Broodstock management, development of rearing systems, and feeding regimes for larvae of the forktail blenny, *Meiacanthus atrodorsalis*

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Jonathan Allyn Moorhead

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Centre for Sustainable Tropical Fisheries and Aquaculture
School of Marine and Tropical Biology
James Cook University

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Abstract

The marine aquarium trade is a growing industry supplied largely by an unsustainable wild fishery, sourcing specimens from poorly managed reefs, which are already under threat from increasing anthropogenic activity and rapid climate change. Research into captive breeding and hatchery rearing of popular marine aquarium fish species, underpinned by robust scientific method, has become a key tool to boost sustainability of the worldwide marine aquarium trade, extend our knowledge and understanding of reef fish biology, and aid coral reef research and recovery.

Among many popular marine aquarium fish groups the family Blenniidae form one of the 10 most traded marine ornamental fish families and are a prime candidate for research into reliable captive culture. However, little literature exists documenting the biology and breeding techniques of species belonging to this family. Within the family Blenniidae the forktail blenny, *Meiacanthus atrodorsalis*, is a species displaying good market value, and high potential for aquaculture, making it an ideal model species for investigation into reliable and repeatable captive culture protocols based in scientific method. This thesis addresses key aspects of the captive culture of *M. atrodorsalis* with particular focus on larval rearing using common hatchery feeds.

Chapter 1 reviews the current state of marine ornamental aquaculture (MOA) relative to the recent growth of the marine aquarium hobby, and the concurrent decline of a vast majority of coral reef enviroments worldwide. While research efforts towards captive breeding of marine ornamental species, stands to supplement or replace the supply of wild caught specimens for the marine ornamental trade, and potentially help boost reef recovery efforts

through restocking, the MOA sector is still in its infancy. This chapter identifies multiple bottlenecks that persist in limiting the progress of marine ornamental aquaculture, and highlights areas where research efforts should be focused to move forward. Major areas in need of increased research efforts include broodstock management, such as the development of specific broodstock diets and broodstock husbandry, spawning induction via hormone technologies that are tailored to the size and sensitivity of small broodstock ornamentals, and comprehensive, species-specific larval rearing techniques, including system design and larval culture conditions as well as larval feeds and nutrition.

In Chapter 2, it was necessary to develop cost effective tank designs to facilitate adequate replication demanded by robust scientific method, while still supporting good growth and survival in *Meiacanthus atrodorsalis*. Four experimental tank designs were developed to help achieve the aims of this thesis and are described in detail. The four designs increase sequentially in scale and include a 3-L and 9-L rounded tank design, a 5-L modified 'planktonkreisel' design and a 100-L cylindricoconical design, all of which can be operated as either static or flow-through systems. The 3-L and 9-L tanks and the 5-L planktonkreisel design are constructed from readily available materials and can be replicated to improve statistical strength while accommodating the requirements of larval fish for gentle flow, mixing, and maintenance of live prey in suspension. The 100-L tank is a technical improvement on existing current designs, using a novel integrated inlet-outlet design to enable the use of a large central outlet filter to increase screen area. This design is scalable and may be applicable for use in a commercial larviculture setting.

While these tank designs were developed primarily to conduct trials on *M. atrodorsalis* larvae, they displayed potential for use in larviculture of other marine fish species not covered in this thesis. The extent to which the designs supported larval growth and survival

of other popular marine aquarium species is presented, highlighting the versatility of these designs to be adapted to a wide range of species and experimental aims.

In Chapter 3, the reproductive behavior and embryonic development of *M. atrodorsalis* was documented, in addition to the growth and development of larvae to settlement stage, using a feeding protocol and live feeds common to fish hatcheries. Courtship and spawning commenced after a series of female and male initiated displays. Adult *M. atrodorsalis* displayed a preference for shelters of single entrance 50-mm PVC pipe, with a 25-mm reduced entrance, for egg laying, while the male took full responsibility for egg care. Eggs were laid individually with a flattened adhesive plate anchoring them to the walls of the provided shelters. Larvae measuring 3.1 ± 0.2 mm standard length (SL) and 0.63 ± 0.0 mm body depth (BD) hatched approximately 181 h post fertilization (PF) at 28 °C, with a mouth gape height and width of 307.3 ± 11.0 and 263.8 ± 5.5 μm, respectively. By firstly feeding rotifers, and then switching to *Artemia* nauplii with enriched *Artemia* metanauplii added later on, newly hatched *M. atrodorsalis* survived and grew, reaching settlement approximately 35 days post hatching (DPH), measuring 13.5 ± 0.4 mm SL and 3.91 ± 0.3 mm BD.

In Chapter 4, two trials were conducted to investigate the survival and growth responses of *M. atrodorsalis* larvae to varying rotifer densities at first feeding, and times to shift from rotifers to *Artemia* nauplii. In the first trial, first feeding *M. atrodorsalis* larvae were offered rotifers at 2, 5, 10 and 20 rotifers mL^{-1}. There was no significant difference in survival and growth between rotifer density treatments, however a density of 10 rotifers mL^{-1} resulted in the best survival (74 ± 6% at 7 DPH) and was recommended and adopted as a balanced choice to avoid under-feeding and wastage. In the second trial prey offered to

M. atrodorsalis was shifted from rotifers (10 mL^{-1}) to *Artemia* nauplii (3 mL^{-1}) on 3, 6, 9 and 12 DPH. While growth was not significantly different between treatments, survival was highest in treatments switched on 9 and 12 DPH (47 ± 14.3% and 47 ± 11.1%, respectively), suggesting that some larvae were able to ingest *Artemia* nauplii beyond 6 DPH, with a majority of larvae demonstrating the ability to adapt to an abrupt switch to the new prey beyond 9 DPH.

In Chapter 5, two trials were conducted with a continued focus on the timing and method of prey switch. These trials investigated the effect a 3-day co-feeding period and use of a specialty AF *Artemia* strain, relative to a standard Great Salt Lake (GSL) strain, had on timing the initial switch from rotifers to *Artemia* nauplii in *M. atrodorsalis* larvae. A third trial was conducted to investigate the time at which larvae of *M. atrodorsalis* could be shifted from AF *Artemia* nauplii to larger enriched GSL *Artemia* metanauplli. A 3-day co-feeding period improved the survival response of *M. atrodorsalis* switched from rotifers to *Artemia* nauplii when undertaken prior to 9 DPH, relative to the trial undertaken in Chapter 4. *M. atrodorsalis* larvae offered GSL *Artemia* nauplii could be transitioned as early as 5 DPH, while those offered the AF *Artemia* nauplii could be transitioned starting as early as 3 DPH when co-feeding was adopted. Larvae fed *Artemia* nauplii of the AF strain showed 17-21% higher survival, 24-33 % greater standard length and body depth, and 91-200% greater dry weight, after 20 days relative to those fed nauplii of the GSL strain. Meanwhile, enriched *Artemia* metanauplii of the GSL strain were shown to be an acceptable alternative to AF *Artemia* nauplii for later larvae, producing similar survival and growth when introduced from 8 DPH.

In Chapter 6, three trials were conducted to establish a benchmark regime for weaning and early growout of *M. atrodorsalis*. The first trial investigated the effect of weaning *M.*

atrodorsalis from live prey to a commercially available marine hatchery diet at 5 different times; starting on 16, 19, 22, 25 and 30 DPH. The second and third trial investigated the effect of 6 different feeding frequencies; 4, 3, 2, and 1 feeds per day, 1 feed every 2 days and one feed every 3 days, and 5 different feed ration sizes; 2, 5, 8, 11 and 15% body weight per day, on survival and growth of *M. atrodorsalis* juveniles. Weaning *M. atrodorsalis* from live prey to the formulated diet was successful at all ages tested. However, fish weaned starting 19 DPH and beyond showed 35-54% greater standard length, 28-56% greater body depth and 121-291% greater dry weight, as well as a 30-49% reduction in deformity. High survival on 49 DPH of between 94-97% was achieved when *M. atrodorsalis* were fed once every 2 days or more frequently from 32 DPH. However, fish fed once per day or more achieved 10-26% greater standard length, 8-29% greater body depth and 54-300% greater dry weight relative to those fed once every 2 days and once every 3 days. Within the feeding rations tested, survival was the highest when fish were fed a ration of 5-15% body weight per day, ranging between 86-93% on 49 DPH. However, there was a clear growth advantage of 18-29% greater standard length, 9-20% greater body depth and 41-160% greater dry weight when *M. atrodorsalis* were fed a ration of 8% body weight per day or more, relative to a ration of 2 and 5%.

In summary, the research presented throughout this thesis describes innovative tank designs to conduct well replicated larval fish research, and goes on to describe reproductive behaviors and techniques, embryo development, and develop a scientifically derived larval feeding protocol for the popular marine ornamental species *M. atrodorsalis*; a model for the Blenniidae family. While larvae and juveniles of *M. atrodorsalis* were found to adapt to variation in feeding densities, time and method of prey switchs, live prey quality, time of weaning, feeding frequency, and ration sizes, a balanced approach to rearing was adopted

in this thesis to synthesize a reliable feeding protocol. *M. atrodorsalis* larvae can be reliably reared with good survival and growth following a feeding protocol that consists of rotifers at a density of 10 mL^{-1} between 0-2 DPH inclusive, followed by newly hatched AF *Artemia* nauplli starting 3 DPH with a 3 day co-feeding period, then enriched GSL *Artemia* metanauplii starting 8 DPH with a 3 day AF *Artemia* nauplii co-feeding period. Weaning onto a formulated diet should take place between 22-25 DPH, after which juveniles are fed at least once per day at a ration of 8% body weight per day. This feeding protocol will produce consistent survival and growth results in *M. atrodorsalis* up to an age of 49 DPH, balancing key hatchery and research metrics such as cost, food wastage, labor intensity and culture complexity. In conclusion this thesis provides a significant contribution to the commercial and research fields of marine ornamental aquaculture providing innovative tank designs for research and commercial scale aquaculture of marine ornamentals and a comprehensive breeding and larval rearing protocol for a popular marine ornamental species, *M. atrodorsalis.*

List of abbreviations and terms

AA	Amino acid
AF	*Artemia franciscana*; a specialty small-strain of *Artemia* used in this thesis
AOI	Aquatic-ornamental industry
ARA	Arachidonic acid
BD	Body depth
BW	Body weight
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPH	Days post hatching
EAA	Essential amino acid
E-GSL	Enriched great salt lake *Artemia* metanauplii
EPA	Eicosapentaenoic acid
FAA	Free amino acids
FCR	Food conversion ratio
FCP	Full-circle planktonkreisel
FRP	Fibreglass reinforced plastic
GnRHa	Gonadotropin-releasing hormone analogue
GSL	Great Salt Lake; a commonly available strain of *Artemia* sourced from the Great Salt Lakes used in this thesis
GtH	Gonadotropin
hCG	Human chorionic gonadotropin
HUFA	Highly unsaturated fatty acid
JCU	James Cook University

List of abbreviations and terms continued…

LH	Luteinizing hormone
MAS	Marker-assisted selection
MOA	Marine ornamental aquaculture
MPK	Modified planktonkreisel
NH_3/NH_3-N	Ammonia
NO_2/NO_2-N	Nitrite
NO_3/NO_3-N	Nitrate
ø	Diameter
PAR	Photosythetically active radiation
PF	Post fertilization
PL	Phospholipid
Prey switch	An abrupt change from one prey type to another
Prey Transition	A gradual change in prey type accompanied by a co-feeding period
PVC	Poly-vinyl chloride
QTL	Quantitative trait loci
SD	Standard deviation
SE	Standard error
SGR	Specific growth rate
SL	Standard length
SS	Super small; referring to a strain of Rotifer used in this thesis
TAA	Total amino acids
TAN	Total ammonia-nitrogen

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Chapter 1: General introduction: Development of captive breeding techniques for marine ornamental fish: A review

1.1 ABSTRACT

The increasingly popular aquarium hobby is fuelling the rapid growth of the aquatic ornamental industry, particularly the trade of marine ornamental species. However, currently there is a heavy reliance on wild caught marine ornamentals to satisfy consumer demand. As public awareness of the plight of marine ecosystems grows, the often destructive and unmanaged exploitation of coral reefs for the marine ornamental trade has raised concerns. Therefore, there is consensus that urgent actions need to be taken to limit destructive exploitation of coral reefs, and to ensure the sustainability of the marine ornamental industry. An obvious and vital action of such efforts would be the development of captive breeding techniques for marine ornamental species, in particular coral reef fish. Research efforts towards captive breeding of marine ornamental species, also known as marine ornamental aquaculture (MOA), stand to supplement or replace the supply of wild caught specimens for the marine ornamental trade, and potentially help boost reef recovery efforts through restocking. However, the MOA sector is still in its infancy, receiving limited research attention, and in turn has experienced very slow development compared to the technical and industrial advances made in foodfish aquaculture. While it is true that at present, multiple bottlenecks have severely limited the progress of MOA, through careful appraisal and adaptation of culture techniques developed for foodfish, and by addressing the specific needs of the MOA, significant progress could be made for the MOA industry. With this objective in mind, this chapter summarizes the major bottlenecks facing the MOA

industry, and highlights weaknesses in the current state of research. Major areas in need of increased research efforts include broodstock management; such as the development of specific broodstock diets and broodstock husbandry, spawning induction via hormone technologies that are tailored to the size and sensitivity of small broodstock ornamentals, and comprehensive, species-specific larval rearing techniques, including system design, and larval culture conditions as well as larval feeds and nutrition.

1.2 INTRODUCTION

The trade of aquatic organisms for home and public aquariums and water gardens, along with associated equipment and accessories, has become a multi-billion dollar industry known as the aquatic-ornamental industry (AOI) (Larkin, 2003; Wabnitz, et al., 2003; Pelicice and Agostinho, 2005; Prang, 2008; Madhu, et al., 2016). On the whole, the most recent estimated global value of the AOI was in the vicinity of 15 billion US dollars (Whittington and Chong, 2007; Prang, 2008). Within the AOI, among a myriad of aquatic organisms traded, freshwater and marine fish species are the most popular and dominant groups (Lecchini, et al., 2003; Moreau and Coomes, 2006). And on a unit weight basis, ornamental fish form the most valuable fisheries commodity in the world (Hardy, 2003).

Over the past decade, technical advances in responsible captive care and life support system technologies have made aquarium care easier and more accessible to common households, especially with respect to maintaining marine organisms (Murray, et al., 2012; Rhyne and Tlusty, 2012). This has resulted in a rapid increase in demand for marine ornamental fish, particularly coral-reef species, which has also contributed to recent rapid growth of the AOI (Chan and Sadovy, 1998; Rinkevich and Shafir, 1998; Sales and Janssens, 2003; Shuman, et al., 2004; Calado, 2006; Livengood and Chapman, 2007; Rhyne and Tlusty, 2012).

Of the estimated 1,500 to 1,600 ornamental fish species commonly traded, near half are marine (Olivier, 2003; Livengood and Chapman, 2007; Whittington and Chong, 2007; Rhyne, et al., 2012). Marine fish differ significantly from freshwater fish when it comes to the volume and value of specimens traded on the ornamental market. In terms of volume, world trade of marine ornamental species makes up less than 10% of total ornamental fish traded; however, owing to their strong unit value, the proportion is higher when estimated as a percentage of world trade by value, with strong trading growth in recent years (Olivier, 2001; 2003; Livengood and Chapman, 2007; Whittington and Chong, 2007).

Another striking difference between marine and freshwater ornamental fish is the origins from which they are sourced. Unlike their fresh-water counterparts that are mostly captive bred (approximately 90%), it is estimated that between 90 to 99% of marine fish species traded on the ornamental market are collected directly from the wild (Chapman, et al., 1997; Ostrowski and Laidley, 2001; Wilson, et al., 2001; Tlusty, 2002; Moe, 2003; Olivier, 2003; Tissot and Hallacher, 2003; Calado, 2006). This is of particular concern considering that a large portion of coral-reef fish species are collected from poorly managed fisheries in the Southeastern Asian and Caribbean region (Rhyne, et al., 2012; Dee, et al., 2014; Fujita, et al., 2014). Countries in these regions, such as the Philippines and Indonesia, use destructive capture methods, including explosives, poisons (such as sodium cyanide), muro-ami and kayakas fishing techniques (involving smashing of corals and rocks to scare fish into nets)(Rubec, 1988; Rubec, et al., 2001a; Wood, 2001a; Ziemann, 2001; Calado, 2006). At the point of collection, the use of such destructive methods inflicts severe and often permanent collateral damage to coral reef ecosystems; destroying reef structure, and killing coral heads and other fish of no collection interest, along with target species (Rubec, et al., 2001b; Wood, 2001a; Mak, et al., 2005; Vaz, et al., 2012). Furthermore, post-collection mortality due to delayed and chronic chemical toxicity, poor handling, and

transport stress compounds the deleterious effects (Rubec, et al., 2001b; Wood, 2001a; Mak, et al., 2005; Vaz, et al., 2012; Tew, et al., 2015; Militz, et al., 2017). Continued exploitation of reefs in a destructive, poorly managed manner, has been reported to reduce the yields of both ornamental and food fish from reef fisheries, ultimately affecting the capacity of exploited reefs to recover (Rubec, et al., 2001a; Ziemann, 2001). In some instances, collection of specimens for the marine ornamental market itself has been directly linked to localized declines in several popular ornamental species (Lubbock and Polunin, 1975; Ziemann, 2001; Tissot and Hallacher, 2003; Shuman, et al., 2005; Militz and Foale, 2016).

Aside from direct exploitation, the world's coral reefs also face the effects of global climate change and increased impacts from anthropogenic activity, highlighting the need for multi-faceted management and recovery programs to curb reef declines and encourage recovery (Rubec, 1988; Wood, 2001a; Bellwood, et al., 2004; Shuman, et al., 2004; Olivotto, et al., 2011; Rhyne, et al., 2012; Militz, et al., 2017; Olivotto, et al., 2017). As a part of effective coral reef management plans, there exists great potential for a marine ornamental reef fish captive breeding program (Wood, 2001a; Ziemann, 2001; Tlusty, 2002; Bellwood, et al., 2004). Such a program could complement coral reef management through controlled releases of hatchery produced fingerlings of heavily exploited species, and provide a significant contribution to scientific knowledge of reef fish biology (Rinkevich and Shafir, 1998; Ostrowski and Laidley, 2001; Wood, 2001a; Ziemann, 2001).

In its present state, MOA is still in its infancy, with a limited number of species being produced at an economically viable scale (Wood, 2001b; Holt, 2003). To date, captive larval rearing successes have been largely limited to small, experimental, or hobbyist enterprises. Additionally, very few scientific publications exist documenting aspects pertinent to captive culture of ornamental reef fish species (Shei, et al., 2010). However,

there is great potential for significant growth in this sector of the ornamental industry, owing to high product value and its significance to coral reef conservation (Murray, et al., 2012).

There are growing interests in marine ornamental aquaculture from the commercial aquarium trading industry, marine conservation, and research community (Watson and Hill, 2006). However, for MOA to grow and reach commercial success, techniques need to be developed to yield high quality and quantity of eggs, and to culture a large quantity of larvae successfully through to a marketable end point (Brooks, et al., 1997; Coward, et al., 2002; Tlusty, 2002; Holt, 2003). This requires robust scientific experimentation, with a view to make such information freely available to the public. The aim of this chapter is to highlight major bottlenecks/barriers to the development of MOA, with focus on the key issues of broodstock management and larval rearing. Aquaculture techniques and methods developed for marine foodfish that have the potential to be adapted for use in MOA will also be reviewed. This chapter aims to elucidate the needs and appropriate avenues by which research progress can be made in order to advance captive breeding techniques for marine ornamental fish species.

1.3 BROODSTOCK MANAGEMENT

In general, broodstock management encompasses all the appropriate measures to enable captive fish to produce a high quantity of fertilized eggs of high quality. It requires competent knowledge in gonad maturation and spawn induction techniques of broodstock fish, and successful egg fertilization procedures (Mylonas, et al., 2010). Marine fish, in particular coral reef fish, occupy diverse habitat niches and display variable and complex reproductive modes and strategies (Brock and Bullis, 2001; Holt and Riley, 2001; Coward,

et al., 2002; Wittenrich, et al., 2007). One of the major challenges MOA faces is the need to accommodate these reproductive strategies in order to produce a diverse range of ornamental fish species that satisfy market demands (Watson and Shireman, 1996; Ostrowski and Laidley, 2001).

Reproduction in broodstock fish encompasses three key developmental stages, i.e., gonad and gamete development (gonadogenesis and gametogenesis), final oocyte maturation, and gamete release (ovulation/oviposition in females and ejaculation of milt in males) (Schreck, et al., 2001; Zohar and Mylonas, 2001; Coward, et al., 2002). In reality, this process is highly complex, involving multiple, and sometimes very different developmental stages. The quality of resulting gametes comes under the influence of a range of variables, including aspects of the behavioral, physical, and chemical environment, and broodstock diet (Sumpter, 1990; Munro, et al., 1999; Coward, et al., 2002; Wittenrich, et al., 2007).

The ability to condition broodstock to maximize gamete quality and output is a major prerequisite to a successful captive breeding program, but at the same time represents a significant bottleneck for both foodfish and marine ornamental fish aquaculture (Ostrowski and Laidley, 2001; Zohar and Mylonas, 2001; Coward, et al., 2002; Mylonas, et al., 2010). The major critical control points in this process include careful broodstock selection, and the combination of appropriate broodstock husbandry, diet, tank design, and manipulative techniques that induce reproductive activity and spawning of the target fish.

1.3.1 Broodstock selection

As a critical first step in obtaining a high quantity of good quality gametes, appropriate broodfish should be chosen (Brock and Bullis, 2001; Schreck, et al., 2001; Olivotto, et al., 2003). At a basic level, broodstock can be selected based on size, age, and external attributes; however, a well-informed choice will also take into account social interaction and behavioral influences on sex and sex change, morphological and biochemical char-

acteristics of the gametes produced by the broodfish, the risk of disease transmission to other broodstock and larvae, and the genetic background of the broodfish. All these factors are important in any foodfish hatchery and, thus, must be considered when culturing marine ornamental fish to maximize hatchery production and efficiency.

1.3.2 Broodstock age and size

In some foodfish species, adult age and spawning history has been reported to have a bearing on egg size and chemical content of eggs produced by females, and sperm quality of males (Vuthiphandchai and Zohar, 1999; Kamler, 2005). Gamete quality generally follows a trend in which very young and old age females produce smaller eggs of lower nutritional content as compared to those of average age (Kamler, 2005). Likewise, very young and old males produce lower quality sperm compared to middle aged males, particularly with respect to sperm concentration and spermatocrit, as well as storage potential and longevity (Vuthiphandchai and Zohar, 1999). At present, these ontogenetic factors are poorly understood in marine ornamental fish. Considering that broodstock supply for marine ornamental fish currently relies almost exclusively on wild caught specimens (Sales and Janssens, 2003; Pavlov and Emel'yanova, 2006), the absence of age and spawning history information concerning wild caught broodfish may limit the possibility of selecting broodfish based on their age. However, creating an database that documents the change in gamete quality with age of marine ornamental species is likely to be useful in the future. With such information, marine ornamental aquaculturists may be able to make more informed choices on broodfish selection and determine when an individual is reaching the ‘retirement’ age. A relatively unique issue related to broodstock selection in marine ornamental aquaculture is collection and capture history. In particular the use of dangerous chemicals such as cyanide to stun and capture marine ornamental fish

is still prevalent in the marine ornamental trade (Mak, et al., 2005; Vaz, et al., 2012). Fish suspected to have been sourced from areas where such practices are common should be avoided as fish exposed to cyanide invariably die or suffer long term adverse effects including reduced growth and swimming performance, inhibited reproduction and poor egg quality (Mak, et al., 2005).

