia feeding stage

The number of food items (*Artemia*) consumed by fish from 5.8 ± 0.1 to 10.0 ± 0.2 mm TL was low when chemoreception was the only sensory system available (SS Dark). There was also a trend of increasing number of prey consumed with increasing complexity of senses available for prey detection; however, the number of prey consumed was only significantly higher in the SW Light treatment (all senses available) compared to the SS Dark treatment (chemoreception only) in fish with mean lengths of 5.8, 7.5 and 10.0 mm (Fig. 14).

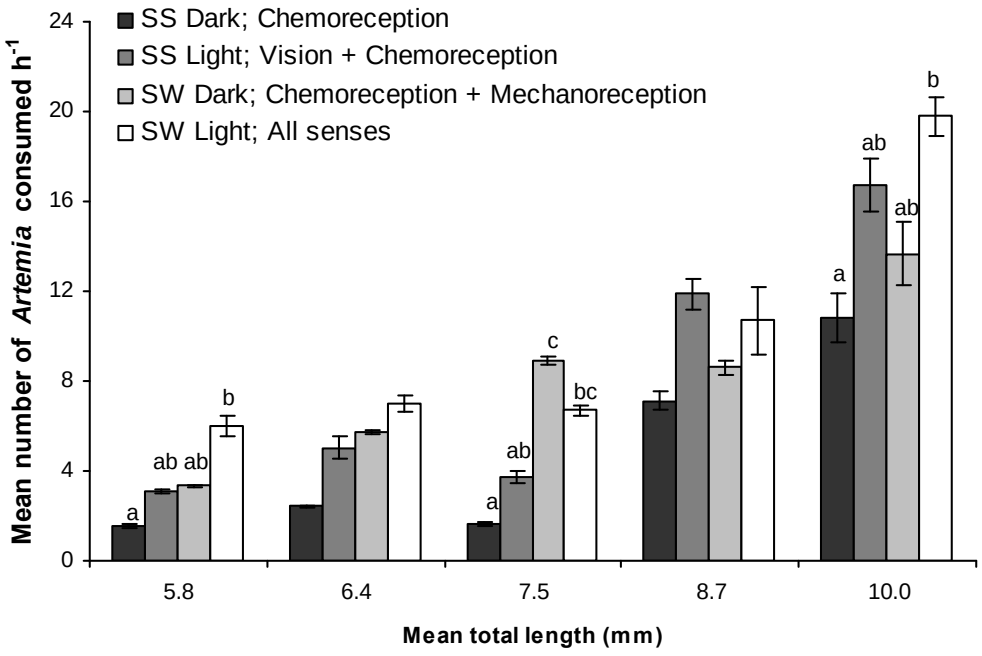

Figure 14: The number (mean ± SEM) of *Artemia* consumed by fish in 1 h. Fish were treated with streptomycin sulphate (SS) or seawater (SW) and fed in the dark or light. Different letters within a sample period indicate significant differences ($P<0.05$; one-factor ANOVA, SNK).

In fish size classes from 5.8 ± 0.1 mm to 7.5 ± 0.2 mm TL the lowest proportion of feeding occurred when chemoreception alone was available (SS Dark); but was significantly lower than all other treatments only in fish with mean lengths of 6.4 mm (Fig. 15). From 8.7 ± 0.2 to 10.0 ± 0.2 mm, there was no significant difference in the number of fish feeding amongst the treatments (Fig. 15).