The size of a marine ornamental broodfish may also be an important selection consideration, as it has been shown to influence fecundity and egg size in many foodfish species (Bromage, et al., 1992; Kolm, 2002; Kamler, 2005). This influence is particularly pronounced in species that display large size variation between reproductively mature females (Kamler, 2005). The positive relationship between egg size/number and broodfish size is considered largely universal among foodfish (Kamler, 2005), which has also been observed in several marine ornamental fish. For example, large females of the banggai cardinalfish, *Pterapogon kauderni,* and the blueband goby, *Valenciennea strigata,* were found to produce more and larger eggs than smaller conspecifics (Reavis, 1997; Kolm, 2002).

1.3.3 Social interaction and behaviour-mediated sex change

When selecting broodstock to establish breeding pairs or groups, size and age must also be considered relative to their potential influences on social behavior (e.g., competition between males for females, agonistic, and territorial behaviors), the social environment and sex change, particularly in social and haremic fish (Warner, 1988; Olivotto, et al., 2017). Social and behavioral factors influence intra-specific interactions and the establishment of social hierarchies, affect the stability of the social environment, stress levels, sex change of individuals, and ultimately the reproductive capacity and likelihood of spontaneous spawning (Warner, 1988; Fox, et al., 1997; White, et al., 2002; Forrester, et al., 2006;

Mylonas, et al., 2010). The pathway and degree to which these interactions may influence reproduction in teleosts depends on the sexual morphology (gonochorism or hermaphroditism), reproductive strategy (monogamy or polygamy), spawning mechanism (such as oviparity and ovoviviparity; Coward, et al., 2002), and mode of reproduction (demersal or pelagic spawners) of the species concerned. Such behavioral interactions may be accentuated in a closed system where an individual is unable to avoid or escape, or find an alternate mating partner or group. For example, sex change is a common phenomenon among coral reef fish; a change in social environment brought about by a sex change event, may expose an individual or group to agonistic behaviors, causing stress, and leading to low reproductive output, poor gamete quality, 'reproductive failure,' or even mortality (Kjesbu, 1989; McCormick, 1998; Nordeide, 2007; Olivotto, et al., 2017).

The collection and/or captive history of an individual is also a point of consideration, particularly with respect to selecting hermaphroditic fish. In the wild, reproductive development and sex change that comes under the control of the social environment and dominant conspecifics, may limit the reproductive opportunity of an individual, or suppress maturation all together (Fox, et al., 1997). However, once collected, the individual may be liberated from the suppressive effects of its social group, and thus, have an opportunity to progress to a reproductively expressive or receptive state. The implications of this are that the individuals' behavior changes from that of a submissive sex or subordinate, to an active and potentially aggressive competitor for reproductive opportunity. This may mean, for example, that larger females of protogynous species will more likely change sex, becoming a 'bachelor male' (Aldenhoven, 1986), and challenge or compete with male conspecifics in an artificial breeding group, particularly if its history included long periods of seclusion from dominant conspecifics. Such a case may necessitate the choice of smaller females to facilitate successful pairing, in favor of a more common situation of selecting larger fish for

greater fecundity and higher quality gametes. For example, attempts to pair similar sized animals of the protogynous sequential hermaphrodite; bicolor angelfish, *Centropyge bicolor*, often resulted in intense aggression, physical damage, or death of one of the animals (personal observation). It appeared that conspecifics of a similar size displayed more aggression towards one-another, in an effort to establish dominance, relative to those that differed significantly in size (personal observation). The reverse scenario may be true when selecting males of species whom are protandrous hermaphrodites.

Even in less aggressive species, the interaction between size and behavior of male and female fish during reproduction could influence gamete output. For example, male size in the paternal mouthbrooding banggai cardinalfish, *Pterapogon kauderni*, influenced the number of eggs provided by the female for incubation. Females were observed to adjust their egg output, or cannibalize excess eggs based on the size of their male partner (Kolm, 2002). Furthermore, male size in wild blueband gobies, *Valenciennea strigata*, was suggested to have a bearing on fecundity and egg survivorship, leading to an active choice of females to leave their partner, if a larger single male was available (Reavis, 1997). Similarly, it has been observed in wild blue devil damselfish, *Chrysiptera cyanea*, that larger sized males experienced greater spawning success, as male size influenced the decision of females to spawn (Gronell, 1989).

In summary, a thorough knowledge of the way in which broodstock size influences social interaction and reproductive behavior, and vice versa, could be critical, and a factor to consider when selecting broodstock of marine ornamental fish.

1.3.4 Gamete quality

Hatchery productivity can be substantially enhanced if the appropriate steps are taken to obtain high quality gametes (Giménez, et al., 2006). On occasion, when a large number of potential broodstock are in breeding condition, selection may be made based on assessment of gamete quality. Assessing gametes identifies readiness for spawning and avoids selecting broodfish with under or over-ripe gametes (Kjørsvik, et al., 1990; Bromage, et al., 1994). The difficulty in obtaining sufficient high quality eggs and larvae is often considered a major limiting factor in foodfish aquaculture, particularly of marine species (Kjørsvik, et al., 1990; Brooks, et al., 1997; Aristizabel, et al., 2009). With respect to assessing fish eggs for quality, high fertilization rate and survivorship of developing eggs, and a good number of healthy larvae at hatching and first feeding are the major criteria (Bromage, et al., 1992; Brooks, et al., 1997; Lahnsteiner and Patarnello, 2005). Apart from broodstock nutrition, many other factors that affect egg quality in fish are not well understood (Brooks, et al., 1997). For example, Callan and Laidley (2010) reported significant reduction in fertilization rate, quality and viability of eggs produced by flame angelfish, *Centropyge loriculus*, when maintaining broodstock in water drawn from a saltwater well, relative to oceanic water, but were unable to pinpoint the causative factors of their observations. Despite the lack of knowledge of both biotic and abiotic factors that affect egg quality in fish, several morphological and biochemical criteria have been used as indicators of egg quality, however, their effectiveness appears to vary with species. Common morphological and physical parameters used include size of egg, egg yolk and lipid vesicle size, egg shape, and cell morphology (e.g., cell symmetry at early cleavage stages), as well as egg buoyancy, dry weight, and larval morphology at first feeding. The biochemical parameters include carbohydrate metabolism and lytic enzyme activity, as well as lipid, amino acid, and/or vitamin content of eggs (McEvoy, 1984; Carillo, et al., 1989; Kjørsvik, et al., 1990;

Bromage, et al., 1994; Kjørsvik, 1994; Shields, et al., 1997; Lahnsteiner and Patarnello, 2003; Lahnsteiner and Patarnello, 2004; Lahnteiner and Patarnello, 2004; Lahnsteiner and Patarnello, 2005; Planas, et al., 2010; Callan, et al., 2011). Depending on species, one or a combination of these measurements may be employed to select female broodstock before larval culture efforts commence.

Although receiving less attention, milt quality, including sperm density, motility, and fertilizing capacity of spermatozoa, has also been shown as an important factor influencing fertilization and hatching success of fish (Kamler, 2005; Pavlov and Emel'yanova, 2006). Pavlov, (2006) described a method for assessing sperm motility in fish, which has potential for use in screening male broodstock candidates. Pavlov and Emel'yanova (2006) further implemented this technique in assessing three reef fish commonly traded on the ornamental market; i.e., the brown tang, *Zebrasoma scopas,* the scissortail sergeant, *Abudefduf sexfasciatus,* and the domino damselfish, *Dascyllus trimaculatus,* but found little difference in sperm motility between species. All three species tested possessed spermatozoa of comparatively low initial velocity, retaining activity for at least 5 min, and showing viability after being stored for several hours below 4.5°C. The technique may be useful for monitoring male performance and assessing sperm quality of other marine ornamental fish, particularly in relation to factors, such as diet, stress, size, and age.

Since gamete quality comes under the influence of multiple factors, including environmental variation, the physiological and endocrinological status of the broodfish, their diet, and genetic factors (Brooks, et al., 1997; Coward, et al., 2002; Aristizabel, et al., 2009), measurements of gamete quality can also serve as an indicator of inappropriate captive conditions or broodfish health. Therefore, attention should be paid to the application of gamete quality monitoring and control techniques in marine ornamental

aquaculture, as an important step towards quality control and the optimization of conditions conducive to reproduction.

1.3.5 Disease and biosecurity

Prevention, rapid detection, and control of disease outbreaks are critical issues in all hatcheries with broodstock being one of the primary control points for such management (Brock and Bullis, 2001; Adams and Thompson, 2006). Pathogens that can be vertically transmitted to gametes from broodstock include bacteria, fungi, parasites, and, more prominently, viruses (Brock and Bullis, 2001). Common pathogens reported to affect marine ornamental fish, potentially posing a threat to MOA, include bacteria such as *Mycobacterium* spp., *Vibrio* spp., *Aeromonas* spp., *Pseudomonas* spp., and myxobacteria, as well as parasites such as *Cryptocaryon, Amyloodinium, Uronema*, *Neobenedenia*, and monogenetic trematodes (Lipton, 1993; Francis-Floyd and Klinger, 2003; Tendencia, 2004; Zanoni, et al., 2008; Koldeway and Martin-Smith, 2010; Pirarat, et al., 2011; Militz and Hutson, 2015). Some of these pathogens are identifiable and treatable (Francis-Floyd and Klinger, 2003), however, avoiding contaminated broodfish is the first line of defence, with gametes being sourced ideally from pathogen-free broodstock (Brock and Bullis, 2001). Although research continues in the development of specific pathogen-free broodstock in many foodfish and shrimp, this is not particularly practical for marine ornamental fish at present. Characteristically, broodstock sourced from wild populations, such as most marine ornamental fish, have been exposed to many endemic pathogens, and are then further exposed to pathogens commonly found in aquarium and aquaculture systems (Brock and Bullis, 2001; Francis-Floyd and Klinger, 2003; Pirarat, et al., 2011). Therefore, considering the lack of certified pathogen-free marine ornamental fish, there is a need for vigilance in identifying new pathogens, and diligence to develop techniques and maintain control over

outbreaks of diseases and their vertical transmission (Brock and Bullis, 2001; Adams and Thompson, 2006).

Apart from inspection for external signs of disease while selecting broodstock, and the use of traditional disease management plans post selection, such as freshwater baths, copper treatments, antihelmenthics, and novel biological controls such as housing broodstock with cleaner shrimp (Militz and Hutson, 2015), there are several technologies that allow more accurate screening and identification of pathogens. These include traditional methods of bacteriology, virology, parasitology, and mycology, which are often supported by histology, for the detection of easily cultured pathogens (Adams and Thompson, 2006; Pirarat, et al., 2011), and more advanced cell cultures, and immunological and molecular methods for more complicated cases (Villena, 2003; Adams and Thompson, 2006). For example, investigation into screening methods for barfin flounder (*Verasper moseri*) broodstock suggested that both a PCR test and ELISA antibody titers could help prevent vertical transmission of viral-nervous necrosis from broodstock (Watanabe, et al., 2000). As breakthroughs are largely made in foodfish disease management and biosecurity, the MOA industry stands to benefit. However, considering the potential of increasing economic importance of MOA, there is also a need for more research into pathologies that affect ornamental fish specifically. Furthermore, efforts to improve biosecurity, diagnostic support, quarantine, and prophylactic and probiotic treatments, should also be made as the MOA sector grows (Francis-Floyd and Klinger, 2003; Zanoni, et al., 2008).

1.3.6 External attributes and genetic selection

As future progress is made in MOA, broodstock selection may inherit an added dimension, that is the quest to both track and maintain genetic pools for conservation purposes (i.e.,

potential for restocking), and add value to the commercialization of aquacultured marine ornamental fish. The commercial arm of the AOI is highly selective in the species that are collected, cultured, and traded (Lecchini, et al., 2006). Species that display vibrant coloration, unique and interesting behaviors and habits, or some degree of novelty, in addition to their suitability for aquaria, are key criteria dictating the value and interests for the species traded and the level of their exploitation (Lecchini, et al., 2006; Sinha and Asimi, 2007; Willis, 2007). Those species that find popularity among consumers are often judged for quality based on appearance and physi- cal attributes, such as coloration and finnage (Watson and Hill, 2006).

Clearly, the MOA has not evolved to the level seen in freshwater ornamental aquaculture; i.e., producing a large range of hybrids and 'fancy' strains of fish. In its present state, MOA is more likely to concentrate on the elucidation and manipulation of non-genetic factors, such as diet and environmental conditions, as these often precede consideration of genetic effects in broodstock selection and management for early stages of development of an aquaculture sector (Butts and Litvak, 2007). However, in the long term and with the future development of the industry, genetic manipulations will inevitably become a very important option.

Traditionally, efforts directed toward genetic improvement of aquaculture broodstock have involved selective breeding techniques, such as selection of favorable phenotypes, crossbreeding, and hybridization (Liu and Cordes, 2004). The first concern for commercial breeders of marine ornamental fish is likely to obtain broodstock with attractive appearance and physical attributes, in the hopes that these attributes will be expressed in their offspring, and, thus, ensure good economic return for culture efforts. The application of modern genetic techniques for broodstock selection will be a valuable tool in the future, particularly as it relates to identifying and mapping monogenic, and polygenic

(quantitative) gene loci associated with desirable phenotypes (Khoo, et al., 2003). Both for foodfish aquaculture and MOA, knowledge of linkages between easily amplified polymorphic DNA markers and polymorphic quantitative trait loci (QTLs), has application in selective breeding for improving traits of cultured species, such as growth, color, patterning, finnage, and disease resistance, and is termed marker assisted selection (MAS) (Poomuang and Hallerman, 1997; Liu and Cordes, 2004). Additionally, as many marine ornamental fish are sexually dimorphic and fetch a different price based on their sex, genetic techniques may be used in the selection of broodstock that display some degree of autosomal sex determination like that of Tilapia, or to produce monosex populations in dioecious species (Piferrer and Lim, 1997; Poomuang and Hallerman, 1997).

On the whole, DNA marker technology and gene manipulations have not impacted aquaculture in a significant way until recently (Liu and Cordes, 2004; De-Santis and Jerry, 2007). There are few publications focused on ornamental fish species in this area of biotechnology, and few if any QTLs have been mapped for marine ornamental fish. However, there is confidence that with continued progress in genomic research, and in particular QTL mapping, MAS will realize significant use for efficient and precise broodstock selection for aquaculture, including marine ornamental fish culture, both for commercial and conservation purposes (Fernando, et al., 1997; Taniguchi, 2003; Liu and Cordes, 2004; Chistiakov, et al., 2006).

1.3.7 Broodstock husbandry and reproduction manipulation

Once broodstock have been obtained, appropriate conditions, including diet and physical conditions, must be identified and maintained to provide an environment that is conducive to sexual maturation and, ideally, natural reproduction (Mylonas, et al., 2010). However, not all fish will spawn naturally under captive conditions. Therefore, as an integral part of

an effective broodstock management plan, invasive techniques may become necessary for obtaining viable gametes, particularly when a species exhibits reduced reproductive activity or spawning asynchrony under captive conditions (Coward, et al., 2002; Mylonas, et al., 2010). While invasive techniques may be required, proper husbandry practices are still a major contributor to the resulting quality of gametes (Kjørsvik, et al., 1990; Brooks, et al., 1997; Coward, et al., 2002). Therefore, appropriate husbandry for broodstock and the need for manipulation or stripping to obtain mature gametes, are not mutually exclusive with respect to obtaining quality gametes.

Considerable attention must be paid to provide appropriate conditions associated with reproduction in marine ornamental fish, particularly in light of the high diversity of living and breeding modes, and strategies displayed among species. The main areas that often encompass broodstock husbandry and reproductive manipulation are the control of environment, diets and feeding, the direct administration of reproductive hormones, and in some cases, invasive stripping techniques (Coward, et al., 2002; Melamed, et al., 2002).

1.3.7.1 The physical and chemical characteristics of the culture Environment

A fish's response to and utilization of a particular habitat is influenced by its interactions with biotic and abiotic factors in the environment, and its ability to exploit the habitat for the acquisition of food, refuge, or territory (Bellwood and Wainwright, 2001; Fulton, et al., 2001; Gill and Angrews, 2001; Wilson, et al., 2001). In the wild, a fish may typically choose an appropriate niche habitat within a broader environment that suits its needs, or be forced to move on or adapt to survive. However, in a captive setting, a fish has very limited freedom to choose its environment. Therefore, with respect to basic husbandry of captive fish, the onus is on the caregivers to provide an environment in which the animal can survive and thrive. This can be achieved through sound knowledge of the biology of the

fish and a careful assessment of its tolerances and preference for certain environmental conditions.

Similarly, reproduction in many fish also comes under the influence of a series of environmental factors, some of which play a specific role during reproduction periods. Therefore, further to their basic husbandry needs, the environmental factors that influence reproductive success need to be identified and if possible replicated. (Coward, et al., 2002). In captivity, many fish species have successfully spawned when the correct physical and chemical environmental conditions are provided (Kodric-Brown, 1988; Clifton, 1995; Peter and Yu, 1997; Gordon and Bok, 2001; Holt and Riley, 2001; Zohar and Mylonas, 2001; Olivotto, et al., 2006a). Among many factors, tank design, substrate, and water conditions are some of the most important considerations.

1.3.7.1.1 Tank design.

The aquaculture industry relies heavily on what has been classified as undomesticated stocks. Therefore, system engineering and successful captive culture often hinges on the animal's ability to cope and adapt to artificial surroundings (Koolhaas, et al., 1999; Rasmussen, et al., 2005; Watson and Hill, 2006). With this in mind, greater success may be achieved in encouraging target species to spawn under controlled conditions if the engineered environment more closely replicates natural conditions in which the animal is commonly found.

At present, both industry and researchers of MOA tends to focus on culture of demersal spawners, particularly members of the families Pomacentridae, Gobiidae, Sygnathidae, and Pseudochromidae (Fig. 1.1). These species tend to form strong pair bonds, produce regular clutches of large eggs, and display some degree of parental egg care (Brown, et al., 2003; Watson and Hill, 2006; Olivotto, et al., 2011; Olivotto, et al., 2017). One reason for such a trend appears to be the ready acceptance of many demersal fish for relatively simple, space

efficient tank designs, and artificial spawning substrate. Clownfish, or anemonefish (*Amphiprion* spp.), are a classic example, which have been shown to spawn in tanks as small as 37 L, attaching their benthic eggs to terracotta pots or tiles, as substitutes for natural rock surfaces (Hoff, 1996; Wittenrich, et al., 2007). However, tank design remains a stumbling block for species diversification of MOA.

In general, tank design has a significant effect on the physical characteristics of the holding environment, and the behavior of the organisms being held (Ross, et al., 1995; Rasmussen, et al., 2005). The influence of tank design on growth performance is a recognized concept in grow-out aquaculture (Ross, et al., 1995; Rasmussen, et al., 2005). It has also been recognized that tanks proven adequate for growth may be inadequate for broodstock conditioning (Ostrowski and Laidley, 2001; Koldeway and Martin-Smith, 2010).

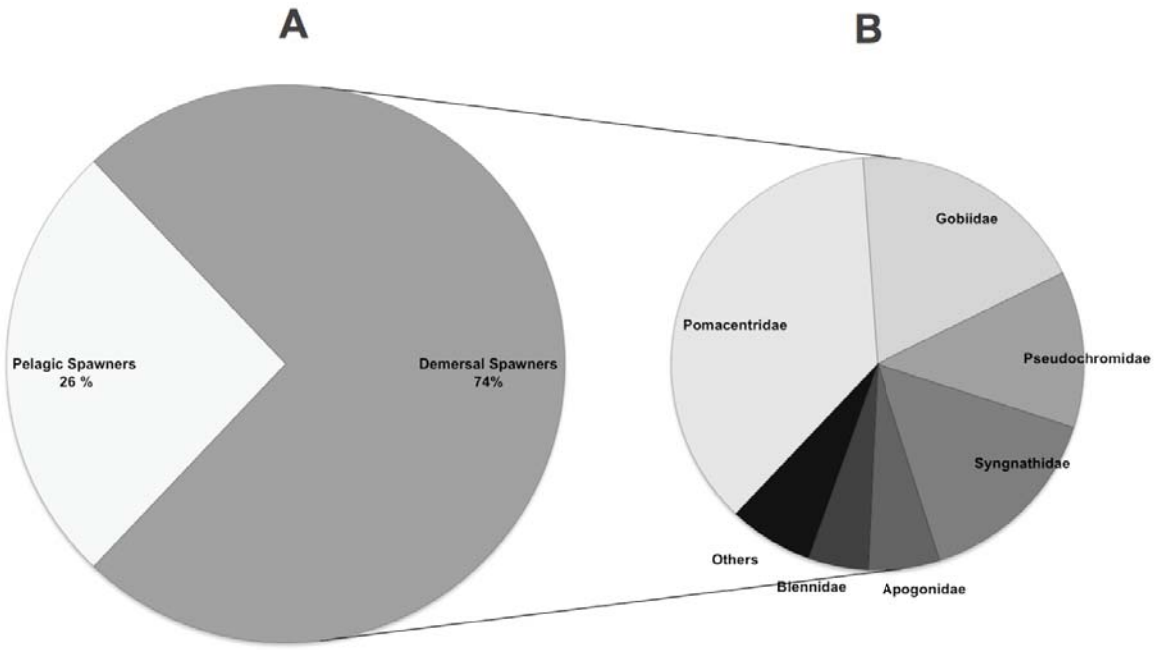

Figure 1.1: (A) Percentage of demersal and pelagic spawning marine ornamental fish species that have been cultured commercially or as a part of research; and (B) proportion of various families within demersal spawning species that have been cultured commercially or as a part of research. Data drawn from 49 sources; principal, Arvelund et al. 2000; Wabnitz et al. 2003.

Tank size, water volume, and depth, as well as stocking density, can all influence the reproductive success of the fish being held (Koldeway and Martin-Smith, 2010; Mylonas, et al., 2010). For example, Planas, et al. (2008) reported that for the Spiny seahorse, *Hippocampus guttulatus*, deep tanks and good plumbing design eliminated the occurrence of gas bubble disease; a commonly occurring ailment in seahorses kept in shallow tanks with fine water bubbles, and facilitated breeding activity. In assessing the specific needs of broodstock fish, it may be useful to examine and collate information recorded from field studies. For example, Lecchini, et al. (2003) pointed out that there are definite patterns in the spatial distributions and abundance of reef fishes, which in many instances are not only explained by biological factors, but also physical factors, such as water depth and hydrodynamic conditions, including water movement and wave exposure. An understanding of a fish's natural distribution and abundance gradients may give insight into its specific physical requirements. Important clues as to the physical requirements of certain reef fish may also be found through understanding their physiological capabilities. For example, studies on the swimming capabilities of wrasses (Labridae) showed that swimming performance, as a function of pectoral fin aspect ratio (pectoral fin aspect ratio = (length of pectoral fin leading edge)^2/total fin area; Fulton et al., 2001), influences their distribution (Bellwood and Wainwright, 2001; Fulton, et al., 2001). When applying this knowledge to the engineering of a captive environment, inferences may be made as to the suitable current regimes that should be created for broodstock. It is unclear whether strong swimming fish may prefer environments of high wave and current energy at every stage of their life history and especially during spawning. However, it may be postulated with some confidence that fish with weak swimming performance (possessing a low fin aspect ratio), may fail to progress through certain stages of reproductive maturity if faced with a stressful, high turbulence environment (Watson and Hill, 2006). It should also be noted that

an environment in which a fish is commonly found might be different to where it spawns. Once gonad maturity is reached, some fish may require a change in wave and current regimes, tank dimensions, or even water quality, to initiate gamete release (Mylonas, et al., 2010). In summary, replication of the physical environment that typifies a species' preferred niche in the wild might reduce stress and encourage broodstock to progress through early gonad development, maturation, and finally gamete release in captivity.