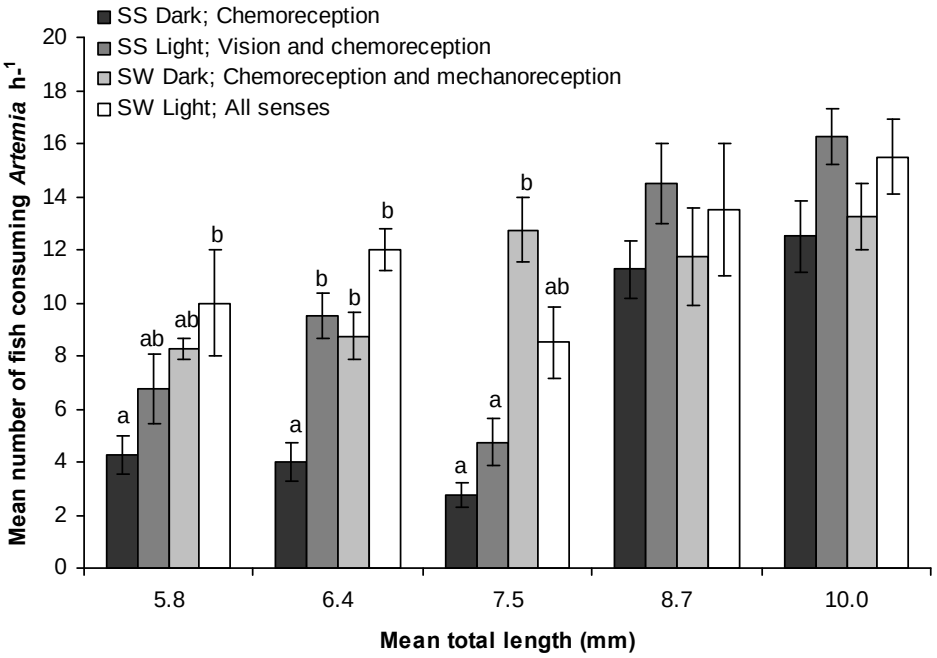

Figure 15: Number of fish (mean ± SEM) consuming *Artemia* in 1 h. Fish were treated with streptomycin sulphate (SS) or seawater (SW) and fed in the dark or light. Different letters within a sample period indicate significant differences (P<0.05, one-factor ANOVA, SNK).

6.3.2.3 Pellet feeding stage

Fish from 10.6 ± 0.3 to 53.3 ± 0.7 mm TL consumed pellets in both light and dark conditions; however, significantly (P<0.05) more pellets were consumed and a higher proportion of fish fed when all senses were available in light conditions (Figs. 16 & 17).

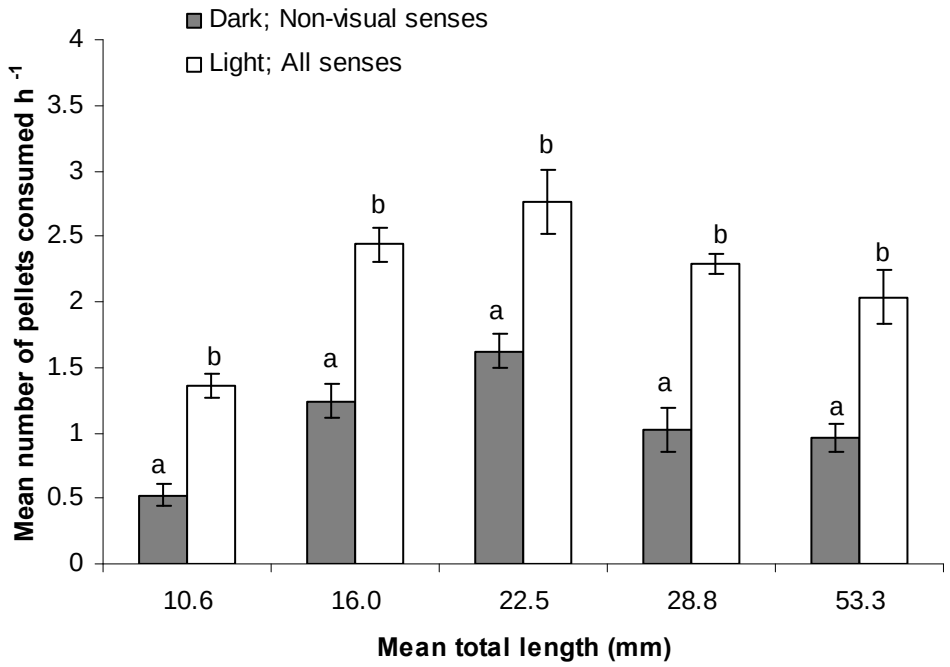

Figure 16: Number (mean ± SEM) of food items (pellet) consumed by fish in 1 h. Fish were fed in the dark or light. Different letters within a sample period indicate significant differences (P<0.05; one-factor ANOVA, SNK).