It has been postulated that as focus shifts from benthic spawning species to more popular and higher value pelagic-spawning marine ornamentals, such as pygmy angelfish (*Centropyge* spp.), surgeonfishes (Acanthuridae) and wrasses (Labridae), a move from shallow tank designs, towards larger and/or deeper designs has to occur (Ostrowski and Laidley, 2001). However, there are conflicting reports in this regard. For example, Job, et al. (1997) recommend a tank size of 1,000 L for the bicolor angelfish, *Centropyge bicolor*, while Olivotto et al. (2006a) noted that a 300 L tank was adequate to elicit natural spawning in the closely related lemonpeel angelfish, *Centropyge flavissimus*, despite the absence of several courtship behaviors seen in the wild (Bauer and Bauer, 1981).

Broadly, despite ongoing development towards larger tank designs to accommodate pelagic spawning species, hatcheries built for marine ornamentals are still much smaller than that used for foodfish, but often require more technologically advanced equipment (Watson and Hill, 2006). Although at present, the culture of some marine ornamental fishes may demand expensive system designs and techniques to elicit spawning, Mylonas, et al. (2010) suggested that the domestication process is likely to facilitate spontaneous spawning in captivity, which may help simplify system design in the future.

1.3.7.1.2 Substrate.

Vast majorities of marine ornamental fish depend upon reefs for food, shelter and for larval settlement. Provisions of suitable substrate that simulate natural reef structure are therefore

very important in marine ornamental aquaculture. More importantly, they are crucial in eliciting a natural spawning response in some fish species, particularly benthic spawning species (Friedlander, 2001; Ostrowski and Laidley, 2001). However, different views exist on substrate type and effectiveness. Natural live substrate has been used successfully to facilitate spawning of marine ornamental fishes in captivity. For example, Olivotto, et al. (2003); Olivotto, et al. (2006a) successfully spawned lemonpeel angelfish, *Centropyge flavissimus,* and the yellow-tailed damselfish, *Chrysiptera parasema* in aquaria with live rocks. And a study on the banggai cardinalfish, *Pterapogon kauderni*, even utilized live sea urchins (*Diadema* sp.), an animal the banggai cardinalfish exploits for protection in the wild (Allen, 2000), during breeding experiments. Despite successes with a natural substrate, such as live rock, their use may not be ideal for commercial production of marine ornamental fish. This is because natural live substrate is likely to carry a range of nuisance and pathogenic organisms that could severely affect broodstock health, and directly or indirectly affect gamete viability, fertilization, and hatching rate (Brock and Bullis, 2001; Ostrowski and Laidley, 2001). In their experiments leading to captive spawning of the flame angelfish *Centropyge loriculus,* and the potter's angelfish, *Centropyge potteri*, Ostrowski and Laidley (2001) made use of artificial structures, such as PVC scaffolding, and noted the ease in which they could be removed, cleaned, and sterilized to reduce the pathogen loading in broodstock holding systems. Similarly, successful conditioning and spawning of marine ornamental species including the puple firefish, *Nemateleotris decora*, barber goby, *elacatinus figaro*, redhead dottyback, *Pseudochromis dilectus*, bicolor angelfish, *Centropyge bicolor*, the blueband goby, *Valenciennea strigata,* and the blue devil damselfish, *Chrysiptera cyanea*, has been achieved with the use of artificial substrate including PVC pipe and terracotta roof tiles (Shei, et al., 2010; Madhu and Madhu, 2014; Madhu, et al., 2016). Since mass production of marine ornamental fish is the aim of

commercial ventures and also for conservation initiatives, it seems obvious that research should weigh toward the development of artificial analogues to natural reef structures for captive breeding.

1.3.7.1.3 Water conditions

Control and manipulation of the physical and chemical characteristics of the culture water is critical for the husbandry and conditioning of broodstock fish (Brock and Bullis, 2001; Schreck, et al., 2001; Sales and Janssens, 2003; Callan and Laidley, 2010). Unlike many foodfish species, most marine ornamentals come from ecosystems that display chemical stability, or 'oligotrophic' conditions (Watson and Hill, 2006; Callan and Laidley, 2010). Broadly speaking, water quality parameters may be roughly drawn from literature that investigates the tolerances of marine fishes. For example, a review by Camargo, et al. (2005) suggests that NO_3-N be maintained at less than 20 mgL^{-1} for most marine fishes. Unfortunately, very few scientific documents detail the specific tolerances of marine ornamental fishes to water quality. It is useful therefore, to draw general guidelines for marine ornamental broodstock based on maintenance conditions described in literature, particularly where successful spawning has occurred (Table 1.1).

Table 1.1: List of species-specific water quality conditions of marine ornamental fish where captive reproduction has occurred

Common Name	Species	Salinity ‰	pH	Temperature (°C)	Ammonia (TAN) mgL^{-1}	Nitrite (NO_2^-) mgL^{-1}	Nitrate (NO_3^-) mgL^{-1}	Dissolved Oxygen mgL^{-1}	Photoperiod (Light: Dark)	References
Clown anemonefish	*Amphiprion percula*	28-34	-	24.0-28.0	-	-	-	-	-	Önal et al., 2008; Gordon and Hecht, 2002
False clown anemonefish	*Amphiprion ocellaris*	26-30	8.0-8.5	25.5 - 30.0	< 0.02	< 0.02	-	-	15h:9h	Furuta et al., 2005; Yasir and Qin, 2007; Avella et al., 2007
Black anemonefish	*Amphiprion melanopus*	33-35	8.0-8.2	27.0 - 30.0	-	-	-	-	14h:10h	Arvedland et al., 2000; Green and McCormick, 1999
Yellowtail anemonefish	*Amphiprion clarkii*	30	8.0-8.2	27.5 – 28.5	< 0.03	< 0.03	-	-	14h:10h	Olivotto et al., 2008a, 2008b., 2010.
Sebae anemonefish	*Amphiprion sebae*	33-35	-	28.0-32.0		-	-	-	-	Ignatius et al., 2001
Skunk anemonefish	*Amphiprion akallopisos*	27-33	8.0-8.1	25.0-31.0	0	0	< 6	-	12-14h:12-10h	Gordon and Bok, 2001
Twoband anemonefish	*Amphiprion bicinctus*	-	-	25.0-26.0	-	-	-	-	12h:12h	Maroz and Fishelson, 1997
Black damsel	*Neopomacentrus cyanomos*	22-30	7.5-8.5	22.0-32.0	< 0.1	-	-	4.5-6.5	12h:12h	Rajasekur et al., 2009; Setu et al., 2010
Yellow tailed damsel	*Neopomacentruc nemurus*	22-28	7.5-8.5	24.0-32.0	< 0.1	-	-	4.5-6.5	-	Rajasekur et al., 2009
Blue damsel	*Pomacentrus caeruleus*	22-28	7.5-8.5	24.0-32.0	< 0.1	-	-	4.5-6.5	-	Rajasekur et al., 2009
Domino damsel	*Dascyllus trimaculeatus*	22-28	7.5-8.5	24.0-32.0	< 0.1	-	-	4.5-6.5	-	Rajasekur et al., 2009
Yellow-tailed damselfish	*Chrysiptera parasema*	28-30	8.2	27.0-28.0	-	-	-	-	13h:11h	Olivotto et al., 2003
Blue Devil damselfish	*Chrysiptera cyanea*	29-37	8.0-8.2	27.5-29.5	< 0.02	< 0.02	< 6	-	Natural	Unpublished data from Author
Semicircle angelfish	*Pomacanthus semicirculatus*	30-33	-	22.8-31.7	-	-	-	-	12h:12h	Leu et al., 2009
Cherubfish	*Centropyge argi*	32-36	8.2	20.0-24.0	< 0.03	< 0.03	-	-	10-13h: 14-11h	Bauer and Bauer, 1981; Holt and Riley, 2001
Orangeback angelfish	*Centropyge acanthops*	27-30	-	20.0-24.0	-	-	-	-	-	Bauer and Bauer, 1981
Twospined angelfish	*Centropyge bispinosus*	27-30	-	20.0-24.0	-	-	-	-	-	Bauer and Bauer, 1981
Orange angelfish	*Centropyge fisheri*	27-30	-	20.0-24.0	-	-	-	-	-	Bauer and Bauer, 1981
Lemonpeel angelfish	*Centropyge flavissimus*	27-36	8.2	20.0-30.0	< 0.03	< 0.03	-	-	13h:11h	Bauer and Bauer, 1981; Olivotto et al., 2006a
Flame angelfish	*Centropyge loriculus*	27-35	-	20.0-27.0	-	-	-	-	-	Bauer and Bauer, 1981; Callan et al., 2011
Blue Mauritius angelfish	*Centropyge debelius*	32	8.2	22.0-24.0	0	0	< 10	-	11-14h:13-10h	Baensch and Tamaru, 2009
Spiny seahorse	*Hippocampus guttulatus*	34-39	7.8-8.3	15.0-25.0	< 0.1	< 0.03	< 10	-	14-15.5h:10-8.5h	Faleiro et al., 2008; Planas et al., 2008, 2010
Bluestriped angelfish	*Chaetodontoplus septrionalis*	30.1-34.5	-	26.1-30.7	-	-	-	-	-	Leu et al., 2010
Long-snouted seahorse	*Hippocampus reidi*	27-30	8.0-8.4	22.0-28.5	< 0.03	< 0.03	-	-	12-14h:12-10h	Hora and Joyeux, 2009; Olivotto et al., 2008c
Lined seahorse	*Hippocampus erectus*	34-36	7.5-8.1	27.5-28.5	-	-	-	6-7	14h:10h	Lin et al., 2008
Coral seahorse	*Hippocampus barbouri*	33-36	7.9-8.2	25.1-26.8	< 0.05	< 0.1	-	-	-	Payne, 2003
Big belly seahorse	*Hippocampus abdominalis*	33-35	7.9-8.2	10.6-19.5	-	-	-	7.73-8.97	15h:9h	Woods, 2000b; Woods, 2000a
Three spotted seahorse	*Hippocampus trimaculatus*	31-34	7.3-7.9	30.2-30.3	0	< 0.07	-	5.95-6.7	16h:8h	Murugan et al., 2009
Common seahorse	*Hippocampus kuda*	32-33	8.3-8.7	26.0-29.3	< 0.05	< 0.05	< 0.05	7.4-7.8	-	Lin et al., 2006, 2007
White's seahorse	*Hippocampus whitei*	-	-	20.0	-	-	-	-	12h:12h	Wong and Benzie, 2003
West Australian seahorse	*Hippocampus subelongatus*	35	8.1-8.3	22.5-23.5	< 0.1	< 0.1	-	~ 7.7-8.5	12h:12h	Payne and Rippingale, 2000
Knysna seahorse	*Hippocampus capensis*	25	-	22.0	-	-	-	-	16h:8h	Lockyear et al., 1997
Tiger Tail seahorse	*Hippocampus comes*	30.94-31.36	8.2-8.3	28.5-29.5	< 0.1	< 0.1	< 0.1	-	13.5h:10.5	Job et al. 2006
Sunrise dottyback	*Pseudochromis flavivertex*	30	8.0-8.2	26.5-27.5	< 0.03	< 0.03	-	-	14h:10h	Olivotto et al., 2006b
Redhead dottyback	*Pseudochromis dilectus*	30-32	8.2-8.2	28.0-30.0	< 0.02	< 0.01	< 0.05	5.2-5.6	14h:10h	Madhu et al. 2016
Cleaner goby	*Gobiosoma evelynae*	30	8.2	25.0	< 0.03	< 0.03	-	-	13h:11h	Olivotto et al., 2005
Blueband goby	*Valenciennea strigata*	29-37	8.0-8.2	27.5-29.5	< 0.02	< 0.02	< 6	-	Natural	Unpublished data from author
Blackline fangblenny	*Meiacanthus nigrolineatus*	-	8.0	23.0-25.0	-	-	-	-	-	Fishelson, 1975
Striped blenny	*Meiacanthus grammistes*	30	8.2	28.0	< 0.03	< 0.03	-	-	13h:11h	Olivotto et al., 2010
Forktail blenny	*Meiacanthus atrodorsalis*	29-37	8.0-8.2	27.5-29.5	< 0.02	< 0.02	< 6	-	Natural	Unpublished data from author
White Tiger goby	*Priolepis nocturna*	32	8.2	30.0	< 0.02	< 0.02	< 20	-	14h:10h	Wittenrich et al., 2007
Barber goby	*Elacatinus figaro*	34	8.2	26.0	<0.02	< 2.0	-	-	12h:12h	Shei et al., 2010; Shei et al., 2012
Red head goby	*Elacatinus puncticulatus*	32.5-33.5	7.85-7.95	25.5-26.5	< 0.25	< 0.25	< 0.25	-	8h:16h	Pedrazzani et al. 2014
Purple firefish	*Nemateleotris decora*	31-33	8.0-8.4	27.0-29.0	< 0.02	< 0.01	< 0.05	4.6-6.2	14h:10h	Madhu & Madhu, 2014
Banggai cardinalfish	*Pterapogon kauderni*	34-36	7.8-8.0	24.5-25.5	0	0	-	-	12h:12h	Vagelli, 1999
Spotfin hogfish	*Bodianus puchellus*	32-36	8.2	22-25.5	< 0.03	< 0.03	-	-	11-13h:13-11h	Holt and Riley 2001
French grunt	*Haemulon flavolineatum*	-	-	23.0-28.0	-	-	-	-	12-14h:12-10h	Barden et al. 2014
Bluehead wrasse	*Thalassoma bifasciatum*	32-36	8.2	22.0-29.0	< 0.03	< 0.03	-	-	10-13h:14-11h	Holt and Riley 2001
Clown wrasse	*Halichoeres maculipinna*	32-36	8.2	25.0-27.5	< 0.03	< 0.03	-	-	11-13h:13-11h	Holt and Riley 2001

When attempting to trigger gonad maturation and/or illicit spawning, manipulations of temperature and photoperiod are often effective for many teleost fish, including marine ornamentals, and these factors can also influence fecundity and gamete quality (Carillo, et al., 1989; Pankhurst, et al., 1996; Peter and Yu, 1997; Koger, et al., 1999; Holt and Riley, 2001; Coward, et al., 2002; Holt, 2003; Kamler, 2005; Mylonas, et al., 2010; Barden, et al., 2014). It has been reported that changes, particularly increases in temperature and photoperiod, that simulate the diurnal and seasonal characteristics of the breeding season, often trigger reproductive activities in fish, particularly for those dwelling in more temperate regions (Hoff, 1996; Richardson, et al., 1997; Boeuf and Le Bail, 1999; Gordon and Bok, 2001; Holt and Riley, 2001). For example, Olivotto, et al. (2006a) reported that manipulation of photoperiod and temperature in captivity resulted in a spawning response from the lemonpeel angelfish, *C. flavissimus*. Research has also shown that spawning periodicity and frequency of various Clownfish (*Amphiprion* spp.) appears dependant on temperature and photoperiod (Hoff, 1996; Richardson, et al., 1997; Gordon and Bok, 2001).

However, in addition to photoperiod, lighting conditions also include light intensity and spectral quality, both of which have received less attention compared to photoperiod. According to Boeuf and Le Bail (1999), light intensity and spectral quality can affect fish growth. Characteristics of light vary with water depth, and the receptiveness and reaction to change in light intensity and spectral quality varies with fish species (Boeuf and Le Bail, 1999). There is no known literature that investigates the effect of light intensity or spectral quality on reproductive performance of broodstock of marine ornamentals. However, this concept may be an important factor in a captive setting as incorrect intensity and spectral quality may stress fish. Conversely, light intensity and

spectra that simulate certain water depths where natural spawning occurs, may be a key factor stimulating gonad maturation and gamete release of some species maintained in shallow broodstock tanks.

For the basic husbandry of marine ornamental fish, a simplistic but effective approach to water quality is to maintain them within the ranges that they experience in the wild. However, considering that water quality ideals and tolerances vary with species, age and associated interactions among parameters (Poxton and Allouse, 1982; Cuenco, et al., 1985; Woods, 2003c), future research taking these factors into account is clearly beneficial.

Although the water quality requirements of marine ornamental fish are generally uncompromising compared to that of many foodfish, the unique economics, and the often significantly smaller systems required for ornamental aquaculture allow the incorporation of expensive, and precise system components to maintain high water quality, while remaining profitable (Watson and Hill, 2006). However, research efforts are required to determine which and how water quality parameters affect aspects of reproductive performance of a particular marine ornamental fish in a captive setting. This should also include chemical inputs that are for therapeutic or prophylactic treatments. Such treatmeants are likely to be applied more regularly in marine ornamental operations as compared to more strictly regulated foodfish operations. For instance copper; an active ingredient in copper sulphate solutions for treating pathogens (Lipton, 1993), has been found to accumulate in tissues, impact physiological and osmoregulatory mechanisms of fish, and at high concentrations, cause death (Dethloff, 1999; Burridge and Zitko, 2002; Grosell, et al., 2004; Oliva, et al., 2007). Although trace levels of copper have been shown to benefit larval culture of false clownfish, *Amphiprion ocellaris*, overdose is likely to have lasting detrimental effects (Furuta, et al., 2005). This highlights the need for caution and the necessity to develop alternative treatments, such as vaccination, biocontrols and probiotics,

which are showing promise in foodfish aquaculture (Gatesoupe, 1999; Vine, et al., 2004; Bondad-Reantaso, et al., 2005; Adams and Thompson, 2006; Militz and Hutson, 2015).

1.3.7.2 Broodstock diet

Compared to foodfish, research related to the dietary nutrition needs of ornamental fish, and the production of diets specifically designed for broodstock is lacking (James and Sampath, 2004b; a; Callan, et al., 2011). Despite the obvious significance of such research, information concerning the nutritional requirements of both freshwater and marine ornamental fish is patchy (Blom and Dabrowski, 2000; Sales and Janssens, 2003; Vijayagopal, et al., 2008; Callan, et al., 2011). The foods used to feed marine ornamentals are often not specifically designed for them, but rather for foodfish (Tamaru, et al., 2001). Formulated diets that are commercially available and claim to be designed for ornamental fish, are generally packed in expensive small packages targeting the hobbyist market, making them economically unsuitable for large-scale operations (Mosig, 200; Chong, et al., 2003). Moreover, the creditability of such claims is questionable. Clearly, for marine ornamental culture, broodstock dietary requirements including food type, ration, feeding frequency, and the nutritional contents of diets, should receive closer attention as it is known to influence the general health, condition, fecundity, and gamete and larval quality of marine fish (Brooks, et al., 1997; Izquierdo, et al., 2001; Kamler, 2005; Donelson, et al., 2008; Callan, et al., 2011; Mejri, et al., 2017; Kloeblen, et al., 2018).

1.3.7.2.1 Food type

On the whole, in order to achieve consistent performance from broodstock, there is an industry wide push in aquaculture to develop formulated feeds. However, at present even for the foodfish industry, few formulated diets completely satisfy the dietary needs

(including palatability and nutritional content) of broodstock fish. As a result, formulated feeds are often used as a supplement to fresh or frozen raw natural foods, such as 'trash fish' (also known as 'industrial fish'), when feeding broodstock fish in the hatchery (Izquierdo, et al., 2001).

Likewise, based on their feeding habits, marine ornamental broodstock fish are generally fed one or a combination of raw or processed natural foods, such as squid, fish, mussels, prawns, *Artemia*, aquatic or terrestrial plant matter, supplemented with formulated diets or cultured live prey (e.g. live *Artemia* or mysis) for conditioning (Job, et al., 1997; Ignatius, et al., 2001; Ostrowski and Laidley, 2001; Olivotto, et al., 2003; Hopkins, et al., 2005; Olivotto, et al., 2005; Olivotto, et al., 2006a; Olivotto, et al., 2006b; Wittenrich, et al., 2007; Callan, et al., 2011). The need to feed broodstock fish with raw or live prey is not only based on a concern for nutritional requirements, but also in response to higher acceptability and palatability of raw or live foods observed over formulated diets. This phenomenon is probably also linked to the wild origins of most marine ornamental broodstock, particularly as their natural diet is made up of live organisms. Members of the family Syngnathidae, including seahorses and pipefishes, are a prime example of marine ornamentals that display strict and almost obligate preference for live or frozen feeds that resemble their natural prey, particularly when the broodstock are sourced from the wild. In freshwater ornamental aquaculture, even for species that are considered fully domesticated, live foods are still fed either exclusively or commonly with formulated diets (Lim, et al., 2003; James and Sampath, 2004a).

This reliance on raw/live feeds for marine ornamental broodstock poses several potential areas for investigation. Firstly, it may be that this reliance dissipates with successive generations of captive breeding, therefore, investigation into how the domestication process may affect preference and acceptability of diet types may prove worthwhile. Secondly,

processed foods that emulate the physical and chemical features of live natural foods may need to be developed. This could include the addition of chemical attractants and the incorporation of visual stimuli to encourage feeding behavior; i.e. arousal, search and consumption (Davis, et al., 2006). Finally, there is a clear disadvantage in the use of live/raw foods, owing to the risk of disease transmission and often variable reproductive performance as a result of inconsistent nutritional values of such diets (Izquierdo, et al., 2001; Sales and Janssens, 2003). Therefore, the development of formulated diets that are not only acceptable but also meet the specific nutritional needs of broodstock fish should be the ultimate goal in marine ornamental aquaculture.

1.3.7.2.2 Ration and feeding frequency

Feeding ration and frequency are also important considerations when conditioning broodstock fish (Izquierdo, et al., 2001). They can ultimately affect the reproductive performance of broodstock and the quality of gametes and larvae produced (Izquierdo, et al., 2001; Donelson, et al., 2008). Food restriction can negatively affect reproduction, resulting in delayed gonadal maturation and spawning (Izquierdo, et al., 2001), increased instances of filial cannibalism of eggs under parental care (Okuda and Yanagisawa, 1996; Okuda, et al., 2004), and the production of smaller larvae that display poor survival (Donelson, et al., 2008). On the other hand, overfeeding can also lead to food wastage, resulting in water quality problems, which could in turn lead to poor reproductive performance (Chang, et al., 2005). Although a safe default is to feed fish regularly to satiation, significant savings in feed and labor costs can be achieved if feeding ration and frequency are optimized, possibly in conjunction with the use of automatic feeders.

1.3.7.2.3 Broodstock nutrition

The ingredient and nutrient composition of broodstock food can significantly affect reproductive performance as well as the quality of gametes and larvae they produce (Izquierdo, et al., 2001; Tocher, 2003; Watanabe and Vassallo-Agius, 2003; Chong, et al., 2004; Khan, et al., 2005; Jaya-Ram, et al., 2008). Unfortunately, this is not a particularly well-studied area in finfish aquaculture (Izquierdo, et al., 2001; Khan, et al., 2005), with a majority of studies focusing on nutritional requirements for somatic growth (Izquierdo, et al., 2001). The lack of studies on foodfish broodstock nutrition is probably related to the high cost, long experimental durations and large facilities required to undertake such studies (Brooks, et al., 1997; Izquierdo, et al., 2001).

Research on foodfish to date has shown that fecundity and gamete quality in finfish can be optimized by maintaining proper protein and lipid levels in their diets (Pustowka, et al., 2000; Emata and Borlongan, 2003; Tocher, 2003; Watanabe and Vassallo-Agius, 2003; Khan, et al., 2005; Kloeblen, et al., 2018). Furthermore, reproductive performance, and gamete and larval quality are influenced by the level of certain essential fatty acids, such as *n*-3 and *n*-6 highly unsaturated fatty acids (HUFA), vitamin E, vitamin C, and carotenoids (Sandes, et al., 1984; Ciereszko and Dabrowski, 1995; Bell, et al., 1996; Dabrowski and Ciereszko, 1996; Asturiano, et al., 2001; Dabrowski and Ciereszko, 2001; Izquierdo, et al., 2001; Coward, et al., 2002; Watanabe and Vassallo-Agius, 2003; Domínguez, et al., 2005; Jaya-Ram, et al., 2008; Furuita, et al., 2009; Callan, et al., 2011; Kloeblen, et al., 2018). For example, females of the freshwater ornamental swordtail, *Xiphophorus helleri*, require a dietary protein level of at least 30% to support reproductive processes (Chong, et al., 2004). A recent study of another freshwater ornamental; the zebrafish, *Danio rerio*, has also shown that a direct supply of dietary HUFA, such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA), to female diets, benefits

reproduction (Jaya-Ram, et al., 2008). In contrast to foodfish and freshwater ornamental fish, little research appears to have been done to elucidate the specific nutritional needs of marine ornamental broodfish. However, recent interest in culturing very high value ornamental species has culminated in a broodstock nutrition study by Callan, et al. (2011) on the flame angelfish, *Centropyge loriculus*, that supports the vital role HUFAs, and in particular ARA, plays in improving egg production and quality in a marine ornamental angelfish.

Out of consideration for cost, it is common practice in freshwater ornamental aquaculture that inert diets formulated for foodfish are used, instead of commercially available ornamental diets, and often the same diet is used for both growout and reproductive conditioning of broodfish (Garciá-Ulloa and Gómez-Romero, 2005). If a specified diet does exist for an ornamental fish, they are often formulated based on extrapolation of formulations for foodfish, and are often too expensive to use at a production level (Sales and Janssens, 2003). For marine ornamentals, a commonly practiced 'rule of thumb' approach is to combine various diets in an attempt to satisfy the nutritional requirements of broodfish. Considering that the nutritional requirements of fish can vary substantially between species and at different life stages, there will be obvious benefits if specific diets could be developed for broodstock of marine ornamental fish based on vigorous research.

At present, there is a lack of high quality formulated foods for ornamental fish (Chong, et al., 2003; James and Sampath, 2004a), it comes as no surprise therefore, that there are numerous problems with the sole use of inert feeds, and that raw and live feeds are considered superior to inert feeds for broodstock conditioning (Degani, 1993; Chong, et al., 2003; Kaiser, et al., 2003; Velu and Munuswamy, 2003; James and Sampath, 2004a; Garciá-Ulloa and Gómez-Romero, 2005; Koldeway and Martin-Smith, 2010; Callan, et al., 2011). There is hence urgent need for research on nutritional requirements of marine

ornamental fish, which in turn will allow the formulation of appropriate diets. It is true that at present research in this area for marine ornamentals lags behind that of foodfish. However, their smaller size, relative ease of maintenance, ability to reach sexual maturity in a shorter time period, and often short spawning intervals, means that studies on broodstock nutrition in marine ornamental fish can be relatively easy, and hence could quickly catch up and serve as ideal models for closely related foodfish species (Callan, et al., 2011).

1.3.7.3 Spawning induction and stripping

In basic terms, final oocyte maturation and ovulation in teleosts requires a surge in the secretion of gonadotropins (GtHs), particularly leutenizing hormone (LH), by the pituitary gland (Brooks, et al., 1997; Ng, et al., 1997; Mylonas, et al., 2010). In some species, environmental manipulation to stimulate final oocyte maturation and ovulation may fail. Firstly, this may be due to the fact that creating the correct set of conditions to elicit the release of gonadotropin by broodstock fish may be unviable or beyond current knowledge (Zohar and Mylonas, 2001; Hill, et al., 2009; Mylonas, et al., 2010). Secondly, fish in captivity may display severe physiological responses to stressors present in captive environments, antagonizing processes leading to ovulation (Schreck, et al., 2001; Zohar and Mylonas, 2001). In such cases, hormone treatments and/or manual stripping may be used to initiate final phases of oocyte maturation and to obtain gametes if they are not released naturally (Schreck, et al., 2001; Zohar and Mylonas, 2001; Coward, et al., 2002; Pavlov, 2006). Even for species that can spawn naturally in captivity, hormonal treatments and stripping can still be used as management tools to enhance hatchery efficiency (Mylonas, et al., 2010).

Fish species that do require hormone and/or invasive treatments to obtain viable gametes generally experience one of following three ‘reproductive problems’: (1) the failure to complete vitellogenesis or spermatogenesis; (2) the absence of final oocyte maturation and/or (3) the absence of spawning (Zohar and Mylonas, 2001). In foodfish aquaculture, many species, including the European eel, *Anguilla anguilla*, the grey mullet, *Mugilis mugilis*, the rainbow trout, *Oncorhynchus mykiss*, and the striped bass, *Morone saxatilis*, encounter at least one of these problems and thus require hormone induction (Bromage, et al., 1992; Zohar and Mylonas, 2001). Freshwater ornamental species, such as the red-tailed black shark, *Labeo bicolor*, and rainbow shark, *Epalzeorhynchos frenatus*, are also examples of species that display the inability to reach final oocyte maturation and/or ovulation under captive conditions, and thus require hormone treatments (Watson and Shireman, 1996; Hill and Yanong, 2002; Brown, et al., 2003; Hill, et al., 2005). Hormone treatments have also helped achieve year-round production for seasonally spawning species in freshwater ornamental aquaculture (Burton, et al., 1998a; Brown, et al., 2003).

With respect to marine ornamental fish, hormone induction has not been used extensively and only a handful of reports exist on the subject (Ostrowski and Laidley, 2001). However, to date, results appear promising. For example, in combination with strip spawning, Moe (1997) utilized human chorionic gonadotropin (hCG) to successfully obtain viable gametes from wild-caught French and grey angelfish (*Pomacanthus* spp.) and Emel'yanova, et al. (2006) induced maturation and ovulation in the scopas tang, *Zebrasoma scopas*, via double injection of the hormone surfagon.

It must be noted however, that many marine ornamental fish are smaller compared to either *Pomacanthus* or *Zebrasoma* species. This will not only add difficulty in injecting, but also cause a higher degree of stress in small fish from handling and delivery of hormones via injection, and thus may hinder or antagonize the hormone induction process (Clearwater

and Pankhurst, 1997; Hill, et al., 2005). To negate these problems, methods of less- or non-invasive hormone administration that have been used in foodfish and freshwater ornamental aquaculture, may be adopted as viable alternatives for marine ornamental fish (Thomas and Boyd, 1989; Burton, et al., 1998b; Roelants, et al., 2000). For instance, Burton, et al. (1998b) demonstrated that *Artemia*-mediated delivery of gonadotropin-releasing hormone analogue (GnRHa) to broodstock of the freshwater cardinal tetra, *Paracheirodon axelrodi*, could successfully induce ovulation, if the *Artemia* were exposed to a 33.3% GnRHa solution for 30-60 min prior to feeding them to the fish. Similarly, hormones could be delivered via formulated diets fed to broodstock fish, and may yield similar results as *Artemia*-mediated delivery with an additional advantage of precise dosage control. Furthermore, investigations by Hill, et al. (2005) into the topical gill application of ovaprim, dissolved in a dimethyl sulfoxide solvent, to the red-fin shark, *Epalzeorhynchos erythrurus*, broodstock also showed that this less invasive method could be effective in inducing ovulation. Such techniques may prove effective if adapted for marine ornamental broodstock conditioning, and thus stand as a potential avenue for further research in marine ornamental aquaculture. Furthermore, for ornamental species that are priced based on the sex of the animal, hormones may also be used for the production of mono-sex populations (Piferrer and Lim, 1997).

Despite their many advantages, hormone induction techniques may have detrimental effects on egg fertilization and hatching rates. Such negative effects are often linked to inappropriate adoption of methods; incorrect dosage and timing of hormone administration/stripping, (Ohta, et al., 1996; Coward, et al., 2002; Hill, et al., 2005; Kamler, 2005) stresses incurred by the administration and failure to fertilize eggs soon enough after ovulation (Mylonas, et al., 1992; Mylonas, et al., 2010). Therefore, hormone induction

techniques and stripping should be used as a last resort only after efforts to induce natural spawning in marine ornamental broodstock fishes prove unsuccessful.

1.4 GAMETE PHYSIOLOGY, EMBRYOLOGY AND LARVAL MORPHOLOGY AT HATCH

Development of captive breeding techniques for targeted aquaculture species requires a sound knowledge of their gamete physiology, embryology and early larval ontogeny (Coward, et al., 2002; Olivotto, et al., 2011). Such baseline studies could help in the establishment and refinement of broodstock management and larval rearing techniques. For instance, developing indices and criteria or benchmarks unique to target species for gauging the quality of gametes, the development rates and characteristics of embryos, or newly hatched larvae, could provide information to help monitor broodstock reproductive health and assess larval quality prior to a rearing attempt (Coward, et al., 2002; Kamler, 2005; Yasir and Qin, 2007; Önal, et al., 2008). Such knowledge may include gamete size, embryonic development stages and durations, larval size and stage of development, endogenous reserves or condition indices at hatch, and functional morphology, such as mouth gape at first feeding and ontogeny of sensory organs, as well as swimming ability of larvae (Brooks, et al., 1997; Kamler, 2005).

Embryology studies may also be extrapolated to refine management techniques. For example, observations of the clownfish *Amphiprion ocellaris* by Yasir and Qin (2007), suggest that the rate of 'body turnover' in late stage eggs dictates hatching success. It is also accepted that the biotic and abiotic environment in which eggs are incubated has a bearing on embryonic development, and eventually the hatching rate and viability of the newly hatched larvae (Brooks, et al., 1997; Skjermo and Vadstein, 1999; Olafsen, 2001). Therefore, baseline knowledge of embryology and early larval ontogeny will help identify

how the environment may affect developing embryos and resulting larvae, helping develop techniques to improve hatching rate and survival of newly hatched larvae (Kamler, 2005). To date, basic information is available for some marine ornamental species with respect to gamete physiology, embryo development and ontogeny of newly hatched larvae (Fishelson, 1976; Green and McCormick, 2001; Gordon and Hecht, 2002; Olivotto, et al., 2003; Olivotto, et al., 2005; Olivotto, et al., 2006a; Olivotto, et al., 2006b; Wittenrich, et al., 2007; Yasir and Qin, 2007; Önal, et al., 2008; Pedrazzani, et al., 2014; Madhu, et al., 2016). Further research to elucidate species-specific developmental characteristics, environmental, physiological and immunological capabilities and tolerances of embryos and newly hatched larvae should benefit the establishment and refinement of rearing techniques.

1.5 LARVAL REARING

Larval rearing has presented itself as a major challenge and a persistent bottleneck for contemporary aquaculture development (Dhert, et al., 1998; Planas and Cunha, 1999; Holt, 2003; Papandroulakis, et al., 2004; Battaglene and Cobcroft, 2007). The need to develop techniques for rearing fish past critical larval stages is essential to success in commercial production of any aquaculture species, and marine ornamentals are no exception (Papandroulakis, et al., 2004). Since research and techniques in larval rearing of marine ornamental fish lag far behind that of foodfish and freshwater ornamentals, these areas should be the main focus of future studies (Holt, 2003). Key issues include system design, physical parameters of the culture environment, and larval feeds and nutrition (Planas and Cunha, 1999; Ignatius, et al., 2001; Holt, 2003; Olivotto, et al., 2003; Papandroulakis, et al., 2004).

1.5.1 Water quality and system design

Like that of broodstock, a general lack of knowledge in the area of water quality requirements for larval culture of marine ornamental fish means that there is a reliance on drawing broad guidelines from literature where larval culture has been successful (Table 1.2)

Table 1.2: List of species-specific water quality conditions for marine ornamental fish in which larval rearing has been attempted

Common Name	Species	Salinity	pH	Temperature (°C)	Ammonia (TAN) mgL^{-1}	Nitrite (NO_2^-) mgL^{-1}	Nitrate (NO_3^-) mgL^{-1}	Dissolved Oxygen mgL^{-1}	Reference
Clown anemonefish	*Amphiprion percula*	30-35	8.1-8.3	24.0-27.0	0-0.02	0-0.01	< 25	~ 6.5-8.0	Gordon et al., 1998; Önal et al., 2008
False clown anemonefish	*Amphiprion ocellaris*	-	-	26.0-27.0	-	-	< 440	-	Frakes and Hoff, 1982; Furuta et al., 2005
Black anemonefish	*Amphiprion melanopus*	-	-	27.0-29.0	-	-	-	-	Green and McCormick, 1999
Yellowtail anemonefish	*Amphiprion clarkii*	30	8.0-8.2	27.5 – 28.5	< 0.03	< 0.03	-	-	Olivotto et al., 2008a, 2008b, 2010
Sebae anemonefish	*Amphiprion sebae*	28-35	8.1-8.6	26.5-33.4	0	0	> 0.2	3.6-6.8	Ignatius et al., 2001; Divya et al. 2011
Yellow-tailed damselfish	*Chrysiptera parasema*	30	-	28.0	-	-	-	-	Olivotto et al., 2003
Hawaiian dascyllus	*Dascyllus albisella*	35	-	25.5-27.5	-	-	-	-	Danilowicz and Brown, 1992
Whitetail dascyllus	*Dascyllus aruanus*	35	-	24.0-28.5	-	-	-	-	Danilowicz and Brown, 1992
Sergeant major	*Abudefduf saxatilis*	35	8.2	28.0	< 0.02	< 0.02	< 10	-	Wittenrich et al., 2012
Semicircle angelfish	*Pomacanthus semicirulatus*	33-35	7.9-8.3	26.0-28.2	-	-	-	5.62-8.09	Leu et al., 2009
Lemonpeel angelfish	*Centropyge flavissimus*	32-36	-	26.0-28.0	-	-	-	-	Olivotto et al., 2006a
Blue Mauritius angelfish	*Centropyge debelius*	-	-	25.0-26.0	< 0.25	-	-	-	Baensch and Tamaru, 2009
Bluestriped angelfish	*Chaetodontoplus septrionalis*	33.0-34.6	8.0-8.3	26.0-28.5	< 0.1	-	-	6.3-8.5	Leu et al., 2010
Redhead dottyback	*Pseudochromis dilectus*	30-32	8.2-8.2	28.0-30.0	< 0.02	< 0.01	< 0.05	14h:10h	Madhu et al. 2016
Forktail blenny	*Meiacanthus atrodorsalis*	34-38	8.0-8.2	26.0-29.0	< 0.02	< 0.02	< 0.5	-	Data from this thesis
Lyretail blenny	*Meiacanthus reticulatus*	34-38	8.0-8.2	26.0-29.0	< 0.02	< 0.02	< 0.5	-	Unpublished data from author
Striped blenny	*Meiacanthus grammistes*	30	8.2	28.0	< 0.03	< 0.03	-	-	Olivotto et al., 2010
White Tiger goby	*Priolepis nocturna*	32	8.2	30.0	< 0.02	< 0.02	< 20	-	Wittenrich et al., 2007
Barber goby	*Elacatinus figaro*	34	8.2	26.0	< 0.02	< 2.0	-	-	Shei et al., 2010
Red head goby	*Elacatinus puncticulatus*	32.5-33.5	7.85-7.95	25.5-26.5	< 0.02	< 0.02	< 0.02	-	Pedrazzani et al. 2014
Purple firefish	*Nemateliotris decora*	-	-	28.0-30.0	-	-	-	-	Madhu & Madhu, 2014
French grunt	*Haemulon flavolineatum*	33-35	-	26.0-28.0	-	-	-	-	Barden et al., 2014

As water quality ideals and tolerances will vary with species and developmental stages, another common and effective approach is to imitate the environmental conditions observed in the wild. For instance, compared to many foodfish and freshwater ornamental species, oligotrophic conditions often characterize the habitats of marine ornamentals, and hence many marine ornamentals are thought to have a low tolerance to ammonia, nitrite and nitrate (Watson and Hill, 2006). However this is no substitute for properly designed research on a species-by-species basis. For example, Frakes and Hoff (1982) recorded a survival of 37 % after 21 days' larval culture of the clownfish, *Amphiprion ocellaris*, when subjecting them to a NO_3-N concentration of approximately 16 mgL^{-1}. However, rate of larval metamorphosis, and subsequent growth and survival were reduced when larvae were exposed to a high NO_3-N level of approximately 100 mgL^{-1} (Frakes and Hoff, 1982). Limited information in this area calls for more research in this field as quantifying larval water quality tolerances and ideals is important for both the establishment, and the refinement of culturing protocols for marine ornamental fish.

System design for larval rearing is another important area that is dependent on many other factors, including rearing density and culture methods (e.g. 'clear' or 'green water'), the level of contact with the external environment (closed/indoors, semi-closed/protected or open/exposed), and the water exchange regimes and rate (static, recirculating or flow through) (Planas and Cunha, 1999; Papandroulakis, et al., 2004). Although there are a large number of areas to cover with respect to system design, a collation of studies in which larvae of marine ornamentals were reared shows the adoption of a key culture system, i.e. the almost exclusive use of a closed greenwater 'mesocosm' system (Table 1.3).

Table 1.3: Summary of culture systems, culture methods, tank background, water flow and photoperiod used for rearing larvae of various marine ornamental fish. Culture method: GW - greenwater; CW- clearwater. Culture system: C - closed; SC - semi-closed; and O - open. Tank background: Bw - black walls, Bb - black bottom; Gw - grey walls; Gb - grey bottom; Ww - white walls; Wb - White bottom; Bgw - Beige walls, Bgb - beige bottom. Water flow: S - static; R - recirculating; and FT - flow through.

Common Name	Species	Culture method	Culture system	Tank background	Water flow	Photoperiod (Light: Dark)	Reference
Clown anemonefish	*Amphiprion percula*	GW	C	BwBb	S	14h:10h	Job et al., 1997
		GW	C	Bw	S	16h:8h	Önal et al., 2008
False clown anemonefish	*Amphiprion ocellaris*	GW	C	BwBb	S	12h:12h	Gordon and Hecht, 2002
		GW[a]	C	BwWb	S	14-16h:10-8h	Hoff, 1996
		GW	C	Bw	R /FT***	24h:0h	Avella et al., 2007
Black anemonefish	*Amphiprion melanopus*	GW	C	BwBb	S	14h:10h	Job et al., 1997
		GW	C	BwBb	S	14h:10h	Green and McCormick, 2001
Sebae anemonefish	*Amphiprion sebae*	GW	C	-	S	-	Ignatius et al., 2001
		GW	C	Opaque FRP (Color unknown)	R	12h:12h	Divya et al. 2011
Yellowtail anemonefish	*Amphiprion clarkii*	GW	C	Bw	R/FT***	14h:10h	Olivotto et al. 2008a, 2010
Spinecheek anemonefish	*Premnas biaculeatus*	GW	C	BwBb	S	14h:10h	Job et al., 1997;
		GW	C	BwBb	S	14h:10h	Job and Shand, 2001;
		GW	C	BwBb	S	14h:10h	Job and Bellwood, 2007
Yellow-tailed damselfish	*Chrysiptera parasema*	GW	C	Covered (color unknown)	S	24h:0h	Olivotto et al., 2003
Whitetail dascyllus	*Dascyllus aruanus*	GW	C	-	FT*	24h:0h**	Danilowicz and Brown, 1992
Hawaiian dascyllus	*Dascyllus albisella*	GW	C	-	FT*	24h:0h**	Danilowicz and Brown, 1992
Ambon damselfish	*Pomacentrus amboinensis*	GW	C	BwBb	S	14h:10h	Job et al., 1997
		GW	C	BwBb	S	14h:10h	Job and Shand, 2001;
		GW	C	BwBb	S	14h:10h	Job and Bellwood, 2007
Chinese demoiselle	*Neopomacentrus bankieri*	GW	C	BwBb	S	14h:10h	Job et al. 1997
Regal Damselfish	*Neopomacentrus cyanomos*	GW	C	Opaque FRP (Color unknown)	-	12h:12h	Setu et al. 2010
Sergeant major	*Abudefduf saxatilis*	GW	C	BwBb	FT (100mL min^{-1})	24h:0H	Wittenrich et al. 2012
Semicircle angelfish	*Pomacanthus semicirculatus*	GW	C	-	S	-	Leu et al. 2009
Lemonpeel angelfish	*Centropyge flavissimus*	GW	C	GwGb	S	24h:0h	Olivotto et al. 2006a
Blue Mauritius angelfish	*Centropyge debelius*	GW	C	BwWb	S	16h:8h	Baensch and Tamaru, 2009
Yellow-striped cardinalfish	*Apogon cyanosoma*	GW	C	BwBb	S	14h:10h	Job et al. 1997
Bluestriped angelfish	*Chaetodontoplus septentrionalis*	GW	C	BgwBgb	S/R ($83mLmin^{-1}$)	-	
Blue-eyed cardinalfish	*Apogon compressus*	GW	C	BwBb	S	14h:10h	Job et al. 1997;
		GW	C	BwBb	S	14h:10h	Job and Shand, 2001;
		GW	C	BwBb	S	14h:10h	Job and Bellwood, 2007
Sunrise Dottyback	*Pseudochromis flavivertex*	GW	C	Bw	R or FT***	-	Olivotto et al., 2006b
Redhead dottyback	*Pseudochromis dilectus*	GW	C	Bw	-	24:0	Madhu et al. 2016
Cleaner Goby	*Gobiosoma evelynae*	GW	C	Bw	R or FT***	24h:0h	Olivotto et al., 2005
Five-lined cardinalfish	*Cheilodipterus quinquelineatus*	GW	C	Bw	S	14h:10h	Job et al., 1997
Forktail blenny	*Meiacanthus atrodorsalis*	GW	C	BwWb	S	24h:0	Unpublished data from author
Lyretail blenny	*Meiacanthus reticulatus*	GW	C	BwWb	S	24h:0	Unpublished data from author
Striped blenny	*Meiacanthus grammistes*	GW	C	Bw	R or FT***	13h:11h	Olivotto et al., 2010
White Tiger goby	*Priolepis nocturna*	CW	C	BwBb	S/R (100mL min^{-1})[b]	14h:10h	Wittenrich et al., 2007
Barber goby	*Elacatinus figaro*	GW	C	BwWb	S	24h:0h	Shei et al., 2010, Shei et al. 2012
Red head goby	*Elacatinus puncticulatus*	GW	C	Bw	-	24h:0h	Pedrazzani et al. 2014
Purple firefish	*Nemateleotris decora*	GW	C	Bw	R (87mL min^{-1})	-	Madhu & Madhu, 2014
Manderinfish	*Synchiropus splendidus*	-	C	-	S	24h:0h	Sadovy et al., 2001
French grunt	*Haemulon flavolineatum*	GW	C	BwBb	FT	-	Barden et al. 2014

* Water exchange of between 12 % and 29 % total tank volume per day directly from the ocean

** Consisted of a 14 h 'High-light' period and a 10 h 'Low-light' period

*** Unclear whether water was recirculated or flow through

[a]Spirulina used to 'green-up' water

[b]Static replicates placed inside a larger water bath with recirculated, filtered water

The mesocosm system is seen to adopt the positive aspects of live algae addition to the culture water, such as system stability, encourageing diverse assemblages of microzooplankton such as ciliates to supplement larval diets, better prey quality and light dissipation, while integrating them with the positives of an intensive system, i.e. maximum control over system water parameters and larval stocking density (Job, et al., 1997; Planas and Cunha, 1999; Papandroulakis, et al., 2004; Tew, et al., 2015). Marine fish larvae have very limited immunological capabilities, relying mostly on nonspecific mechanisms and phagocytosis, to respond to challenges posed by obligate and opportunistic pathogens (Skjermo and Vadstein, 1999; Olafsen, 2001). Therefore, it is important to note that studies have indicated that live algae greenwater (and/or the microbial 'cocktail' it introduces), as a part of mesocosm systems, may antagonize some bacteria types (such as opportunistic pathogenic bacteria) through the production of antibiotics, and possibly promote bacteria that benefit larvae (Gatesoupe, 1999; Hargreaves, 2006; Vine, et al., 2006). Such beneficial bacteria, known as probiotics, may act directly on larvae as an ingested probiont that enhances intestinal micro-flora; or indirectly as either a passive bio-control of pathogenic and toxin-releasing bacteria or as a bio-filtration enhancer (Gatesoupe, 1999; Hargreaves, 2006; Vine, et al., 2006). A complete understanding of the effects of microalgae on larviculture has not yet been attained (Van der Meeren, et al., 2007). Marine ornamental research in this sense therefore should contribute to knowledge concerning the benefits of greenwater and the harnessing of these benefits for the development of efficient rearing systems.