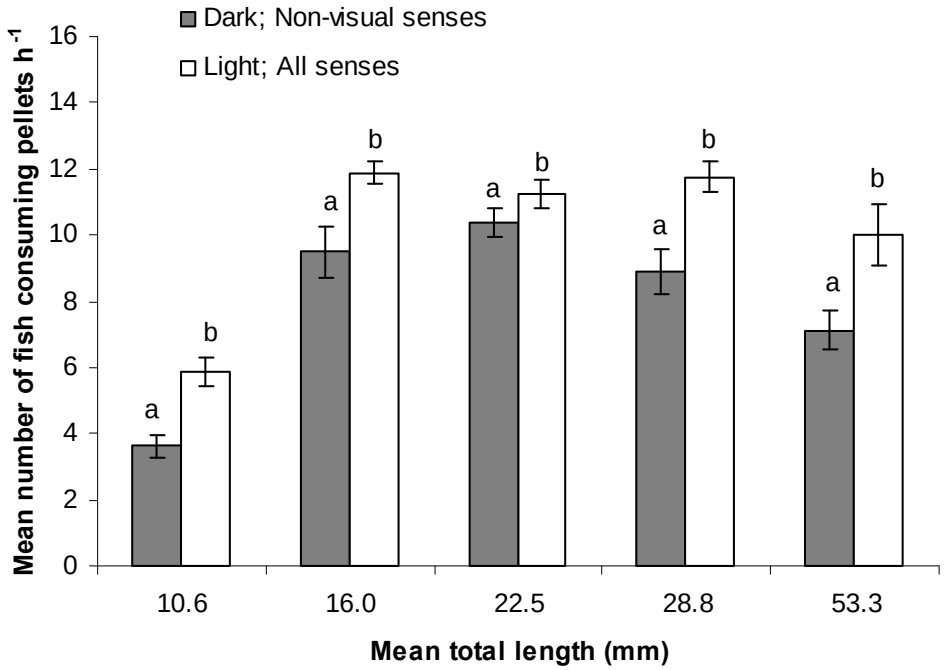

Figure 17: Number of fish (mean ± SEM) consuming pellets in 1 h. Fish were fed in the dark or light. Different letters within a sample period indicate significant differences (P<0.05; one-factor ANOVA, SNK).

6.4 Discussion

The sensory organs available to young mulloway for feeding vary with development. This study has established that vision is the dominant sensory system for feeding by early mulloway larvae as the proportion of larvae feeding and the number of food items consumed improved in light conditions when visual senses were available, compared with dark conditions when only non-visual senses were potentially available. This is common for marine teleost larvae, and many studies have reported that larvae require light at the onset of exogenous feeding (Blaxter, 1986; Poling and Fuiman, 1997; Cox and Pankhurst, 2000; Cobcroft and Pankhurst, 2003). A study into the importance of vision and mechanoreception of another sciaenid, the Atlantic croaker (*Micropogonias undulates*), reported variations in the role of the sense organs during ontogeny and similarly to mulloway, vision was the dominant system in larvae (Poling and Fuiman, 1997).

Vision remained the dominant sensory system for feeding by mulloway larvae from 5.0 mm TL; however, the proportion of larvae feeding in darkness increased when the mechanosensory lateral line was intact. The contribution of this sensory system appeared to increase when larvae began feeding on *Artemia* (5.8 mm), likely reflecting proliferation and growth of neuromasts of the developing mechanosensory lateral line system but which may also have been influenced by the more pronounced hydrodynamic signal created by *Artemia* appendage beating compared with rotifers. Superficial neuromasts are present at or soon after hatching in marine fish larvae, usually comprising very few sensory cells, the mechanosensory hair cell, and adjacent support cells. The mechanosensory hair cell is responsible for detecting motion or movement, and comprises displacement-

sensitive ‘hairs’, which are arranged asymmetrically and project from the apical surface of the hair cell. Each hair has a specific directional sensitivity and can detect the direction of a hydrodynamic stimulus (Pankhurst, 2008). Supporting cells surround the hair cells and secrete a gelatinous mass called the cupula, which can amplify water disturbances and bend the sensory hairs of the cell to stimulate sensory neurons and elicit a mechanosensory response (Kunz, 2004). The role of mechanoreception in teleost larvae has been well documented and studies of marine fish larvae have found that while the primary role of superficial neuromasts is to elicit an escape response for predator avoidance (Yin and Blaxter, 1987; Blaxter and Fuiman, 1989), some species are also capable of detecting the water disturbances created by prey items (Jones and Janssen, 1992). The present study is similar to one by Jones and Janssen (1992), who described an improvement in feeding ability of mottled sculpin (*Cottus bairdi*) larvae in the dark when superficial neuromasts were intact, while Diaz et al. (2003) observed improved feeding rates in seabass (*Dicentrarchus labrax*) when live prey was available compared with frozen prey and also attributed this to the role of superficial neuromasts. The sensory epithelium of individual superficial neuromasts continue to develop during ontogeny; this being reflected in an increase in the number of sensory hair cells present in each neuromast. During the later portion of the larval period superficial neuromasts gradually sink into the dermis becoming enclosed in a rigid canal but maintaining communication with the hydrodynamic environment adjacent to the fish via numerous canal pores (Webb, 1989; Poling and Fuiman, 1997; Coombs et al., 2002). An increase in receptivity to water disturbances created by prey items has been reported at this time (Jones and Janssen, 1992; Cobcroft and Pankhurst, 2003; Webb and Shirey, 2003; Pankhurst, 2008).

In larvae greater than 5.0 mm TL, vision and mechanoreception continued to be utilised in live prey detection; however, feeding success was also improved in larvae with only chemoreception available once the average larval length reached 8.7 mm. Evidence of a role for chemoreception continued when fish were offered pellets, and while feeding success of fish offered pellets was higher in light conditions compared with dark conditions, the presence of feeding in the dark on these inert food items suggests a continued contribution of the olfactory and/or gustatory chemosensory organs to feeding. Jones (1992) described three phases of food searching behaviour in relation to chemical stimuli; an initial arousal phase, where the fish is alerted to the presence of food; a search and exploratory phase, where the fish attempts to locate the food source; and finally a consumption phase. This process of detection, exploration and consumption is likely to be a comparatively more time consuming feeding process than when visual stimuli are present and the relatively short feeding duration of these trials (1 h) may have been inadequate to assess the full potential of chemoreception to feeding in larval and juvenile mulloway.

This study has determined that while vision is the dominant sensory modality utilised for prey detection and consumption by larval and juvenile mulloway, an increasing reliance upon non-visual senses was evident during development, firstly with the recruitment of mechanosensory and later chemosensory organs to feeding behaviour. These changes were reflected in the results of previous chapters, where early larval mulloway were unable to feed in dark conditions and suffered complete mortality by 6 days after hatching (2.8 mm TL) (Chapter 3). However, advanced larval mulloway (>4.5 mm TL) were observed feeding on live prey in dark conditions and had improved survival compared with

other light regimes (Chapter 3), providing further evidence that the contribution of the mechanosensory lateral line had improved sufficiently to allow advanced larvae in the trial to detect the hydrodynamic disturbances created by rotifers and *Artemia*. In Chapter 5, juvenile mulloway (up to 8.9 cm) were also observed feeding in the dark on pellets, and this could be attributed to the increase in the chemosensory organs in fish of this age. In the wild, larval mulloway disperse into lower estuaries and move into the complex environment of the upper reaches as juveniles (Silberschneider and Gray, 2008). The increasing availability of non-visual senses as ontogeny proceeds may be an adaptive feature of mulloway to enhance feeding opportunities within these habitats.

Chapter 7

General Discussion

7.1 Introduction

Mulloway (*Argyrosomus japonicus*) has the potential to be a viable aquaculture species in temperate climates around the world and has been produced in Australia as a food fish and for recreational fish stocking programs (Silberschneider and Gray, 2008). Production capacity has been restricted by the availability and high costs of fingerlings, which have occurred in part from limited information on the biological and physical requirements of the species. A reduction in the production costs for mulloway fingerlings combined with an increase in production yield are likely to improve the viability of mulloway as an aquaculture species.

The principal objectives of this thesis were to investigate the optimal physical conditions and biological requirements for intensive rearing in mulloway hatcheries and to subsequently develop efficient culture protocols for each stage of hatchery production. A series of trials were conducted with the eggs, larvae and fingerlings of mulloway spawned from captive, first-generation broodstock and were designed to investigate five key areas of research:

1) Egg disinfection (Chapter 2)

2) Embryonic development (Chapter 2)

3) Sensory function relating to feeding (Chapter 6)

4) Optimal light requirements (Chapters 3 and 5)

5) Optimal feeding regimes (Chapters 4 and 5)

Light and feeding regimes are interrelated and are dependant on age and the level of sensory development achieved. Therefore, these hatchery conditions were investigated using multi-factor feeding trials that allowed ontogenetic development to be examined. The results of these trials were then used to develop hatchery protocols that promote both growth and development during each stage of hatchery production.

7.2 Egg disinfection and embryonic development

An important constraint to the yield of a production run is variability in the quality of eggs (Lahnsteiner and Patarnello, 2003). Attempts to rear eggs of poor quality are often futile and can add to overall production costs by increasing the resources usually allocated to egg incubation and larval rearing. In addition to the quality of eggs, pathogens such as nodavirus, present in the ovaries of brood fish, have been reported to affect egg development (Grotmol et al., 1999) and can later infect the larvae (Munday and Nakai, 1997). In this study, a series of investigations were completed to develop guidelines that will assist hatchery operators to reduce the chance of hatchery infections associated with viruses and bacteria and to assess the quality of mulloway eggs (Chapter 2). An ozone disinfection protocol was

developed to minimise the vertical transfer (from broodstock to progeny) of aquatic pathogens. The most suitable ozone CT value (Concentraion [mg l^{-1}] x Time [min]), treatment temperature and egg age at the time of treatment were investigated and it was determined that mulloway eggs should be maintained in 22°C water and exposed to ozone (CT = 1) early in the development process to reduce the chance of pathogens affecting the development of the embryo. A reference guide of the embryonic development of mulloway was also created to assist hatchery operators to identify normal or abnormal development and to assess egg quality.

In summary, mulloway eggs should be maintained in 22°C water and exposed to ozone (CT = 1) early in development. A visual guide to the development of mulloway embryos is now available and can be used to assess the viability of mulloway eggs. It is expected this information will reduce hatchery costs by promoting good egg quality through appropriate disinfection techniques, reducing the vertical transfer of pathogens from broodfish to progeny, and by allowing operators to more accurately identify non-viable batches of eggs before significant resources are allocated to them.