The eyes of first feeding marine fish larvae are an important sensory organ for prey identification and capture, as most marine fish larvae are visual feeders (Naas, et al., 1996). Therefore, lighting conditions (including intensity, spectral quality and photoperiod) are an important environmental parameter that needs to be considered when rearing marine fish

larvae (Naas, et al., 1996; Boeuf and Le Bail, 1999). Unfortunately, few studies record light intensity or manipulation of light spectrum used in larval culture of marine ornamental fish. There is evidence to suggest that the effect of these parameters may be significant. For instance, it has been reported that on mass-scale rearing of the three-spotted seahorse, *Hippocampus trimaculatus*, survival of pelagic stage juveniles subjected to 3 different light intensities was the highest at a light intensity of 2000 lux (Murugan, et al., 2009). Peña, et al. (2004) concluded that larval feeding incidence of the spotted sand bass, *Paralabrax maculatofasciatus*, increased with an increasing light intensity from 0 to 700 lux. Job and Shand (2001) found that larvae of ochre striped cardinalfish, *Apogon compressus*, the ambon damselfish, *Pomacentrus amboinensis*, and the spinecheek anemonefish, *Premnas biaculeatus* appear well adapted to wavelengths between 493-507 nm experienced in shallow coral reef waters. Also, an extrapolation on the findings of Job and Bellwood (2007), concerning ultraviolet sensitivity of coral reef fishes, is that prey perception by larvae may be improved by manipulating light wavelengths.

With respect to photoperiod, it appears that the light durations investigated by most authors in rearing marine ornamental fish larvae are either emulating natural summer conditions or are extended up to 24 h (Table 1.3). Several studies have investigated the effect of varied photoperiod on larval fish culture (Barahona-Fernandes, 1979; Tandler and Helps, 1985; Duray and Kohno, 1988; Barlow, et al., 1995; Martin-Robichaud and Peterson, 1998; Downing and Litvak, 1999; Arvedlund, et al., 2000). Many have concluded that photoperiods longer than 14 h light resulted in increased survival and growth (Barahona-Fernandes, 1979; Tandler and Helps, 1985; Duray and Kohno, 1988; Barlow, et al., 1995; Arvedlund, et al., 2000). However, Martin-Robichaud and Peterson (1998) reported that for striped bass, *Morone saxatilis*, a short photoperiod of 8h light :16h dark appeared to facilitate better swim-bladder inflation in early larvae, leading to greater survival as compared to a 16h light :8h dark photoperiod. Meanwhile, results in support of extended

photoperiods did appear to vary between species and sometimes between different developmental stages of the same species. There also appears to be some conjecture as to the effectiveness of unnatural constant light phases (24h light: 0h dark) used for culture. The findings of Arvedlund, et al. (2000) led to the argument that the anemonefish, *A. melanopus*, larvae benefited from an extended photoperiod, but may require a dark period to optimize growth. Meanwhile, in foodfish, Barlow, et al. (1995) concluded that an extended light period from 16 to 24 h results in better growth of barramundi, *Lates calcarifer*, but also noted that these fish still displayed a circadian feeding rhythm with periods of feeding inactivity. Better survival and growth was found with a 24 h light period compared to natural photoperiods for larval rabbitfish, *Siganus guttatus*, and gilthead seabream, *Sparus aurata* (Tandler and Helps, 1985; Duray and Kohno, 1988). Further to these findings for foodfish, more recent research on the ornamental yellow-tailed damselfish, *Chrysiptera parasema*, noted that a 24 h light period was indispensable for successful rearing of larvae (Olivotto, et al., 2003). Consequently, the constant light period appears to have been adopted by these authors as a standard photoperiod in studies on larvae culture of various marine ornamental reef fish, including the cleaner goby, *Gobiosoma evelynae*, the sunrise dottyback, *Pseudochromis flavivertex*, and the lemonpeel angelfish; *Centropyge flavissimus* (Olivotto, et al., 2003; Olivotto, et al., 2005; Olivotto, et al., 2006a; Olivotto, et al., 2006b). However, caution ought to be exercised with attempts to compare among studies or to formulate a 'best practice' of photoperiod for different species, as responses to photoperiod seem species specific and linked to associated variables, including light intensity and spectral quality (Boeuf and Le Bail, 1999; Downing and Litvak, 1999; Trotter, et al., 2003).

Tank color has also been shown to have an influence on larval survival and growth, and coloration of juvenile stages (Yasir and Qin, 2009). It is suggested that appropriate backgrounds may provide the right contrast for better prey visualization and hence

improved capture efficiency (Job, et al., 1997; Martin-Robichaud and Peterson, 1998; Green and McCormick, 2001). For example, Peña, et al. (2005) reported that although feeding incidence was not affected by tank color in *Paralabrax maculatofasciatus*, feeding success (number of prey in the digestive tract) was improved by a darker tank. Furthermore, many fish larvae display phototaxis, which dictates their response to light, including reflected light off tank walls (Naas, et al., 1996). Phototaxis responses have been reported to be linked to mortality and jaw malformation of larvae of striped trumpeter, *Latris lineata*, as a result of 'walling' behavior (Cobcroft, et al., 2001; Cobcroft and Battaglene, 2009). For culture of marine ornamental larvae, standard practice appears to be that of using black walled tanks (Table 1.3). This appears to follow the conclusion that black walled tanks best emulate natural conditions experienced by pelagic larvae in the wild, and tend to prevent or reduce the negative affects of 'walling' (Naas, et al., 1996; Cobcroft, et al., 2001; Green and McCormick, 2001; Cobcroft and Battaglene, 2009). However, the effect of tank color appears to vary depending on the species being investigated and the light intensity used. For example, Downing and Litvak (1999) concluded black walled tanks impaired growth of larval haddock, *Melanogrammus aeglefinus*, at low light intensity, as compared to a white tank background. Similarly, Woods (2000) reported better attack rate and prey capture success of the planktonic stage juveniles of pot-bellied seahorses, *Hippocampus abdominalis*, in clear jars compared to black and white background jars. However, in an attempt to build on the findings of Woods (2000), Martinez-Cardenas and Purser (2007) used a different photoperiod, light intensity and tank volume for their experiments, and found no significant difference in growth or survival of the planktonic stage *Hippocampus abdominalis* when culturing them in clear, white, yellow, red, blue and black background tanks. This suggests that caution again needs to be exercised when attempting to formulate a 'best practice' for tank color, as this is

likely to be species specific and stage specific, and involve consideration of light intensity, spectrum, photoperiod, tank shape and size, and the interactions between these parameters. Clearly, the interactions between light characteristics, tank color and design, and water turbidity (such as the use of greenwater) are all likely to influence the performance of larvae and make it a very complex issue. However, as a 'rule thumb', lighting conditions that maximize contrast between prey and the surrounding environment, promoting regular feeding incidence and feeding success, as well as limiting the occurrence of light trapping and walling phenomena, are the underlying goals of light manipulation in larval rearing of marine fish (Downing and Litvak, 1999).

1.5.2 Larval diet

Larval diets are critical to the success of larval culture of fish species and there are many factors that determine whether a diet is suitable or not for a particular stage of larvae of a target species. These factors include both physical characteristics, such as size, shape, buoyancy, mobility and coloration of diets as well as chemical features, including inclusion levels of various nutrients, enzymes and attractants. During larval ontogeny, it is well recognized that high mortality occurring at the onset of exogenous feeding of larvae represents a stumbling block for the development of both marine ornamental and foodfish aquaculture (Watanabe and Kiron, 1994; Rønnestad, et al., 1999; Holt, 2003; Yúfera and Darias, 2007). Multiple papers and literature reviews have addressed this issue, with feed type, size, density and provision of correct nutrition being the major focuses (Yúfera and Darias, 2007), of which will be discussed in the following section.

1.5.2.1 Feed types, sizes and density

Gut analyses of wild caught marine fish larvae have revealed a wide range of ingested prey, including various life stages of copepods, protozoans, dinofagellates and larvae of various marine organisms (Watanabe and Kiron, 1994; Holt, 2003; McKinnon, et al., 2003). In

contrast, captive larval rearing of marine fish has relied mainly on two traditional live prey; the rotifer, *Brachionus* spp. and the nauplii of brine shrimp *Artemia* spp. (Watanabe and Kiron, 1994; Battaglene and Fielder, 1997; Sargent, et al., 1997; Wullur, et al., 2009). The major advantage of rotifers and *Artemia* is the relative ease of mass-production, however, as they are not natural prey for many marine fish larvae, the larval culture of some species, particularly reef fish, has been hindered by the inadequacy of these two live foods (Rodriguez and Hirayama, 1997; Schipp, et al., 1999; Holt, 2003; McKinnon, et al., 2003; Olivotto, et al., 2008a; Olivotto, et al., 2008b). Difficulty in raising tropical reef fish larvae, of both foodfish and ornamental species, commonly arises due to their typically small size, small mouth gape and simple digestive system at the time of first feeding (Rønnestad, et al., 1999; Holt, 2003; McKinnon, et al., 2003). For example, in larvae of the tropical groupers and snappers (both prized foodfish species), small mouth gape dictates the size requirement of first foods to be less than 100 μm. Despite the development of super small-strain rotifers (SS-rotifers; *Brachionus rotundiformis*) that can be ingested by the larvae, survival is still poor if these rotifers are used alone (Schipp, et al., 1999; McKinnon, et al., 2003).

With respect to marine ornamentals, similar problems exist in larval rearing (Ostrowski and Laidley, 2001). To date, among the few marine ornamentals that have been successfully cultured, a majority of them are characterized either by the possession of a large yolk sac, relatively large larvae and mouth gape sizes, or advanced development at hatching or first feeding (Leis, 1991; Fisher, et al., 2000). However, many marine ornamental fish larvae are very small at hatching and first feeding (< 3 mm), and therefore require foods that are smaller than even the smallest strain of *Brachionus* rotifers (Holt, 2003). Furthermore, the low ingestion rates of rotifers, the traditional live prey used for first feeding of fish larvae, is also evident in some marine ornamental larvae, with cases of refused ingestion being observed despite its acceptable size (Young, 1994; Ostrowski and Laidley, 2001).

There are several examples of successful use of alternatives to *Artemia* and rotifers, such as various copepod species, wild zooplankton, ciliates and dinoflagelates, in research scale rearing of early larvae. However, at present, in one way or another, potential replacements or supplements to rotifers for early larvae have repeatedly fallen short of prerequisites that make them feasible for mass production and industrial use, and a similar situation exists for the development of inert microdiets to totally replace *Artemia* nauplii for older larvae (Rodriguez and Hirayama, 1997; Støttrup and Norsker, 1997; Holt, 2003; Olivotto, et al., 2011). This unavoidably affects the development of marine ornamental aquaculture. Among various options, copepods appear to have better potential to supplement or replace rotifers and *Artemia* for marine ornamental larval culture, given the fact that marine ornamentals generally produce far fewer larvae compared to most foodfish species, and given recent development and refinement of mass culture techniques, particularly for tropical copepod species (Schipp, et al., 1999; Marcus and Murray, 2001; McKinnon, et al., 2003; Milione and Zeng, 2007; Camus and Zeng, 2008; Milione and Zeng, 2008; Camus and Zeng, 2009; Olivotto, et al., 2011). The use of copepods has been shown to be effective in addressing problems of size, digestibility, attractiveness and nutritional value in the culture of various foodfish (van der Meeren, 1991; McEvoy, et al., 1998; Schipp, et al., 1999; Rajkumar and Kumaguru Vasagam, 2006) as well as some marine ornamental species (Ignatius, et al., 2001; Olivotto, et al., 2006a; Olivotto, et al., 2008a; Olivotto, et al., 2008b; Baensch and Tamaru, 2009; Leu, et al., 2010; Olivotto, et al., 2010a). Additionally, marine ciliates, even smaller in size, have been suggested as a potential live prey that may bridge the gap between first feeding and the acceptance of rotifers or copepod nauplii (Nagano, et al., 2000a; Nagano, et al., 2000b; Olivotto, et al., 2005; Setu and Ajithkumar, 2010). Laboratory trials on very small marine ornamental larvae, such as the palette surgeonfish, *Paracanthurus hepatus*, and the cleaner goby, *Gobiosoma evelynae*, and the regal damselfish, *Neopomacentrus cyanomos*, as well as the seven-band grouper,

Epinephelus septemfasciatus, have shown some promise in this respect (Nagano, et al., 2000a; Nagano, et al., 2000b; Olivotto, et al., 2005; Setu and Ajithkumar, 2010). However, the low nutritional value of ciliates in some of these trials was apparent (Nagano, et al., 2000a; Nagano, et al., 2000b), and there is a need for research on their culture techniques to ensure a consistent supply. Therefore, more work on mass production techniques of ciliates, and investigation into the potential to manipulate their nutritional value to satisfy the needs of first feeding larvae are required before they become a viable option.

Another interesting development has been investigation into alternative rotifer species to the genus *Brachionus*. Chigbu and Suchar (2006) reported the successful isolation and mass culture of the marine rotifer *Colurella dicentra*, which displayed an average lorica length of 93μm and a width of 49μm, while Wullur, et al. (2009) documented the culture of the rotifer *Proales similis*, which displayed an average body length and width of 83μm and 40μm respectively. These rotifers are substantially smaller than *B. rotundiformis*, the super small *Brachionus* strain commonly used for feeding tropical fish larvae, which have an average lorica length and width of 134μm and 102μm respectively (Wullur, et al., 2009). With the use of similar culture methods, these rotifers can be cultured to achieve comparable densities to that of *Brachionus* rotifers. However, although Wullur, et al. (2009) found a higher feeding incidence of seven-band grouper, *Epinephelus septemfasciatus*, larvae on *Paoles similus* as compared to *B. rotundiformis*, there was no clear evidence that larval survival was improved. Hence, there are doubts as to whether the use of these alternative rotifer species would increase the likelihood of successfully culturing small-mouthed fish larvae. Further investigation is obviously required to verify the potential of these alternative rotifer species for the culture of small-mouthed fish larvae.

In the past decades, inert formulated microdiets have attracted substantial interest because of their stable nutrient composition, low production cost and off-the-shelf convenience. While they represent the ultimate goal in larval culture (Yúfera, et al., 1999; Langdon, et

al., 2007), the current state of development sees them largely unstable in water, displaying poor buoyancy and residence time in the water column. They are often unattractive to fish larvae, and unable to contribute to or promote enzyme activity in larval guts (Gordon, et al., 1998; Chong, et al., 2002; Kanazawa, 2003; Langdon, et al., 2007). As a consequence, formulated diets have mostly had limited success in completely replacing live feeds in the culture of early larvae of marine fish species, including ornamentals (Gordon, et al., 1998; Yúfera, et al., 1999; Cahu and Zambonino Infante, 2001; Kanazawa, 2003). For example, Gordon, et al. (1998), and Gordon and Hecht (2002) concluded that even for larvae of the anemonefish *A. percula*, a relatively hardy species for larvae culture, the earliest time for weaning onto a formulated diet without significantly affecting survival was 7-9 days after hatch. Nevertheless, with the continuous development and improvement of microdiets, they may eventually replace live feeds, resulting in significant cost savings to hatcheries by phasing out costly and labor-intensive live feed production (López-Alvarado, et al., 1994; Cahu and Zambonino Infante, 2001; Langdon, et al., 2007).

Although not necessarily a bottleneck to the larval culture of marine ornamental fish, prey density could be a consideration in larval rearing of certain species, and can have a significant bearing on feeding success, water quality, and ultimately, larval survival and growth (Houde, 1975; 1978; Duray, et al., 1996; Puvanendran and Brown, 1999b). Prey density optima and thresholds appear to be both species and stage specific (MacKenzie and Kiørboe, 1995; Puvanendran and Brown, 1999b; Laurel, et al., 2001) and depend on factors such as larval density (Houde, 1975; 1977) and mobility, foraging strategies employed by larvae (MacKenzie and Kiørboe, 1995; Laurel, et al., 2001), light and turbidity levels (Grecay and Targett, 1996; Peña, et al., 2005), and water movement in the rearing system (Sundby and Fossum, 1990; MacKenzie and Kiørboe, 1995). In marine ornamental fish larvae culture, while prey densities are often mentioned, they appear to vary depending on the author, and variables that may interact to affect their efficacy are rarely taken into

account. Hence, caution should be taken in committing to a certain prey density and assuming it is a ‘cover-all’ for all species, stages and systems.

1.5.2.2 Larval nutrition

The early life stages of marine fish are often characterized by rapid growth, physiological development, and high rates of food intake and metabolism. These challenges have to be achieved with reliance on a set of rudimentary digestive organs with low capacities to process and assimilate nutritional resources (Segner, et al., 1993; Watanabe and Kiron, 1994; Izquierdo, et al., 2000; Cahu and Zambonino Infante, 2001; Kim, et al., 2001; Rønnestad, et al., 2003; Yúfera, et al., 2003; Morais, et al., 2004; Aragão, et al., 2007; Rønnestad, et al., 2007). Therefore, upon diets being ingested by fish larvae, whether a diet can be digested and provide sufficient nutrition for sustaining larval development arise as a critical issues (Kanazawa, 2003; Kolkovski, et al., 2004a). A thorough understanding of the digestive capacity and function of larvae, from the onset of exogenous feeding, will form the basis of successful diet formulations, particularly with respect to reducing reliance on live foods (Rønnestad, et al., 2007). Many marine larvae have a rudimentary, relatively undifferentiated gut, and possess a limited ability to efficiently process complex food materials in both natural and formulated diets (Kim, et al., 2001; Rønnestad, et al., 2007). It appears that patterns of enzyme production and activity vary during fish ontogeny, are species-specific, and have been reported to be under pre-programmed genetic control, and influenced by diet quality and quantity (Péres, et al., 1998; Cahu and Zambonino Infante, 2001; Kim, et al., 2001; Aragão, et al., 2007). Larval enzymatic capacity is thus an important factor that dictates the nutritional suitability of a particular diet for marine larvae (Izquierdo, et al., 2000; Cahu and Zambonino Infante, 2001; Kim, et al., 2001) and requires species-specific and stage-specific research. In addition to larval enzymatic capacity,

among a host of topics associated with the nutritional make-up of larval diets, there are several major nutrient components that have attracted particular attention and are likely to be important for marine ornamental fish, including lipids (especially essential fatty acids), protein, vitamins and minerals, as well as carotenoids.

1.5.2.2.1 Lipids

Lipids form an important part of marine fish eggs, are the sources of the essential fatty acids (EFA), and together with free amino acids (FAA), form the most important energy sources for developing embryos and larvae (Rainuzzo, et al., 1997; Izquierdo, et al., 2000; Cahu and Zambonino Infante, 2001; Kanazawa, 2003; Tocher, 2003). It has been suggested that the lipid composition found in marine fish eggs, should approximately indicate the lipid requirement of marine fish larvae (Sargent, et al., 1999). Of various lipids in the diets of marine larvae, phospholipids (PL) and highly unsaturated fatty acids (HUFA), are two lipid components that appear to have received the highest attention in past research (Cahu and Zambonino Infante, 2001; Kanazawa, 2003; Tocher, 2003; Glencross, 2009).

The essentiality of PL in the diet of marine fish larvae is well established and they have been suggested as a superior source of HUFA as compared to neutral lipids (Izquierdo, et al., 2000; Cahu and Zambonino Infante, 2001; Kanazawa, 2003; Tocher, 2003; Tocher, et al., 2008; Cahu, et al., 2009). Although the roles that various PL fractions play is not yet clear, dietary PL broadly improves digestive functions and skeletal development of fish larvae, enhancing their development, growth and survival (Cahu and Zambonino Infante, 2001; Tocher, et al., 2008; Cahu, et al., 2009). Both the dietary source and classes of PL, and the criteria by which their effects are measured, play a role in determining the optimal PL level in marine fish larvae (Cahu, et al., 2009). Clearly, much work is still to be done to elucidate the specific functions of various fractions and classes of

PL, and the optimal dietary requirement for the larvae of various fish species, particularly in regards to marine ornamental fish species.

Aside from PL, a large body of research has focused on the nutritional values of essential fatty acids. This research has shown clear evidence of the importance of HUFAs for larval marine fish culture (Sargent, et al., 1997; Planas and Cunha, 1999; Sargent, et al., 1999; Kanazawa, 2003; Tocher, 2003; Glencross, 2009). Among HUFAs, of particular value to fish larvae are docosahexaenoic acid (DHA; 22:6*n*-3) and eicosapentaenoic acid (EPA; 20:5*n*-3) of *n*-3 HUFA, and arachidonic acid (ARA; 20:4*n*-6) of the *n*-6 HUFA (Rainuzzo, et al., 1997; Planas and Cunha, 1999; Sargent, et al., 1999; McKinnon, et al., 2003). For the culture of most larval fish, effective delivery of HUFA is achieved via enrichment of rotifers or *Artemia* using emulsified fish oils or HUFA rich microalgae (Watanabe, 1993; Ozkizilcik and Chu, 1994; Southgate and Lou, 1995; Barclay and Zeller, 1996; Southgate, 1996; Brown, et al., 1997; Harel, et al., 2002). Like in many foodfish species, the importance of essential fatty acids to marine ornamental larvae has been clearly demonstrated, achieving better survival and growth (Olivotto, et al., 2003; Olivotto, et al., 2005; Olivotto, et al., 2006a; Olivotto, et al., 2006b; Avella, et al., 2007; Olivotto, et al., 2008b), as well as decreasing anomalies in the development of the central nervous system (Avella, et al., 2007). Furthermore, of additional interest to the marine ornamental industry are findings that suggest levels of DHA, EPA and ARA in larval diets could effect their pigmentation, which is of critical importance in determining the sale value of many ornamental species (Rainuzzo, et al., 1997; Copeman, et al., 2002; Bell, et al., 2003). For example, Avella, et al. (2007) highlighted a clear correlation between fatty acid enrichment and reduced incidence of miss-bands in the cultured clownfish *Amphiprion ocellaris*, although the underlining mechanisms for such a correlation need further investigation.

1.5.2.2.2 Protein

As the source of amino acids for tissue growth and energy, protein is another critical nutrient to the larvae of marine fish (Watanabe and Kiron, 1994). However, unlike in juveniles and adult fish, it has been suggested that in the absence of HCl- and pepsin-secretion in first feeding larvae, no preparatory acid denaturation of ingested proteins and low proteolytic activity are common for early larvae (Rønnestad, et al., 1999; Helland, et al., 2003; Rønnestad, et al., 2003; Aragão, et al., 2007). This renders alkaline enzymatic attack of complex proteins difficult or inefficient for the larvae, limiting their access to the total amino acid (TAA) pool provided in their diet (Rønnestad, et al., 2003). Therefore, the supply of hydrolyzed protein and/or essential amino acids (EAA), particularly free amino acids (FAA), to diets of marine larvae appears to have greater importance than complex proteins (Rønnestad, et al., 1999; Cahu and Zambonino Infante, 2001; Aragão, et al., 2007). Many amino acids (AA) are important as metabolic fuel, form the basis of body protein synthesis and are chemo-attractants to fish larvae (Kolkovski, et al., 1997; Rønnestad, et al., 1999; Cahu and Zambonino Infante, 2001; Rønnestad, et al., 2003; Brown, et al., 2005; Aragão, et al., 2007). Although many marine fish larvae lack the ability for acid hydrolysis of complex proteins, and appear to have a slow rate of nutrient absorption, it has been reported that they have a high capacity to digest protein hydrolyzate, and a high retention efficiency of amino acids, of which FAA can be readily absorbed without digestion (Cahu and Zambonino Infante, 2001; Morais, et al., 2004; Rønnestad, et al., 2007). This may explain why marine larvae tend to have a high preference for live preys, which are known to be high in FAA, and display better survival when fed them, as opposed to formulated diets (Helland, et al., 2003; Morais, et al., 2004; Rønnestad, et al., 2007). Considering that AA are the building blocks for proteins and an important energy source, they need to be provided in the appropriate levels and ratios to maximize protein synthesis while also providing adequate metabolic fuel (Aragão, et al., 2007). Therefore, there is a need to

develop methods to manipulate the AA profile in both live and formulated larval diets to satisfy the daily requirements of marine fish larvae.