7.3 Optimal rearing protocols for larval and juvenile mulloway

Some of the most scrutinised parameters for improving hatchery rearing protocols include photoperiod, light intensity (Sayer, 1998; Boeuf and Le Bail, 1999, Fielder et al, 2002), and feeding requirements (Person Le Ruyet, 1993; Kolkovski, 2001; Shaw et al., 2003). The biology and ecology of each species of fish is unique, which is an adaptive feature of aquatic organisms to create a niche within their environment, and can allow for increased feeding opportunities and

reduced competition in the wild (Aplin, 1998). It is because of the unique requirements of each species that optimal culture conditions must be investigated separately for each aquaculture candidate to increase the efficiency, and therefore the profitability, of production. This study investigated the light and feeding requirements of early larvae, advanced larvae and juvenile fish, and examined some of the biological mechanisms underpinning the results.

7.3.1 Early larvae

As with many species, the amount of light provided to mulloway larvae during critical stages of development can affect ontogeny and growth (Boeuf and Le Bail, 1999), and is therefore directly related to the production efficiency of the species. There are key developmental milestones that early larvae must achieve in order to grow and survive. These milestones include swim bladder (SB) inflation and buoyancy (Battaglene and Talbot, 1990) and the onset of exogenous feeding (Yúfera and Darias, 2007). The optimal photoperiod and light intensity for good growth, SB inflation and survival was investigated for early mulloway larvae (Chapter 3) (less than 4 mm) and it was determined that length, survival, food consumption and swim bladder (SB) inflation were impacted upon by photoperiod, but not by the range of the light intensities tested (1 or 10 μmol s^{-1} m^{-2}). It was determined that early larvae require light to feed, as those held in long light photoperiods displayed good growth and survival while those held in 24 hours of darkness suffered complete mortality by the sixth day after hatching (dah). In contrast, SB inflation was most improved in photoperiods that provided some darkness, which is not uncommon for Australian marine larval species (Battaglene

and Talbot, 1994; Trotter et al., 2003), and it was suggested by Martin-Robichaud and Peterson (1998) that this may be an adaptation for predator avoidance.

The mortality suffered by larvae held in complete darkness appeared to be directly related to food consumption as these fish were seldom observed with food in their gut. This is typical of early marine finfish larvae and can be attributed to restricted visibility and a reduced chance of encountering prey (Nwosu and Holzlöhner, 2000; Puvanendran and Brown, 2002). These results provided evidence that early larvae are visual feeders, requiring light to feed and survive. This outcome was then tested in Chapter 6, which examined the contribution of vision, mechanoreception and chemoreception in food detection of early larvae. This trial determined that while prey are occasionally consumed in the dark, possibly due to osmoregulatory drinking (Huse, 1994), larvae consumed prey items much more frequently when light, and therefore vision, was available. Mechanoreception and olfaction did not appear to be as important for early larval feeding, and it is likely that these sensory systems were still undeveloped.

In summary, this research has determined that early mulloway larvae require some light to feed as they rely on vision to assist with prey capture. They also require some darkness to promote SB inflation and therefore it is recommended that larvae are maintained in 12 h of light and 12 h of darkness until SB inflation is complete. It is expected that this light regime will promote optimal growth rates and ontogenetic development in larvae and is the most efficient light regime for culturing early mulloway larvae. Once SB inflation has been achieved, it is recommended the light regime be increased to 18 h to increase the available feeding time. It is expected that production costs may be reduced with this more efficient protocol as the time that larvae remain in the hatchery is likely to be shortened.

7.3.2 Advanced larvae

While the critical milestones for early larvae include first feeding and SB inflation, those for advanced larvae include growth rate, survival and improved feeding opportunities. Feeding opportunities can be directly related to the light regime applied, and can depend on the period and intensity of light, as well as the suite of sensory mechanisms available. This relationship between developing complex sensory systems and diversification of food type was considered when determining optimal light and feeding regimes.

The ability of advanced mulloway larvae (greater than 4.5 mm) to detect food and survive in different light conditions was examined (Chapter 3) and, similarly to early larvae, it was determined that the light intensities tested (1 or 10 μmol s^{-1} m^{-2}) did not have an affect on growth or survival. However, in contrast to the early larvae trial, no significant differences in growth were observed at the end of the trial between fish in any of the photoperiods tested (24, 18, 12, 6 or 0 h of light). In addition, survival was highest in fish held in complete darkness. This data provided strong evidence that advanced larvae were no longer dependent on vision for feeding and may have been utilising other sensory systems to assist with prey capture.