1.5.2.2.3 Vitamins, minerals and carotenoids

The effects of vitamins on fish larvae, and the way they are metabolized and stored, can relate to their solubility characteristics, i.e. water-soluble or fat-soluble, and their concentration in the diet and culture media (Furuita, et al., 2009). The functions of vitamins are very diverse and some of them must be provided within optimum ranges to avoid pathologies related to deficiency or hypervitaminosis (Dedi, et al., 1995; Furuita, et al., 2009). Despite the importance of maintaining vitamins within narrow optima for fish, the vitamin requirements of most larval fish are largely unknown (Kanazawa, 2003; Furuita, et al., 2009). Vitamin requirements that have been investigated for fish larvae include Vitamin C, Vitamin A, Vitamin E and riboflavin (Dedi, et al., 1995; Merchie, et al., 1996; Merchie, et al., 1997; Gapasin, et al., 1998; Cahu, et al., 2003; Kanazawa, 2003; Souto, et al., 2008; Furuita, et al., 2009). Among various vitamins, ascorbic acid (Vitamin C) and retinoic acid (related to Vitamin A) have been shown to affect larval development, survival and pigmentation in larvae of marine fish species, such as the Japanese flounder, *Paralichtys olivaceus*, the milkfish, *Chanos chanos*, the barramundi, *Lates calcarifer*, the Japanese eel, *Anguilla japonica*, and the turbot, *Scophthalmus maximus* (Dedi, et al., 1995; Merchie, et al., 1996; Merchie, et al., 1997; Gapasin, et al., 1998; Cahu, et al., 2003; Kanazawa, 2003; Glencross, 2006; Furuita, et al., 2009). Similarly, riboflavin was reported to improve growth and survival of white sea bream, *Diplodus sargus* (Souto, et al., 2008), while Vitamin E (largely in the form of α-tocopherol) is also considered important as it can act as an antioxidant, protecting HUFA from oxidative damage (Stephan, et al., 1995; Furuita, et al., 2009).

As inorganic compounds, various minerals are required by all animals, and like vitamins, often need to be maintained within strict ranges for normal cellular metabolic activities (Watanabe, et al., 1997). Minerals serve many biological and physiological functions, including contributions to skeletal formation, maintenance of colloidal systems, regulation of acid-base equilibrium, and form important components of hormones and enzymes (Watanabe, et al., 1997; Bury, et al., 2003; Nguyen, et al., 2008). Depending on the duration and degree of deprivation, mineral deficiencies can cause biochemical, structural and functional abnormalities (Watanabe, et al., 1997; Nguyen, et al., 2008; Matsumoto, et al., 2009). The specific requirements of fish for minerals have been sparsely researched, particularly as most minerals are either minor or trace nutrients in terms of dietary requirements (Watanabe, et al., 1997; Bury, et al., 2003). Multiple factors, including diet characteristics, mineral chemical state and the waterborne mineral concentration, could affect the bioavailability and level of absorption of a particular mineral to fish larvae (Watanabe, et al., 1997; Bury, et al., 2003). For marine fish larvae, dietary minerals that are considered highly important include zinc, manganese, iron, cobalt, selenium and iodine (Watanabe, et al., 1997). For example, both zinc and manganese are considered essential nutrients for normal growth and skeletal development in larvae of the red seabream, *Pagrus major*, and have been found to further contribute to stress tolerance in larvae (Nguyen, et al., 2008). Meanwhile, Olivotto, et al. (2003); Olivotto, et al. (2006a) added potassium iodine (KI) to the culture water of larvae of *Centropyge flavissimus* and *Chrysiptera parasema* to promote metamorphosis and settlement.

Finally, carotenoids have recently received attention in the growout of freshwater ornamental fish, as they are recognized as important for skin coloration of fish (Guoveia, et al., 2003; Garciá-Ulloa and Gómez-Romero, 2005; Wallat, et al., 2005; Sinha and Asimi, 2007). They are likely to be significant for growout stages of marine ornamental fish as coloration is clearly an important value criteria for marine ornamentals. Carotenoids can

also play important roles as pro-vitamin A, antioxidants, immunoregulators, and are suggested to have a function in disease resistance (Domínguez, et al., 2005). Carotenoids have been reported to be a beneficial nutrient to marine fish larvae. For example, dietary supplementations of carotenoids, such as astaxanthin and *β*-carotene, have been reported to benefit larvae of salmonids, the Japanese and the spotted parrotfish, *Oplegnathus fasciatus* and *Oplegnathus punctatus*, by promoting better survival, growth and increased resistance to bacterial and fungal diseases (Johnson and An, 1991; Christiansen, et al., 1995; Tachibana, et al., 1997; Domínguez, et al., 2005). Furthermore, astanxanthin has also been incorporated into formulated diets to improve visibility of the food particles to the fish larvae (Cahu and Zambonino Infante, 2001).

Though generally only small quantities are needed, vitamins, minerals and carotenoids form important dietary ingredients for marine fish larvae. However, further research is clearly needed to determine the dietary requirements of these nutrients as well as to elucidate the underlining mechanisms of their beneficial effects for the larvae of marine ornamental fish.

1.6 CONCLUSION

The MOA industry is in its infancy and its development is constraned by a lack of specific research and the presence of multiple bottlenecks. Tapping into the growing knowledge base that exists for foodfish has allowed the rudimentary development of the industry. This review has surveyed the available knowledge, and identified areas in which future research can be directed to improve the captive culture techniques of marine ornamental fish. Areas identified to be of particular importance are:

- The optimization of broodstock diet and in particular nutrition in order to maximize reproductive performance

- Investigation of non-invasive hormone delivery techniques for small size marine ornamental broodstock species that fail to spawn naturally under captive conditions
- Optimization of system design and physical parameters for larvae that maximize feeding response, health, survival and growth, and decrease production costs
- Exploitation of alternative, nutritive live prey and developing their mass culture methods for feeding larvae with small mouth gapes at first feeding
- Development of nutritionally balanced formulated feeds that for marine larvae that optimize growth and survival, and produce attractive pigmentation

As interest in marine ornamental aquaculture grows, research findings in the above areas stand to contribute significantly to the development of an economically viable marine ornamental aquaculture industry, and to the overall knowledge base of fish aquaculture, general biology as well as coral reef conservation.

1.7 M. ATRODORSALIS: A MODEL SPECIES FOR MARINE ORNAMENTAL AQUACULTURE RESEARCH

The family Blenniidae form one of the 10 most traded marine ornamental fish families (Green, 2003; Wabnitz, et al., 2003; Olivotto, et al., 2010a; Olivotto, et al., 2017). Blenniids are generally small, demersal or semi-demersal, and commonly reside on reefs (Ditty, et al., 2005). Their eggs are normally substratum-attached with multiple egg cohorts being laid in a nest from several females (Fishelson, 1975; Fishelson, 1976; Tellock and Alig, 1998; Ditty, et al., 2005). Blenniids are commonly territorial and display paternal egg care (Fishelson, 1976; Ditty, et al., 2005). While species within this family have been successfully bred and reared in captivity, and are known to accept common hatchery prey such as rotifers and *Artemia* (Olivotto, et al., 2010a), there has been few attempts to investigate and develop a reliable culture protocol for any of its members using common hatchery prey and formulated diets.

Distributed in the Indo-Pacific, the forktail blenny, *M. atrodorsalis*, also known as the eye-lash harp-tail blenny, is a species in the Blenniid family displaying high market value, and good potential for captive culture. However, little literature exists documenting its biology, captive breeding and larval rearing techniques (Tellock and Alig, 1998). For this reason, *M. atrodorsalis* was chosen as the model species in this thesis to investigate and develop new and reliable techniques for its culture.

1.8 AIMS AND THESIS OUTLINE

The aim of this thesis is to develop scientifically derived captive culture techniques for the reliable production of *M. atrodorsalis* in commercial and research settings. The thesis focuses on developing a sound knowledge of captive reproduction, embryo development and incubation, and a benchmark feeding protocol for *M. atrodorsalis* up to early juvenile

and growout stages. Successful breeding and larval rearing in particular represent the greatest risk and contain the largest bottlenecks to successful captive culture of any marine ornamental species. Therefore, the outcomes of this thesis will enable production of a reliable supply of *M. atrodorsalis* for commercial production and facilitate more focused research to address the key areas of research identified in section 1.6.

Chapter 2 describes 4 tank designs that were developed to carry out the experiments presented in this thesis. The primary aim was to provide a low volume environment that satisfied the environmental requirements of the fragile pelagic life stages of marine ornamental fish species, including *M. atrodorsalis*, while conducting trials focused on feeding and diets. To satisfy the requirements for robust systematic investigations, and achieve relevant, replicable and comparable research outcomes, the designs had to be compact, versatile, scalable and repeatable, allow behavioral observation, and provide a homogenous physical environment. The secondary aim of this chapter was to ensure the tank designs were cost effective, and easy to construct using commonly available materials.

Chapter 3 describes the reproductive behavior and embryonic and larval development of the forktail blenny, *M. atrodorsalis*. The first aim was to observe and document adult behavior of *M. atrodorsalis* in a captive setting particularly as it relates to courting, reproductive behavior, changes in sexual dimorphism and parental investment in egg care. The second aim was to document key aspects and metrics related to embryo development and first hatching in *M. atrodorsalis*, including the appearance of major structures, endogenous reserves, mouth gape and larval size at hatching. The final aim was to document larval development to settlement using common hatchery prey, rotifers and *Artemia*, to establish baseline growth and development metrics in *M. atrodorsalis*.

Chapter 4 documents the early larval stages of *M. atrodorsalis* as it relates to feeding and foraging behavior and capabilities on traditional hatchery live prey of rotifers and *Artemia*. The first aim was to determine the optimal rotifer density to offer *M. atrodorsalis* at the onset of exogenous feeding. The second aim was to determine the best time to implement a switch (defined in this thesis as an abrupt changefrom one prey type/size to another) from rotifers to *Artemia* nauplii.

Chapter 5 evaluates how the transition from rotifers to newly hatched *Artemia* nauplli in *M. atrodorsalis* can be changed by implementing a co-feeding period and offering nauplii of a specialty *Artemia* strain. It also investigates the timing to transition (in this thesis transition refers to a gradual change in prey using a co-feeding method) from newly hatched *Artemia* nauplli to *Artemia* metanauplii that are larger and more competent swimmers, and can be improved nutritionally via enrichment. The first aim was to determine whether a 3-day co-feeding transition period from rotifers to *Artemia* nauplii facilitated an earlier transition time in *M. atrodorsalis* larvae compared to the outcomes of Chapter 4. The second aim was to determine whether the use of a specialty 'AF' *Artemia* strain had an effect on the timing of a switch and transition from rotifers to *Artemia* nauplii, and on the growth of *M. atrodorsalis* compared to the traditional Great Salt Lake (GSL) *Artemia* strain. The final aim was to determine the best time to implement a transition from the newly hatched *Artemia* nauplii to enriched *Artemia* metanauplii.

Chapter 6 investigates weaning *M. atrodorsalis* from live prey to a formulated diet, and the effect of different feeding frequencies and ration sizes of the formulated diet on the survival and growth of juvenile stages. The first aim was to determine the best time to wean *M. atrodorsalis* from live prey to a common marine hatchery diet. The second and third aims were to determine an acceptable feeding frequency and ration size to offer *M. atrodorsalis* juveniles in early growout stages to achieve good survival and growth while minimizing

feed wastage and labour. Ultimately this chapter aimed to establish a benchmark weaning and feeding regime using a commonly available marine fish formulated diet.

Chapter 7 provides a summary and discussion of results, and makes recommendations for the use and application of thesis outcomes. It also examines future directions that build on results for *M. atrodorsalis,* and their application in other marine ornamental fish species.

1.9 ABOUT THIS THESIS

A majority of the data and outcomes of this thesis have already been published in peer reviewed scientific articles (see appendix B). However, to meet the comments and requirements of the reviewers and editors, parts of the data were combined, separated or reordered. This made it difficult to place the publications in the thesis in the exact format they were published. Therefore to ensure this thesis had adequate and logical flow, sections of publications were re-ordered. Additionally, in order to reduce repetition in the introduction and materials and methods sections, identical introductory statements and materials and methods were first included in detail and then referred to in subsequent chapters. Therefore, in some chapters the reader will find that the introduction, and materials and methods sections are truncated, covering only information and descriptions that have not been presented in previous chapters.

Chapter 2: Research-scale tank designs for the larval culture of marine ornamental species, with emphasis on fish

2.1 ABSTRACT

A key challenge facing scientists in marine ornamental aquaculture is the successful design and operation of tanks that accommodate fragile pelagic larvae, whilst allowing for adequate replication, behavioral observation and systematic investigation.

To address this challenge four experimental tank designs are described that have been used successfully in developing research methodologies for the larval rearing of marine ornamental species. The four designs increase sequentially in scale and include a 3-L and 9-L rounded tank design, a 5-L modified 'planktonkreisel' design and a 100-L cylindricoconical design, all of which can be operated as either static or flow-through systems. The 3-L and 9-L tanks and the 5-L planktonkreisel design are constructed from readily available materials and can be replicated to improve statistical strength while accommodating the requirements of larval fish for gentle flow, mixing, and maintenance of live prey in suspension. The 100-L tank is a technical improvement on existing current designs, using a novel integrated inlet-outlet design to enable the use of a large central outlet filter to increase screen area. This design is scalable and may be applicable for use in a commercial larviculture setting.

These tank designs provide a versatile and compact option for studies relating to larviculture of marine ornamental species, and may also be useful for other marine fish species.

2.2 INTRODUCTION

It is widely accepted that captive production of marine ornamental species should play a key role in the sustainability of the growing aquarium hobby, and the conservation and

restoration of the world's coral reefs (Rubec, 1988; Ziemann, 2001; Moorhead and Zeng, 2010; Olivotto, et al., 2011; Calado, 2017; Olivotto, et al., 2017). To date, the captive culture of marine ornamental species has shown potential to meet the growing demand of the aquarium industry, and provide a management tool to directly restore coral reefs, through hatchery releases (Ziemann, 2001; Calado, 2017). Progress in scientific research of marine ornamental aquaculture (MOA) can significantly underpin efforts to build and sustain the aquarium trade, and extend our knowledge and understanding of the life cycles, and the reproductive and larval biology of coral reef species. The broader benefits of research-scale MOA are already aiding research into new areas such as multi-generational adaptation of coral reef fish to climate change (Donelson, 2011; 2012; Miller, 2012; Donelson, 2014), in which ornamental fish, such as clownfish, are bred and reared in temperatures and carbon dioxide concentrations projected for the future. Such research will give important insight into the resilience of these and other coral reef fishes to changing ocean conditions. This highlights the far reaching benefit MOA may have, beyond meeting the demand for a luxury commodity, in informing and shaping our scientific understanding of coral reef resilience, recovery and restoration, and assisting the application of effective management and conservation strategies.

However, while the benefits of growing the MOA industry and research field are clear, and pressure mounts from conscientious consumers and environmental groups to increase the captive production of marine ornamental fish beyond a 'supplementary' source of specimens, the MOA industry and research field are lagging (Ostrowski and Laidley, 2001; Alencastro, 2004; Moorhead and Zeng, 2010; Olivotto, et al., 2011). In particular, bottlenecks associated with larviculture, particularly at a small research scale, continue to be a key limitation to the development of MOA (Calado, et al., 2008; Moorhead and Zeng, 2010; Olivotto, et al., 2011). Larviculture research has not yet delivered technology break-

throughs that have translated to reliable hatchery production, highlighting the need for greater research effort in the field (Moorhead and Zeng, 2010).

To date investigations into the larval rearing of marine ornamental species has been carried out in a range of systems and tank types and sizes, from glass beakers up to 5,000-L fibreglass reinforced plastic (FRP) tanks. However, there has been no concerted effort across the field to design versatile, repeatable rearing units for the culture of marine fish larvae. With the notable exception of restricted studies on marine ornamental decapod crustaceans (Calado, et al., 2003; Calado, et al., 2008), and a marine fish (Wittenrich, et al., 2012), there have been few attempts to collectively improve or standardize tank designs with respect to marine ornamental aquaculture research, in order to generate data that is comparable across the research field.

Among other criteria, size, shape and versatility are key considerations when developing a suitable standardized tank unit for research. Good tank designs for larvae should ultimately strike a balance between the benefits of small tanks, in sparing plankton use, and larger tanks that offer better chemical stability, allow for adequate replication, but also emulate as close as possible the tank size and designs that are used in a commercial setting (Kolkovski, et al., 2004a). The ‘scalable gap’ between research-scale systems and commercial systems is smaller for marine ornamental aquaculture primarily because a vast majority of the fish traded produce a small number of eggs per spawn, but also because the ornamental market deals in a high value commodity that is driven more by variety of species, rather than large volumes of a specific species or genera (Moorhead and Zeng, 2010; Knop and Moorhead, 2012). Therefore, successful marine ornamental aquaculture requires a considerably smaller infrastructure footprint than commercial food fish aquaculture, to be economically feasible (Watson and Hill, 2006; Moorhead and Zeng, 2010). Consequently, commercial-scale systems can closely emulate research-scale systems. However, a challenge faced by the marine ornamental aquaculture industry is the demand for a variety of species to

populate multi-species ‘community’ aquariums. It is increasingly evident that the demands and requirements of the larvae of marine ornamental species are as diverse as the specimens demanded by the market. Therefore, formulating a tank or system design that is small and yet satisfies the environmental demands of all marine ornamental species is not possible. This chapter describes four tank designs, a 3-L, 9-L, 5-L and 100-L design, that have been used to rear larval fish of marine ornamental species available on the marine ornamental market. These tank designs were developed to provide a homogenous and repeatable larval rearing environment to achieve relevant, replicable and comparable research outcomes in the subsequent chapters of this thesis.

2.3 MATERIALS AND METHODS

2.3.1 3-L Tank Design

The 3-L tank design (Fig. 2.1) was developed to conduct live-prey feeding experiments on larvae of ornamental species with low fecundity. It was also developed to resolve the challenge of conducting adequately replicated experiments in a small physical area whilst providing an adequate environment for larval growth and survival, and to reduce live food and algae wastage that can occur when culturing a small number of fish in a large tank.

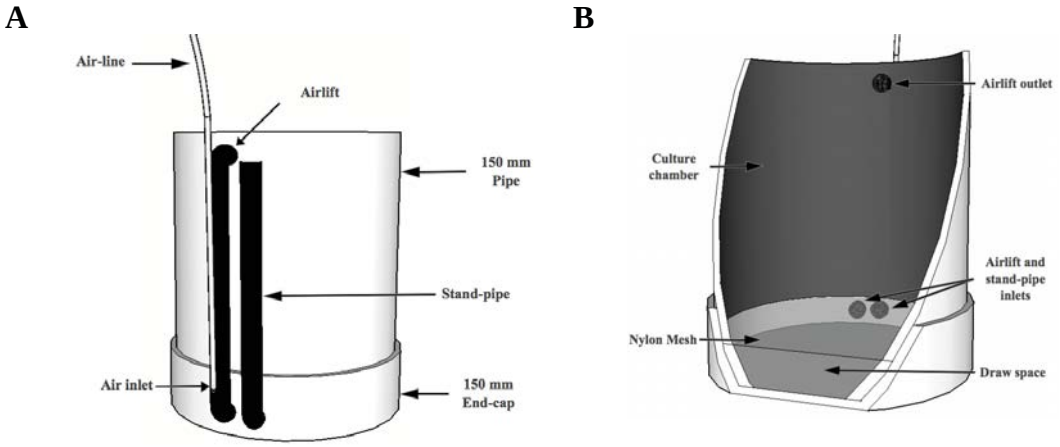

Figure 2.1: 3-L tank design. (A): External view showing the airlift assembly, the external standpipe that regulates the water level in the tank, and the ø150 mm pipe attached to the ø150 mm end-cap forming the main body of the tank. (B): Cross-section view showing the location of the nylon mesh that separates the draw space and the culture chamber, the airlift and stand-pipe inlets located under the mesh in the draw space, and the airlift outlet that returns water drawn from the draw space to the culture chamber.

The 3-L design is made from commonly available PVC and LDPE plumbing and garden irrigation fittings and pipe (Fig. 2.1 and Appendix A). The tank has a 'draw-space' separated from the culture chamber by nylon mesh of appropriate size (300-600μm aperture size mesh is recommended) (Fig. 2.1B). The airlift on the tank (Fig. 2.1A) draws water from the draw-space and returns it to the waters surface in the culture chamber (Fig. 2.1B) creating gentle continuous recirculation within the tank itself. The flow rate of recirculating water can be manipulated by adjusting the rate of airflow into the airlift. A stand-pipe on the tank sets the water level in the tank maintaining it at a constant volume (i.e. standpipes can be cut to size to produce an exact 3-L volume), and allowing passage of effluent water to exit the unit, during a water exchange, or when operated as flow-through. Being located below the mesh floor in the draw-space (Fig. 2.1B), the inlets of the airlift and standpipe are kept away from the fragile larvae in the culture chamber, preventing them from being drawn into the airlift and standpipe assemblies.

The sides and the bottom (below the mesh floor) of the tank unit can be painted to reduce light reflection and prevent 'walling' behaviour (Job, et al., 1997; Planas and Cunha, 1999; Cobcroft, et al., 2001; Papandroulakis, et al., 2004). These self-recirculating tank units can be placed onto a drip-tray or placed into a water bath, and can be operated as static or flow through systems. Their small size is favorable for good replication, providing statistical strength to experiments. One disadvantage of these units is that the mesh screen is not removable, however, these units have run satisfactorily for long periods (up to 20-30 days) without need for cleaning due to clogging or fouling of the mesh. If removal of detritus from the draw space is required, this can be removed by passing 4 mm diameter (hence forth diameter will be represented by the symbol 'ø') tube down the stand-pipe and siphoning the detritus out.

2.3.2 9-L Tank design

The 9-L tank (Fig. 2.2) was developed as a volume ‘step-up’ from the 3-L design, allowing for higher larval stocking densities while still being highly replicable. As for the 3-L design it was required to provide gentle uniform water movement, and facilitate non-invasive *in-situ* water exchange, and easy visual observation of larvae from above.

This tank design works on a similar concept to the 3-L tank design but can be constructed more quickly and easily from commonly available materials (see table A2.1 in Appendix A for approximate materials cost and construction time). When in operation the central airlift (Fig. 2.2A) gently circulates water from the draw-space at the bottom of the unit to the surface, creating a flow pattern that radiates towards the walls of the tank when viewed from above. The water then down-wells along the tank walls and toward the mesh floor. This creates a gentle flow pattern for larval fish rearing. In a similar manner to the 3-L tank design, manipulating the flow-rate of air provided to the central airlift can control how gentle or vigorous the water flow in the unit is. The walls of the tanks can be painted to achieve a desired colour, however considering these buckets were available to our laboratory in a variety of colours, painting was not required.

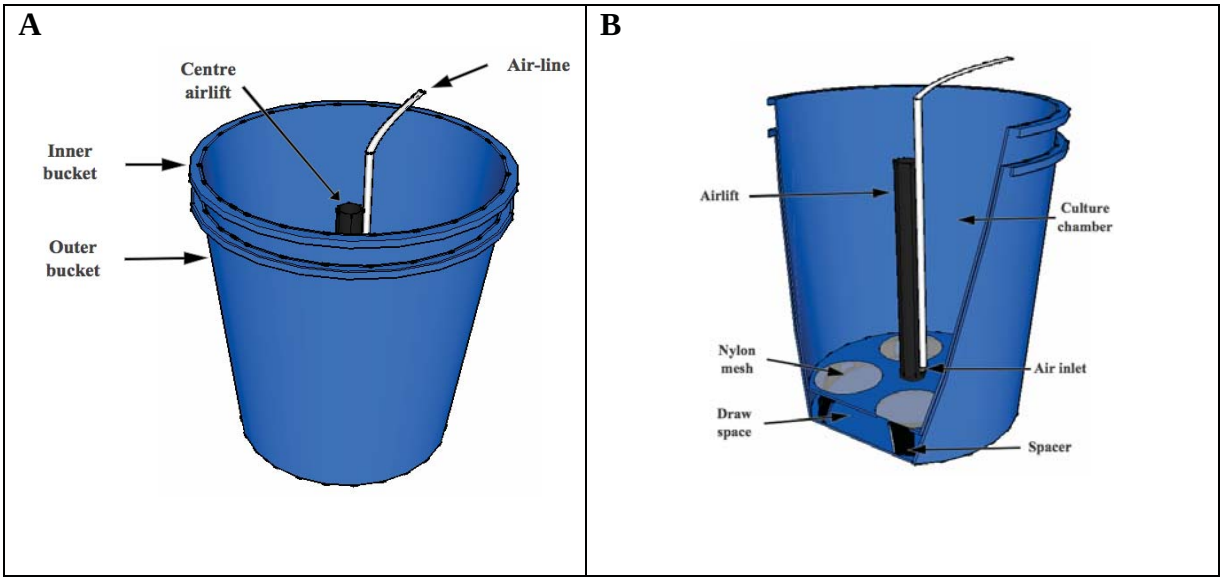

Figure 2.2: 9-L tank design. (A): External view showing the unmodified outer bucket, the inner bucket forming the culture chamber, and the centre airlift and airline, that draws water from draw space formed between the buckets, back to the waters surface of the inner bucket (culture chamber). (B): Cross-section view showing the draw space created by the plastic spacer, on which the inner bucket is supported, the modified inner bucket that forms the culture chamber with large holes covered by nylon mesh, and a full view of the centre airlift.

Using pre-manufactured components that need little cutting or modification means that there is little variability in the volume or operation of these units.

Again these 9-L tank designs are self-recirculating and can be placed onto a drip-tray or placed into a water bath, and operated either as static or flow through. Their larger size allows them to be stocked with more larvae compared to the 3-L design, whilst still being small enough to achieve good replication for statistical strength in experiments. Much like the 3-L tank design, mesh in this 9-L design is permanently glued in place (Fig. 2.2B). However, mesh sizes as small as 150 μm have been used and did not significantly foul or clog before the end of larval rearing trials. If detritus removal is required from the draw space, a flexible ø4 mm tube can be passed down the central airlift and used to siphon the detritus. Alternatively, if being operated in a water bath, the outer bucket (Fig. 2.2A) can be removed whilst retaining the larvae in water in the inner bucket, and replaced with a clean outer bucket.

2.3.3 5-L Modified 'planktonkreisel' (MPK) design

The need for a more powerful viewing tool to observe the swimming and feeding behaviors of larval fish, led to the design of a modified planktonkreisel (MPK) (Fig. 2.3), based on the 'full-circle' planktonkreisel (FCP) described by Greve (1968); 1970); 1975). The MPK was developed primarily as a unit with the same functionality as the 3-L and 9-L designs that would allow for greater *in-situ* observation of larvae, particularly from side-on. However, considering the successful use of the full circle designs for the maintenance of sensitive gelatinous plankton and crustacean larvae (Raskoff, et al., 2003; Goldstein and Nelson, 2011), the MPK was also developed to create a suitable environment for the larviculture of more sensitive marine ornamental fish.

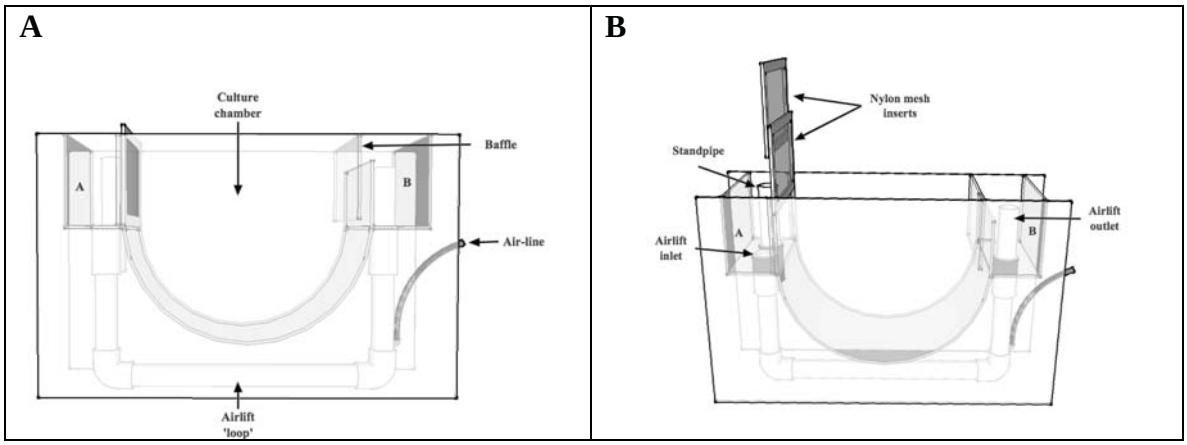

Figure 2.3: Modified planktonkreisel tank design. (A): Showing the half-circle culture chamber, the airlift loop, that draws water from compartment 'A' to compartment 'B', and the baffle that directs the water downwards into the culture chamber. (B): Showing the interchangeable mesh inserts, the standpipe and airlift loop inlet in compartment 'A' and the airlift loop outlet in compartment 'B'

The water drawn from compartment 'A' fills compartment 'B' via the 'airlift loop' and then cascades gently into the culture chamber, directed downwards by a baffle (Fig. 2.3). In compartment A there is also a standpipe that maintains water level in the unit and allows water to overflow from the unit when operated as flow-through. In order to maintain the larvae inside the culture chamber of the MPK a mesh insert, consisting of nylon mesh (of a desired size) glued to a small plastic frame, is placed between the culture chamber and

compartment ‘A’ (Fig. 2.3). Using food dye or algae paste injected into the suction side of the airlift, the observed water flow from compartment ‘B’ follows the curved floor of the culture chamber down to the bottom, and then up towards the waters surface, eventually creating a circular flow pattern. An air inlet is located on the vertical section of the airlift loop connected to compartment ‘B’ (Fig. 2.3). The flow rate of water through the airlift and thus the water movement speed inside the culture chamber can be controlled by manipulation of air injection rate. The airlift allows individual planktonkreisel units to be run as static or semi-static systems (i.e. receiving periodic flushing of new water) while still keeping constant gentle flow within each unit. This design can also be operated as flow-through simply by directing flow of new water into compartment ‘B’. In-flowing water mixes with recirculating water in compartment ‘B’ before it reaches the culture chamber. Old water in the culture unit then flows out of the standpipe located in compartment ‘A’ (Fig. 2.3B) either into a drip tray, water bath or a plumbed-in drainage system connected to the standpipe.

The MPK design allows larvae to be viewed both from above and side-on without moving them to observation tanks, eliminating the stress of handling. To accommodate larvae that are prone to ‘walling’, the unit can be constructed of dark coloured acrylic, or covered with removable panels or coloured plastic laminating paper.

2.3.4 100-L upwelling cylindriconical design

The 100-L tank was developed primarily to bridge the ‘scale-gap’ between research tank sizes and commercial tank sizes, for marine ornamental larviculture (Fig. 2.4). Its design is based on the 270-L FRP design originally documented by Kolkovski, et al. (2004b), but with an altered aspect ratio (tank height/diameter of 1 compared to 1.6) and lower volume to be more economical on space. The reduced volume was chosen to suit replicated

laboratory experiments, but still be large enough for batch-culture of low fecundity species in a commercial hatchery setting. While keeping the important features and functionality of the original design, for gentle water flow via an upwelling inlet design, modifications were made to the 100-L design to address the issue of rapid fouling of outlet-screens. The larger screen area also spreads the suction force of water flowing through the screen to the standpipe, reducing the risk of larvae being drawn into contact with the screen, which can be fatal (Fig. 2.4B and Fig. 2.5).

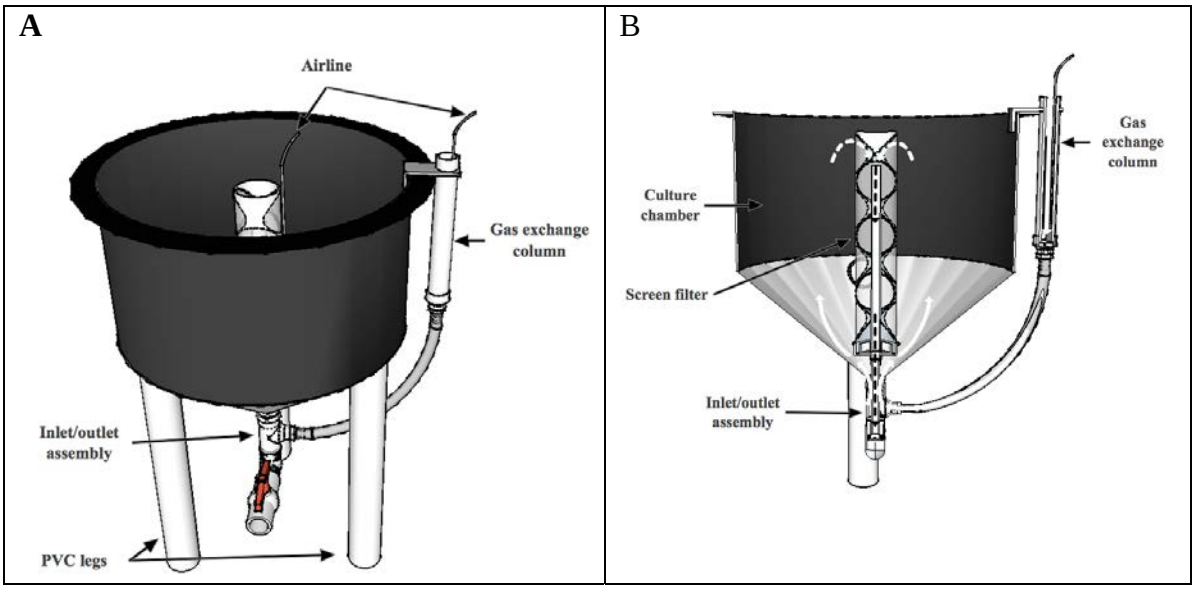

Figure 2.4: 100-L tank design. (A): External view showing the aerated gas exchange column connected to the inlet/outlet assembly via a ø25 mm tube, PVC legs supporting the tank, and airlines that lead to the aeration ring at the bottom of the central stand-pipe filter, and the gas exchange column. (B): Cross-section showing the path of water flow; Water flows into the gas exchange column and into the culture chamber (arrows with solid lines), then through the mesh of the screen filter and out the stand-pipe in the centre of the screen filter assembly (arrows with dashed lines).

When operated as flow through or during periodic flushing, water is directed into the gas exchange column, which then flows down to the inlet-outlet assembly. The water then up-wells from the central drain into the culture chamber of the tank (Fig. 2.4B). Water then exits through the central outlet screen filter that also fits into the inlet-outlet assembly, then out of a ø50 mm PVC valve to drainage (Fig. 2.4B). This design allows for a much larger area of screen filter to be used, compared to the tank design of Kolkovski, et al. (2004b), and limits interference with the flow dynamics set up in the tank by the upwelling water.

During operation, a new filter screen can be put in place before removing the old one. The standpipe at the centre of the filter screen assembly can be cut to any size to operate the tank at any desired water level. This is particularly useful when a limited live food supply is available but an acceptable prey density is still required. For example, when running trials with first feeding fish larvae, using pure strains of cultured copepods, which are difficult to culture in large densities, these tanks can be run at a reduced water level (giving a reduced culture volume of 30-50 L) facilitating higher copepod densities. As prey availability increases and/or the larvae grow, the tank water level can be increased by installing a longer stand-pipe. To create a gentle air curtain around the screen filter a porous tube air-ring is wrapped around the base of the 100 mm PVC end-cap of the base assembly (Fig. 2.5A). This air curtain helps prevent the filter screen from clogging, keeps fragile larvae away from the filter screen and aids the upwelling flow dynamic in the tank. Considering the large surface area created by the screen filter, and the ease at which filters can be changed out, the air curtain is not essential to the successful operation of the tank design during flow through operation. However, operation of the air ring is recommended if the tank is operated as a semi-static system, to create a gentle up-welling water motion.

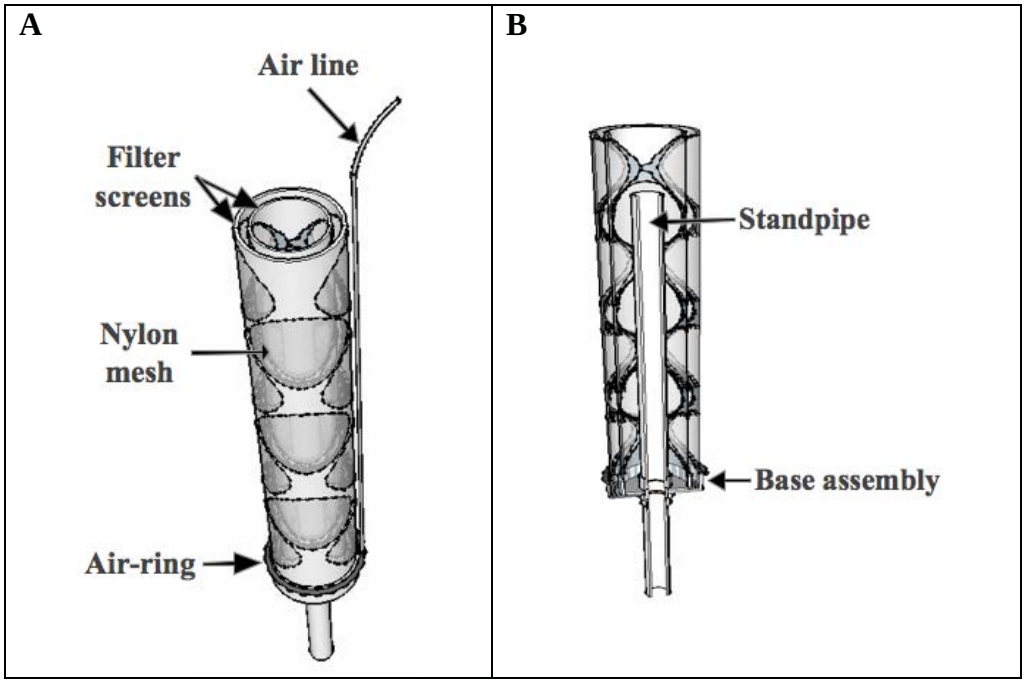

Figure 2.5: Filter screen assembly used for the 100-L tank design. (A): External view of the assembly showing both of the interchangeable filter screens in place covered with nylon mesh and the air ring that creates an air curtain over the filter screen. (B): Cross-section of assembly showing the central standpipe and the base assembly, with a ø90 mm PVC end cap glued into a ø100 mm PVC end cap, in which the filter screens fit.

2.4 RESULTS AND DISCUSSION

All four tank designs described have been used for both pilot studies and formal scientific studies on a number of marine ornamental species (Table 2.1). The 3-L and 9-L tank designs in particular have been used the most owing to the ability to perform highly replicated studies on low fecundity species, and the 5-L (MPK) and 100-L large-scale designs support more challenging and sensitive larvae through critical larval life stages.

2.4.1 3-L and 9-L tank designs

Both the 3-L and 9-L tank designs have been highly effective in well-replicated experiments on low fecundity ornamental fish. These micro-recirculating tanks maintain a uniform environment and prevent the aggregation of live prey and settling of algal pastes. Water exchange is performed easily; eliminating the need to handle or stress larvae while

replacing old water, live prey and algae paste, when the tanks are placed in a water bath linked to a recirculating system.

Table 2.1: Summary of the species of marine ornamental fish that have been cultured in each tank design.

Tank Design	Common Name	Species	Age/stage
3-L Design	Clown anemonefish[a]	*Amphiprion percula*	Newly hatched - Juvenile
	Fire anemonefish	*Amphiprion melanopus*	Newly hatched - Juvenile
	Green Manderinfish[b]	*Synchiropus splendidus*	Newly hatched - Juvenile
	Blue Devil Damselfish	*Chrysiptera cyanea*	Newly hatched - 6 DPH
	Bicolor Blenny	*Ecsenius bicolor*	Newly hatched - 8 DPH
	Pyjama Cardinalfish	*Sphaeramia nemoptera*	Newly hatched - Juvenile
	Forktail Blenny[c]	*Meiacanthus atrodorsalis*	Newly hatched - Juvenile
9-L Design	Clown anemonefish	*Amphiprion percula*	Newly hatched - Juvenile
	Green Manderinfish	*Synchiropus splendidus*	Newly hatched - Juvenile
	Blue Devil Damselfish	*Chrysiptera cyanea*	Newly hatched - 6 DPH
	Forktail Blenny	*Meiacanthus atrodorsalis*	Newly hatched - Juvenile
MPK	Green Manderinfish	*Synchiropus splendidus*	Newly hatched - Juvenile
	Blue Devil Damselfish	*Chrysiptera cyanea*	Newly hatched - 14 DPH
	Forktail Blenny	*Meiacanthus atrodorsalis*	Newly hatched - Juvenile
	Banggai Cardinalfish	*Pterapogon kauderni*	Juvenile
100-L Design	Clown anemonefish	*Amphiprion percula*	Newly hatched - Juvenile
	Green Manderinfish	*Synchiropus splendidus*	Newly hatched - Juvenile
	Bicolor Blenny	*Ecsenius bicolor*	Newly hatched - 16 DPH
	Pyjama Cardinalfish	*Sphaeramia nemoptera*	Newly hatched - Juvenile
	Ambon Damselfish	*Pomacentrus amboinensis*	Newly hatched - 11 DPH
	Blue Devil Damselfish	*Chrysiptera cyanea*	Newly hatched - Juvenile
	Forktail Blenny	*Meiacanthus atrodorsalis*	Newly hatched - Juvenile
	Banded Pipefish	*Doryhampus dactyliophorus*	Newly hatched - Juvenile

Note: Data collected from the use of these tank designs is not presented here, but can be found in current and future publications. Data on fish species that is currently published is represented by superscripts next to their common names:
[a]McLeod et al. 2013; [b]Zeng et al. 2014; [c] Moorhead & Zeng 2011

The 3-L design has been successfully used to study the larviculture of the forktail blenny, *Meiacanthus atrodorsalis* and the Green Mandarinfish (*Synchiropus splendidus*) to settlement stages, and has been adopted for studies into the physiology and effects of climate change on larval Clown anemonefish, *Amphiprion percula* (McLeod, et al., 2013; McLeod and Clark, 2016)(Table 2.1). This tank design was particularly effective when investigating early larval stages (0 to 10 days post hatch) of *M. atrodorsalis* to quantify the

effect of rotifer density on larvae, and determining the age at which *M. atrodorsalis* larvae had the capacity to transition to larger prey, such as *Artemia* nauplii (Moorhead and Zeng, 2011). While obtaining accurate daily larval counts is difficult in larger tanks, the 3-L design is small enough and shallow enough to quickly count larvae with non-invasive visual surveys. This is particularly beneficial when attempting to count small, inconspicuous larvae of species such as *S. splendidus* (Zeng, et al., 2014).

The 9-L tank is simpler to construct and operate than the 3-L design (Refer to Appendix A, Table A2.1 for approximate build times), and allows for simple, non-invasive visual inspection of larval health, behaviour and survival, and gentle replacement of old water and live prey. The 3-L and 9-L tanks were successfully used in experiments that focused on refining the feeding regime of later larvae of *M. atrodorsalis* (8-30 days post hatch) and long-term larval rearing pilot studies of *S. splendidus.*

2.4.2 Modified 'planktonkreisel' (MPK) design

The MPK has similar functionality and ease of use along side the 3-L and 9-L tank designs. Interestingly, the MPK design has a distribution of circular flow velocities, with flow being more gentle towards the middle and top of the tank and faster along the curved floor, which fish larvae appear to respond to. During pilot studies, larvae were often observed to position themselves in the tanks in an area of preferred water velocity, which was generally away from the faster flow on the tank floors.

The MPK has potential as a tool to rear more challenging, sensitive marine ornamental larvae beyond early larval stages, up to settlement and post larval ages. In particular, recent pilot studies supported growth and development of *C. cyanea* to flexion and pre-settlement ages (Table 2.1), which has previously only been achieved in larger tank designs (Gopakumar and Santhosi, 2009). This design has also been successfully used for *in-situ* observation of larval feeding in *M. atrodorsalis* and *S. splendidus* (Table 2.1), allowing for

side-on viewing of larval swimming and feeding patterns, without the need to move the larvae to observation tanks. In a similar manner to the 3-L and 9-L designs, the MPK design has been used with a simple water bath recirculating system, and uses less live feeds and algae than larger tanks, when employing the conventional green water culture method. Considering that full-circle planktonkreisels are successful with the culture of lobster larvae (Goldstein and Nelson, 2011) and maintenance of various zooplankton (Raskoff, et al., 2003), the MPK design described here may also have potential for small-scale larviculture and replicated experiments on fragile crustacean larvae, such as marine ornamental decapod crustaceans. It is the flow patterns set up in these tank designs that reduce turbulence and damaging contact with the tank walls (Kittaka, 1997; Calado, et al., 2003; Raskoff, et al., 2003). This is particularly important in crustacean larviculture as many of the zoeal stages of crustaceans have fragile appendages that can easily be damaged, affecting their capacity to feed (Calado, et al., 2003). Therefore, the MPK may prove to be a versatile design for a wide range of fish and larval culture studies, for the marine ornamental sector. However, systematic testing is needed to prove its value in these areas.

2.4.3 100-L upwelling cylindriconical design

The upwelling 100-L cylindricoconical design has supported good larval survival and growth in pilot studies on 8 marine ornamental species (Table 2.1). It has been used as a communal larviculture tank prior to replicated experiments on later stage larvae in the smaller tank designs for low fecundity fish, such as *M. atrodorsalis* and *S. splendidus* (Table 2.1). It has also been used for the successful larviculture of *C. cyanea* to metamorphosis and settlement, previously only achieved in large tanks (Gopakumar and Santhosi, 2009; Gopakumar, et al., 2009b). Additionally, it has been used for the pilot-scale rearing of the Pyjama cardinalfish (*Sphaeramia nemoptera*), Banded Pipefish (*Doryhampus*

dactyliophorus), Clown Anemonefish (*Amphiprion percula*), Bicolor Blenny (*Ecsenius bicolor*) and the Ambon Damselfish (*Pomacentrus amboinensis*; Table 2.1).

This tank design bridges the 'scale gap' between research and commercial scale aquaculture as its overall size is small enough to replicate in a small laboratory area, yet may also benefit commercial scale culture. With the use of many 100-L tanks a marine ornamental hatchery may supply low volumes of multiple high-value species in a small physical area. However, the design may also be enlarged to increase production, or to accommodate fish larvae that are difficult to rear in smaller tanks, such as the sergeant major, *Abudefduf saxatilis* (Wittenrich, et al., 2012).