A subsequent assessment of the contribution of the sensory organs to feeding in advanced larvae revealed an increase in the role of mechanoreception (Chapter 6), compared with early larvae. Typically, the mechanosensory lateral line is undeveloped at the time of hatching for many marine teleost species (Pankhurst, 2008), with only a few superficial neuromasts present on the head and trunk (Cobcroft and Pankhurst, 2003); however, these neuromasts quickly proliferate,

enabling the larvae to detect hydrodynamic disturbances (Pankhurst, 2008). For mulloway larvae, it is possible that the number and size of superficial neuromasts increased in fish between the lengths of 2.8 and 4.5 mm, and were sufficient to allow larvae to detect hydrodynamic disturbances created by live prey items. This was the period between complete mortality of early larvae kept in dark conditions (2.8 mm) and the commencement of the advanced larvae trial (4.5 mm), where larvae were feeding and surviving in dark conditions. The assessment of which sensory organs are involved in feeding determined that chemoreception can also contribute to feeding success of larvae greater than 8.7 mm in length, and subsequently enhance the overall capabilities of advanced larvae to feed in the dark.

The increased feeding ability of advanced larval mulloway in dark conditions is likely to have contributed to high survival rates, but it does not explain why survival was better in 24 h darkness than in other photoperiods. Cannibalism was reported by Battaglene and Talbot (1994) to be a significant contributor to mortality of larval mulloway. However, larval mulloway kept in the dark when food was in short supply displayed reduced cannibalism and agonistic behaviour (Battaglene, 1996), and this may explain why survival was improved for larvae maintained in dark conditions.

This research provides evidence that it may be suitable to rear advanced mulloway in dark conditions, and possibly increase production output. However, it was observed that larvae reared in dark conditions were lighter in colour compared with those held in light conditions, with visibly less pigmentation. It is unknown how this will affect pigmentation of market size fish and the sale-ability of the final product. Pigmentation anomalies have reduced marketability of Australian snapper (*Pagrus auratus*; Doolan et al., 2007), another temperate marine finfish, and

therefore it is recommended that larvae are maintained in 18 h of light to promote good growth until further investigations into the effects of darkness on mulloway pigmentation are completed.

To increase our understanding of the biological requirements of the species, the feeding behaviour of advanced larvae was examined further to assess optimal weaning techniques (Chapter 4). One of the aims of this study was to reduce the amount of *Artemia* used to rear larval mulloway as this food type can contribute significantly to hatchery costs and the supply can be unreliable (Sorgeloos et al, 2001; Callan et al, 2003). It was determined that mulloway larvae could be weaned directly from large strain rotifers (*Brachionus plicatilis*) to inert pellets without the use of *Artemia*; however, this significantly reduced growth. Larvae fed *Artemia* in combination with rotifers and pellets also weaned successfully but displayed better growth rates, and these growth rates were maintained when larvae were fed a reduced ration of *Artemia*. The trial also established that introducing pellets early or late in larval development did not lead to differences in weight or TL. Therefore, the next research step aimed to examine preference selection by mulloway larvae of different food types.

Preference selection trials have been used to streamline the weaning process for a variety of aquaculture species, including greenback flounder (*Rhombosolea tapirina*; Shaw et al., 2003), Asian catfish (*Pangasius bocourti*; Hung et al., 2002), walleye (*Stizostedion vitreum*; Mayer and Wahl, 1997) and Atlantic halibut (*Hippoglossus hippoglossus*; Olsen et al., 2000). Mayer and Wahl (1997) discussed preference selection in terms of the profits gained, as fish can select strongly for prey types that improve growth and survival. Previously at the PSFI hatchery, mulloway larvae have been offered *Artemia* from 4 mm onwards as the species'

large mouth gape enabled them to consume *Artemia* from this size; however, the first mulloway preference selection trial determined that advanced larvae made a transition from preferentially selecting rotifers to *Artemia* at approximately 5.2 mm. If larval fish are offered a larger food source too early, the period of weaning and the transition between one food type and the next may be extended. An extended weaning period can increase feeding costs (Hart and Purser, 1996) as more than one food type is being offered simultaneously.

The appropriate transition period of larvae from *Artemia* to inert pellets also required consideration and at the PSFI hatchery this has typically occurred when larvae are approximately 8 mm. However, the second prey selection trial indicated that this weaning regime may also have been occurring earlier than was appropriate for mulloway larvae as the results determined that larvae selected inert pellets in preference to *Artemia* once the mean average length was 10.6 mm. Oslen et al. (2000) determined that less digestible prey types were not selected by Atlantic halibut (*H. hippoglossus*), and it is possible that smaller mulloway larvae selected *Artemia* in preference to pellets as they were easier to digest. The previous practice of introducing pellets to larvae of 8 mm rather than 10.6 mm could have increased the feeding costs as the period during which larvae were offered two food types may have been extended.

In summary, this study has determined that the most suitable light regime for advanced larval mulloway is 18 h of light; however, the physiological impacts of keeping larval mulloway in complete darkness should be investigated further as this may enhance survival and production yield. Mulloway larvae should be offered rotifers until they reach 5.2 mm in length, and should then be weaned onto enriched *Artemia*. Once the mean larval length reaches 10.6 mm, larvae can be offered an

inert microdiet. It is expected that these protocols will improve growth rates, reduce feeding costs and increase overall production yield by increasing feeding opportunities and feeding efficiency.

7.3.3 Juvenile mulloway

The production goals for juvenile fish are similar to those for advanced larvae, which are to promote good growth and survival. By this stage fish have metamorphosed (Battaglene and Talbot, 1994) and are well developed. This study examined the optimal light and feeding regime of juvenile mulloway in two feeding trials. Firstly, the photoperiod and feeding interval requirements of juvenile mulloway were studied and it was determined that fish grew poorly in 24 h of light. It was also determined that growth was poor when fish were fed a ration of food (10% body weight) only once in a 24 h period, in all of the photoperiods tested (Chapter 5); however, fish grew equally well when fed once every 1, 3, 6 or 12 h if the total quantity of food offered remained consistent. This indicated that for the amount of food offered (10% body weight), juvenile mulloway were able to digest food at a consistent rate and were able to consume a similar amount of food regardless of the feeding interval, except when they were fed only once every 24 h. From these results it appeared that the gastric evacuation rate of juvenile mulloway required further investigation. It was determined that when juvenile mulloway were fed to satiation, the amount of food remaining in their stomach was half of the initial amount consumed after 10 h and was not completely evacuated until 20 h post-feeding. This provided further evidence that juvenile mulloway will digest food at a consistent rate, supporting the idea that juvenile mulloway only require feeding

twice daily. This is useful information as it may reduce labour costs and may help reduce food wastage.

The design of this feeding trial did not allow for one of the treatments to be provided all allocated feeds in the light. Fish in this treatment were held in 12 h of light and were fed every 12 h. Because feeding commenced 30 min after the lights were turned on, there were not enough hours of light to allow fish in this treatment to be fed twice. This presented an excellent opportunity to examine the ability of juvenile mulloway to feed in dark conditions and therefore, the second feed was provided to these fish 30 min after the lights had been turned off. The weights of these fish were heavier than that of fish fed only once in the light, providing evidence that fish were feeding in the dark by non visual means.

Typically, the ability of marine fish to detect and consume food in the dark improves with age and can be linked to an improvement in the capacity of the sensory systems (Pankhurst, 2008). Juvenile mulloway were examined for the contribution of the sensory systems in feeding (Chapter 6) and it was observed that when fed an inert pellet diet, chemoreception was a contributing sensory system and may have allowed fish to identify the presence of and consume food in dark conditions.

In summary, juvenile mulloway should be kept in 18 h of light and fed twice daily to satiation. It is expected that this light and feeding regime will increase feeding efficiency and reduce costs, including those associated with labour.

7.4 Future research direction

This research has developed efficient rearing protocols for mulloway hatcheries, including embryonic development and egg disinfection, larval and juvenile rearing strategies, and has also investigated the contribution of the sensory systems to feeding. In addition, the study has highlighted future research directions which will contribute to the viability of the species as an aquaculture candidate.

7.4.1 Embryonic development and egg disinfection

In this study, the embryonic development and tolerance of mulloway eggs to ozone was examined. This is useful; however, the concentration of ozone required to inactivate a known virus, the Australian bass nodavirus, has not yet been determined. This information would be useful to ensure the virus is not transferred to the hatchery following ozone treatment.

This study also determined that of the CT values tested a CT of 1 was the highest value that did not negatively impact hatching. The next value tested was a CT of 5, and this value did have a negative impact on hatching. No CT values between 1 and 5 were tested and it is possible that mulloway eggs could be exposed to a higher CT value than 1, without negatively affecting hatching. This would further increase protection of the eggs against pathogens.

7.4.2 Larval rearing

This research has determined that advanced larvae held in 24 h of darkness achieved increased survival rates compared with fish that were exposed to some light. This is an important observation that may help increase the viability of the species in aquaculture; however, as the physiological effects of maintaining larvae of this age in complete darkness are unknown, it is important to investigate this aspect of cultivation further. In particular, the effects of complete darkness on ontogeny and sensory development, as well as the pigmentation of advanced larvae and the subsequent pigmentation of market size fish need to be investigated.

Advances in efficient weaning strategies have been achieved, including an improved understanding of optimal feeding protocols, and a reduction in the quantity of *Artemia* required during the weaning process. Further advances may be possible if new feed technologies and weaning diets are incorporated into future research plans as they become available.

7.4.3 Juvenile rearing

This study has determined that juvenile fish should be maintained in 18 h of light and fed once every 12 h. It has also been determined that juvenile fish are able to consume pellets during darkness. The effects of incorporating feeds during darkness should be investigated further to determine if any benefits in growth or survival can be achieved.

7.4.4 Sensory development

The contribution of the sensory organs to feeding has been assessed using a series of trials that included chemical ablation of the mechanosensory lateral line. This has provided an important insight into the contribution of each sensory system to feeding; however, further information could be obtained using microscopic examination of sensory organ morphology to provide a developmental profile of each sensory system coincident with the functional shifts in sensory performance identified in this study. This may enable further refinements in lighting and feeding regimes to be implemented.

7.5 Conclusions

The major outcomes of this thesis are:

- Mulloway eggs should be incubated at 22°C and exposed to ozone early in development (CT = 1) to reduce vertical transfer of pathogens.
- A reference guide of embryonic development in mulloway was developed to assist hatchery operators to assess egg viability and identify anomalies in development.
- Early larval mulloway are dependent on vision for feeding and cannot survive in complete darkness. However, some darkness is required for SB inflation and therefore larvae should be maintained in 12 h of light until SB inflation has been achieved.

- Advanced mulloway larvae can feed in dark conditions and sensory separation trials have determined this may be because of an increase in the contribution of the mechanosenory lateral line.
- Advanced mulloway larvae have improved survival when held in complete darkness; however, it is recommended advanced larvae are maintained in 18 h of light until changes in pigmentation of larvae held in complete darkness are examined.
- Advanced mulloway larvae require some *Artemia* during the weaning process. Growth is reduced in larvae not offered *Artemia*.
- Advanced mulloway larvae selected *Artemia* in preference to rotifers (*Brachionus plicatilis*) once the mean average length was 5.2 mm and pellets in preference to *Artemia* once the mean average length was 10.6 mm.
- Juvenile mulloway grow best in 18 h of light; however, they are also able to feed in dark conditions. Growth is poor in juvenile mulloway maintained in 24 h of light.
- Juvenile mulloway do not require feeding more frequently than twice daily if fed to satiation.
- Significant savings have been made as a result of this research, and current production costs are estimated to be around $1.05 for mulloway fingerlings produced at the PSFI, noting that it is difficult to estimate the costs associated with infrastructure.

These outcomes are summarised in Fig. 18.

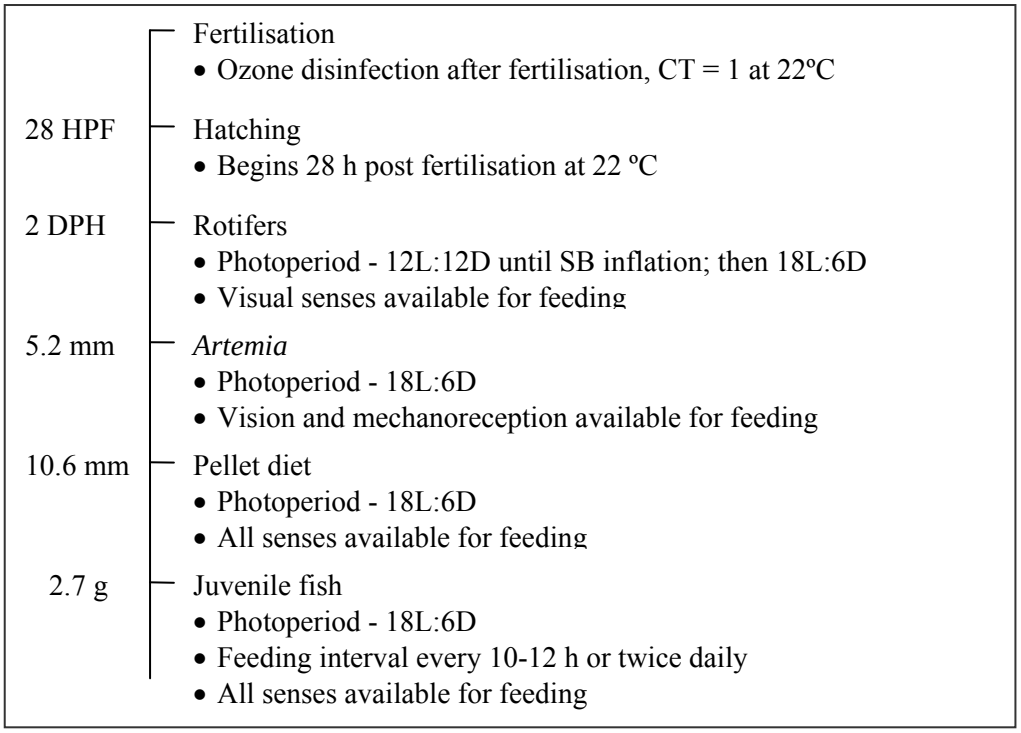

Figure 18: Timeline of mulloway cultivation in the hatchery.

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