2.5 CONCLUSION

Developing and optimizing the design of larval rearing systems is a key priority for MOA research. Effective research-scale tank designs will help unlock critical bottlenecks that to-date, have limited the progress of the marine ornamental aquaculture sector and scientific research. The four tank designs presented here are simple to construct and operate, and provide a compact, versatile, scalable and repeatable tool to accommodate complex experimental designs. They also span a range of volumes for research ranging from small, highly replicated studies, to larger 'industry-scale' experiments. The larvae of several ornamental fish species have been reared in these tank designs suggesting that they are suitable for use in larviculture, and therefore, may facilitate further larval rearing successes, and aid focused larval rearing studies for sensitive marine ornamental fish, and potentially crustacean larvae.

Chapter 3: Breeding of the forktail blenny, M. atrodorsalis: Broodstock management, embryonic development and larval rearing

3.1 ABSTRACT

The present study describes the reproductive behavior and embryonic development of the forktail blenny, *Meiacanthus atrodorsalis* and documents the growth and development of larvae to settlement stage. Two harems of six *M. atrodorsalis* began spawning about 3 months after acquisition, with egg clutches discovered regularly throughout the study period. Observations of courting behavior suggested that the female initiated courtship with spawning commencing after a series of male displays and courtship encounters. Female Adult *M. atrodorsalis* displayed a preference for shelters of single entrance 50-mm PVC pipe, with a 25-mm reduced entrance, for egg laying, while the male took full responsibility for egg care. Eggs were laid individually with a flattened adhesive plate anchoring them to the walls of the provided shelters. Larvae measuring 3.1 ± 0.2 mm standard length (SL) and 0.6 ± 0.0 mm body depth (BD) hatched approximately 181 h post fertilization (PF) at 28 °C, with a mouth gape height and width of 307.3 ± 11.0 and 263.8 ± 5.5 μm, respectively. A common hatchery feeding protocol consisting of firstly feeding rotifers, and then transitioning to *Artemia* nauplii with enriched *Artemia* metanauplii added later on was employed to rear newly hatched *M. atrodorsalis* to gather baseline growth and development metrics in this species. Larvae were confirmed to be accept rotifers soon after hatching, and *Artemia* after 5 DPH, reaching settlement approximately 35 DPH, measuring 13.5 ± 0.4 mm SL and 3.9 ± 0.3 mm BD.

3.2 INTRODUCTION

Very few marine ornamental fish are cultured at a commercial scale and there is a lack of literature that details their biology or culture techniques. A logical approach to the development of culture protocols for marine ornamental fish is to assess the viability of candidates for culture. Key practical considerations include the mode of reproduction and spawning frequency, fecundity, and relative ease of larviculture (Watanabe and Kiron 1994; Rønnestad *et* al 1999; Holt 2003; Wittenrich 2007a; Yúfera and Darias 2007). In particular, it is suggested that assessment of early life histories, embryology, early ontogeny, and first feeding be part of baseline studies to determine culture viability, and direct avenues for further research (Coward, et al., 2002; Mylonas, et al., 2010; Olivotto, et al., 2017). It is these areas in which problems are often experienced when attempting to culture a new aquatic species (Coward, et al., 2002). Therefore, prior to significant culture efforts being made, research directed in these areas can provide important information to overcome bottlenecks, and to ensure successful operations (Coward, et al., 2002; Kamler, 2005; Yasir and Qin, 2007; Önal, et al., 2008).

Of the 10 most traded families of marine ornamental fish, members of the family Blenniidae find popularity among hobbyists owing to their suitablility for community aquariums, striking colouration and interesting behaviours (Green, 2003; Wittenrich, et al., 2007). Members of the genus *Meiacanthus* sp. are particularly popular yet poorly studied (Olivotto, et al., 2010a). Therefore, as the first part of a series of laboratory studies aiming to form a scientifically derived larval rearing protocol for a popular *Meiacanthus* species, *M. atrodorsalis*, this chapter describes the reproductive behavior, embryonic development and larval development of this species. Ultimately this chapter aims to develop baseline development and growth metrics for further investigation into refining the larval culture of this species using standard hatchery techniques and resources.

3.3 MATERIALS AND METHODS

3.3.1 Broodstock husbandry

Altogether 12 adult *M. atrodorsalis* were obtained from a commercial collector in 2008, and maintained until October 2012. Two harems of 6 adults were arbitrarily assigned to two 300-L tanks, and each fish was tagged with a coloured elastomer; injected into the muscle near the tail, in order to distinguish between individuals during observation of reproductive behavior. Several types of shelters were provided for the fish to spawn, including half terracotta pots, 50-mm and 25-mm open PVC pipes, 50-mm capped PVC pipes with a single 25-mm reduced entry hole, and a clamshell. The broodstock tanks were located outside undercover, and formed part of a temperature controlled recirculating system, which received a natural photoperiod for Townsville, Queensland, Australia, of 11 to 13 hrs daylight per day. Temperature was maintained between 26.5 to 29.5 °C, salinity 29 to 37 ‰, pH 8.0 to 8.2, NH_3, NO_2 < 0.02 ppm, and NO_3 < 10 ppm. Feeding occurred twice daily in the morning (0700 to 0800 h), and later afternoon (1700 to 1800 h). All fish were fed a home-made gelatin-bound wet diet, consisting of a blended mix of fresh fish, squid, mussel and prawn meat, with an added multi-vitamin and mineral supplement.

3.3.2 Reproductive behavior and embryonic development

Once egg deposition was first observed, notes on reproductive behavior and nest care were taken twice daily, prior to feeding in the morning, and after feeding in the afternoon, for one month. Video footage was also taken at various times to capture courting behavior, the extent of parental nest care, and behavior changes during egg incubation.

In response to heightened activity, indicative of imminent spawning, nest sites were monitored and checked regularly for deposition of eggs. To obtain an estimate of egg

production in harems, spawning shelters were checked each morning and photographed with a digital camera (Olympus stylus 720 SW) and then analyzed using computer software (Adobe photoshop CS3 version 10.0), to determine the number of eggs spawned per shelter.

To monitor and describe embryonic development, a random sample of 10 eggs was removed from two separate clutches, and placed individually in 5-mL plastic capped vials, linked to a temperature controlled (28.0 ºC) recirculating system. A gentle flow (50-mL min^{-1}) of 50 µm filtered, UV sterilised water (salinity 34-36 ‰, pH 8.2, NH_3, NO_2 and NO_3 undetectable), passed into the vials via a glass pipette inserted through a small hole drilled into the cap of each vial. Water exited through a larger hole in the cap, covered with 180-µm mesh. Maintaining eggs in this way allowed the development of individuals to be documented all the way through to hatching. To confirm that the sampled embryos placed under laboratory conditions were developing at a homogeneous rate to the embryos that remained under parental care, 5-10 eggs were periodically obtained from the source clutch, and observed under a microscope for comparison. Development stages were examined and documented under both a dissecting microscope (Leica MZ 125) fitted with a digital camera (Leica DC 300), and a compound microscope (Leica DMLB), also equipped with a camera (Olympus DB25). Examinations were made every 1-2 h for the first 13 h, then every 6 h until hatching. Embryo measurements were taken with calibrated computer software (Leica IM 50, version 1.2 Release 19) and other measurements, such as heart rate (from when it was first discovered), were measured for each of the 10 embryos incubated under laboratory conditions.

For the measurement of mouth gape of newly hatched larvae, ten larvae were randomly sampled at hatching. They were then preserved (10% buffered formalin), cleared, and stained, according to Taylor and Van Dyke (1985), for determination of mouth-gape height and width. Mouth gape height was determined using the formula:

$GH = \sqrt{(UJL^2 + LJL^2)}$

where UJL is the upper jaw length, LJL is the lower jaw length, and GH is gape height (Shirota, 1970; Wittenrich, et al., 2007). Gape width was recorded as the distance between the left and right postero-ventral tips of the articular bones of the jaw (Kiorboe, et al., 1985).

3.3.3 Live prey production

Live feed used for rearing *M. atrodorsalis* larvae were the SS-type rotifer *Brachionus rotundiformis,* and newly hatched GSL *Artemia* nauplii (INVE technologies, Thailand LTD). The rotifers were fed live *Nannochloropsis* sp. To enrich *Artemia*, newly hatched nauplii were placed in a container for 16 h before enriching with Algamac 3050 (Aquafauna Bio-marine, Inc.) for 8 h prior to feeding to larvae. When rotifers were used as the feed, a standard volume of concentrated microalgae, *Nannochloropsis* sp. (Reed Mariculture, California, USA) was added to the water until the bottom of the culture vessels could not be seen, equating to approximately 4 million cells/mL. This was done to both dissipate light and maintain the nutritional value of the rotifers (Job, et al., 1997; Planas and Cunha, 1999; Papandroulakis, et al., 2004).

3.3.4 Larval development and growth

To describe larval development, larvae from two clutches were used. Newly hatched larvae were placed in a 60-L tub and cultured using a feeding protocol established in this laboratory of unenriched rotifers fed from 0 to 15 DPH and *Artemia* fed from 5 to 35 DPH. Temperature was maintained between 26 to 29 °C, salinity 35 to 37 ‰, pH 8.0 to 8.3, NH_3 <0.25, NO_2 < 0.05 ppm, NO_3 < 20 ppm. Throughout rearing light regime was maintained at 24 L: 0 D as recent studies showed a survival and growth benefit of this light regime for several ornamental fish, including the yellow-tailed damselfish, *Chrysiptera parasema,* the

cleaner goby, *Gobiosoma evelynae*, the sunrise dottyback, *Pseudochromis flavivertex* and redhead dottyback, *P. dilectus*, and the lemonpeel angelfish, *C. flavissimus* (Olivotto, et al., 2003; Olivotto, et al., 2005; Olivotto, et al., 2006a; Olivotto, et al., 2006b; Madhu, et al., 2016). To describe larval development and growth, at least 10 larvae from each clutch were sampled on 0, 2, 5, 10, 15, 25 and 35 DPH, anaesthetized with clove oil, and photographed under a dissecting microscope (Leica MZ 125) fitted with a digital camera (Olympus DB25). Images were then imported to Adobe photoshop (CS3 version 10.0) to measure larval SL and BD.

3.3.5 Measurements

All measurements are expressed as a mean ± standard deviation (SD).

3.4 RESULTS

3.4.1 Reproductive behavior and parental egg care

The first spawning event occurred 96 days after acquisition. During this period, changes in appearance and behavior of some individuals were observed. Dominant males became distinguishable from females by the growth of substantially longer dorsal and ventral edges of the caudal fin, which were not initially present at acquisition. The dominant male regularly displayed himself to other fish in the group, flaring his dorsal, anal and caudal fins. More aggression was also shown to certain individuals in the harem, likely to be subordinate males, but not towards others. Those individuals thought to be females were free to swim in and out of the dominant males' territory and inspect his shelters at a distance. New egg clutches were consistently discovered in the morning, before 11 am. Spawning in *M. atrodorsalis* was preceded by courting behavior and was captured on video. The female was observed to venture close to the males preferred shelter, and in response, the male proceeded to flare his fins and chase the female away. This occurred

several times in a short period before the female swam into the male's shelter, followed by the male. The two stayed in the shelter for approximately 45 minutes. Periodically, the male was observed to take sentry at the entrance to the shelter before re-entering and doing a sweep of the inside of the shelter, likely releasing sperm in the process.

A majority of egg clutches were laid in the capped PVC pipes with a single small entry hole, indicating a preference of these shelters for spawning. During the incubation period, the male showed equal aggression to all fish in the harem, excluding them from entering the egg laden shelter. However, on several occasions, more eggs were found in the same shelter 2 to 6 days after the initially observed spawning event, indicating that either a second female, or the same female spawned a second time with the dominant male. After what appeared to be a second and sometimes third egg clutch laid, new egg clutches were generally not discovered for another 5 to 14 days. In several instances, clutches of eggs laid in a single shelter from different females could not be confidently differentiated, and therefore only total egg counts per shelter were recorded. During the experimental period as little as 315 and up to 2,575 eggs of a similar developmental stage were found in a single shelter. The egg-guarding male was regularly observed hovering at the entrance to the shelter and entered it every 10 to 40 seconds for a period of up to 20 seconds. The shelter type limited observations of egg care, without disturbing the male, however, video footage indicated that the male positioned himself laterally against the eggs and agitated them with rapid movements of his pectoral fins and slow movements of the tail.

3.4.2 Embryo and larval development

Newly laid eggs: Eggs were laid individually, attached to the inside surface of the spawning shelter. They were spherical, with the proximal end of the chorion flattened against an adhesive plate, securing the egg to the shelter wall. The average diameter of eggs

measured was 991.8 ± 11.9 µm, equating to an egg volume of approximately 0.5 mm^3 (assuming a perfect sphere). Each egg contained a large, yellow-orange yolk, averaging 902.3 ± 18.9 µm in diameter, with many oil globules. Yolk volume was approximately 0.4 mm^3. The way in which eggs were laid made it difficult to identify or describe initial stages of embryo development. The orientation of the animal pole of the embryo was almost completely obscured by the adhesive plate when viewing the egg proximally and when viewed distally, it was completely obscured by the large yolk, obscuring the view of the firt two cleavage stages.

Cleavage and blastula stage: The earliest stage of embryonic development that could be observed was the 8-cell stage (Fig. 3.1A), identified about 70 min post fertilization (PF). Approximately 6 h PF, views proximally through the adhesive plate indicated that the embryos had reached 'high' stage with cells arranged in a distinctive high mound at the animal pole. Identification of embryo stage became more difficult as cells became smaller and the blastoderm flattened over the yolk, marking the beginning of epiboly.

Gastrula stage: At 25 h PF, epiboly stage was completed (Fig. 3.1B). A small mound on the yolk surface indicated that movement of deep cells had occurred, producing an obvious localized thickening of the germ ring and the embryonic shield (Fig. 3.1B).

Elaboration of body plan: By 37 h PF, some embryos appeared to have rotated, allowing a better view of developing structures (Fig. 3.1C). At this stage the head and tail of the embryo could be identified. Optic primordia were present and eye-lens development began (Fig. 3.1C). Most embryos showed 8 to 12 somites.

Approximately 12 h later (49 h PF), the yolk size decreased (Fig. 3.1D) while the notocord, flanked by 18 to 20 chevron-shaped somites, was well defined (Fig. 3.1D). Pigmentation was also observed with melanophores spotting the mid-lateral surface of the tail and the yolk. There was also evidence of by formation of the otic placodes. Meanwhile, the formation of the heart was observed in front of the head (Fig. 3.1D). Heart contractions,

and flow of blood cells were easily observed with heart rate at 93 ± 9 beats per minute (bpm).

At 61 h PF, the yolk size decreased further to a kidney shape while increased surface pigmentation in the form of stellate melanophores was observed. Increased pigmentation was also evident in the eyes, indicating iridophore proliferation on the retinas (Fig. 3.1E).

By 73 h PF, a majority of embryos had orientated themselves with their ventral side oriented away from the adhesive plate (dorsal side oriented down), with the tail extending well beyond the yolk (Fig. 3.1F). The retinas of the eyes became darker, and blood volume had obviously increased (Fig. 3.1F). Blood could be seen travelling down the dorsal aorta and through a net of vessels over the yolk. The heart had also differentiated into two distinct chambers. Heart rate was at 107 ± 13 bpm and remained steady until just prior to hatching

Between 84 and 85 h PF, the head and eyes were substantially larger. The eyes were no longer translucent and took on a metallic sheen. The tail extended over the head of the embryo with some pigmentation displayed. Increased pigmentation was also observed on the yolk surface.

Along with an increase in head and eye size at 109 h PF, the yolk had noticeably receded. The embryo periodically moved within the egg, and smaller oil globules in the yolk had coalesced into a few large ones. Evidence of the dorsal and ventral cartilages of the jaw were observed. Xanthophores spotted the snout and bordered the eyes.

By 121 h PF, the color of the yolk lightened. Cartilages of the jaw were well defined with movement of the mouth being observed periodically (Fig. 3.1G). Increased pigmentation was observed on the head and tracing the cartilage of the jaw.

At 145 h PF, all major structures, such as tail, head, heart, jaw and fins, appeared well developed, with the eyes heavily pigmented. At this time some embryos were observed to turn over, revealing their dorsal side (Fig. 3.1H). Dense melanophores were observed

dorsally, posterior to the head and fully developed otic vesicles were also observed, each containing two visible otoliths (Fig. 3.1H).

At 157 h PF, the yolk appeared much reduced with a single oil globule. Pigment was observed on the lower jaw and the ventral surface around the anus (Fig. 3.1I). Soon-to-hatch larvae displayed erratic, agitated movement inside the egg and an increased heart rate of 171 ± 25 bpm.

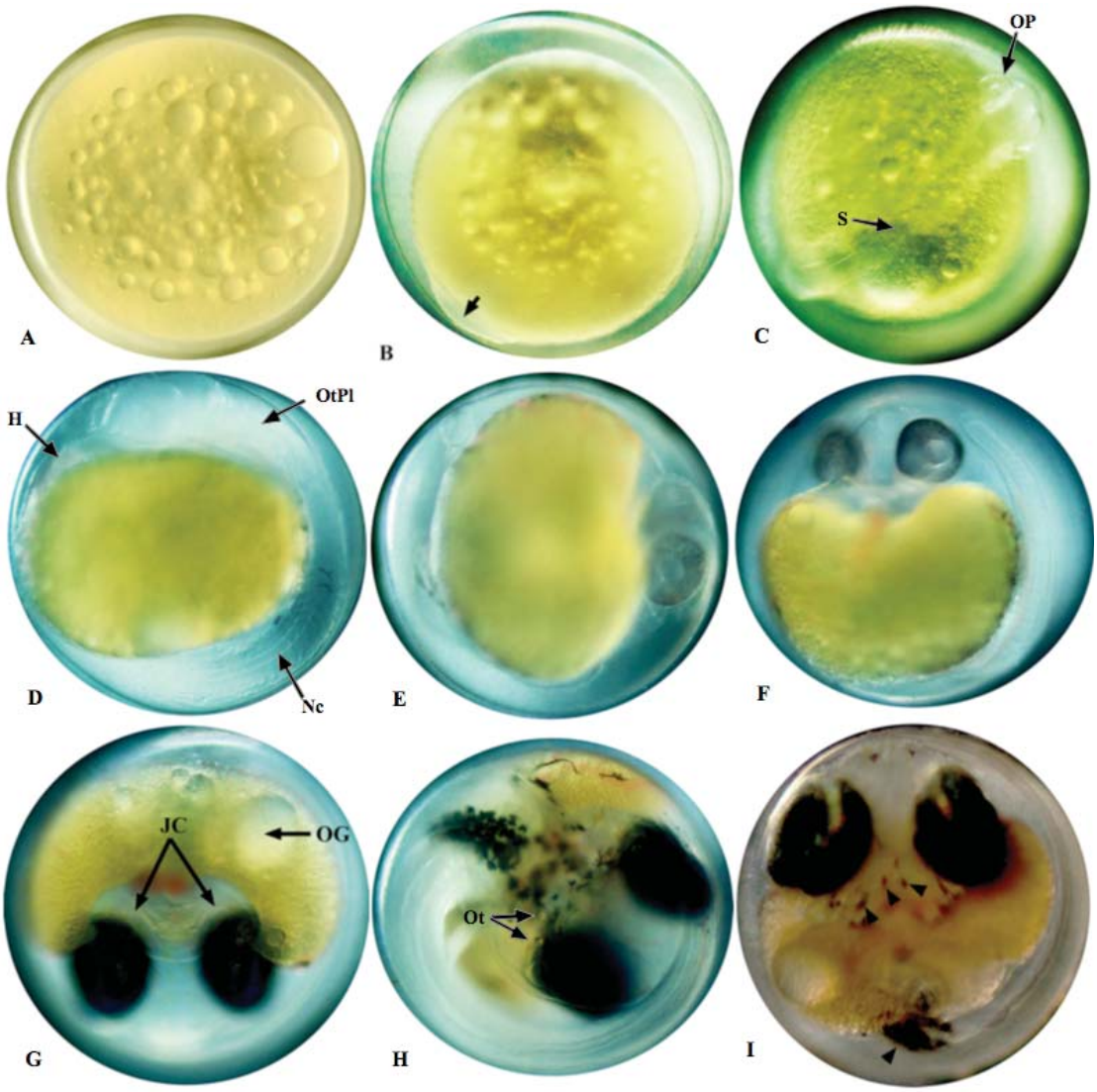

Figure 3.1: Embryonic development of *M. atrodorsalis* from 8 cell stage to hatching. All photographs taken at 100 x total magnification (TM). (***A***) 8 cell stage (70 min post fertilization (PF)) viewed proximally at the vegetal pole; showing a large yolk dotted by many oil globules. (***B***): 25 h PF; showing a localised thickening of the germ-ring (arrow) indicative of the formation of the embryonic shield. (***C***): 37 h PF; showing the head, with optic primordia (OP), and tail with somites (S). (***D***): 49 h PF; notochord (Nc), otic placode (OtPl), heart (H) and first signs of pigmentation visible. (***E***): 61 h PF; showing darkening indicative of iridophore proliferation on the retina. (***F***): 73 h PF; kidney-shaped yolk is obvious, red blood can be seen in large vessels, and eyes have darkened. (***G***) 121 h PF: a lighter yolk colour and large oil globules (OG) and jaw cartilages (JC) are visible. (***H***): 145 h PF; pigment cells are obvious around the head and dorsal surface just posterior to the eyes. Otic vesicles with otoliths (Ot) are also visible. (***I***):157 h PF; showing a much reduced yolk and pigment along lower jaw surface and around ventral surface of the tail (arrows), posterior to the yolk.

Hatching and larval development: Hatching occurred soon after dark approximately 181 h PF with larvae breaking free of the chorion; tail first. Larvae attempting to hatch from dislodged eggs that had been removed for observation could not free the head from the chorion. This indicated the need for eggs to be attached to a firm surface so larvae could lever the head free of the chorion. Pigmentation was found mainly on the head and the dorsal-posterior surface of the yolk (Fig. 3.2A). Newly hatched larvae measured 3.05 ± 0.12 mm SL (Table 3.1) and still possessed a small yolk reserve and a finfold (Fig. 3.2A). A majority swam near the water surface with obvious feeding activity observed within an hour of hatching. The jaw appeared well developed and open, and a functional gut was present. Average mouth gape height and width of newly hatched larvae was 307.3 ± 34.7 and 263.8 ± 17.4 μm, respectively.

Approximately 12 h post hatching, most larvae appeared to have exhausted their remaining yolk reserve. Approximately 2 DPH, healthy larvae displayed active foraging behavior; a gut full of rotifers, increased pigmentation, and well developed eyes (Fig. 3.2B). On 5 DPH, the head of larvae appeared to be undergoing anteroposterior elongation (Fig. 3.2C). At this stage larval length measured approximately 3.78 ± 1.06 mm (Table 3.1). Beyond 5 DPH larvae were observed to make strikes at *Artemia* nauplii, and increasingly take preference to this prey by 7 DPH. At 10 DPH they displayed an increased number of stellate melanophores at the end of the tail and along the dorsal edge of the tail (Fig. 3.2D). Pelvic fins were also observed at this stage. Between 10 and 15 DPH, larvae began to develop caudal and pectoral fin rays and began notochord flexion (Fig. 3.2E). Approximately 25 DPH, a majority of larvae appeared to change behavior, staying close to the tank walls and bottom, suggesting they were entering metamorphosis and approaching settlement. Fin rays were observed on the dorsal, caudal and along the full extent of the anal fin, however, adult coloration was not obvious (Fig. 3.2F). On 35 DPH, a majority of

larvae were considered to have settled and began displaying adult coloration (yellow tail and blue head) and behaviors. The distinctive fork tail was also evident (Fig 3.2G).

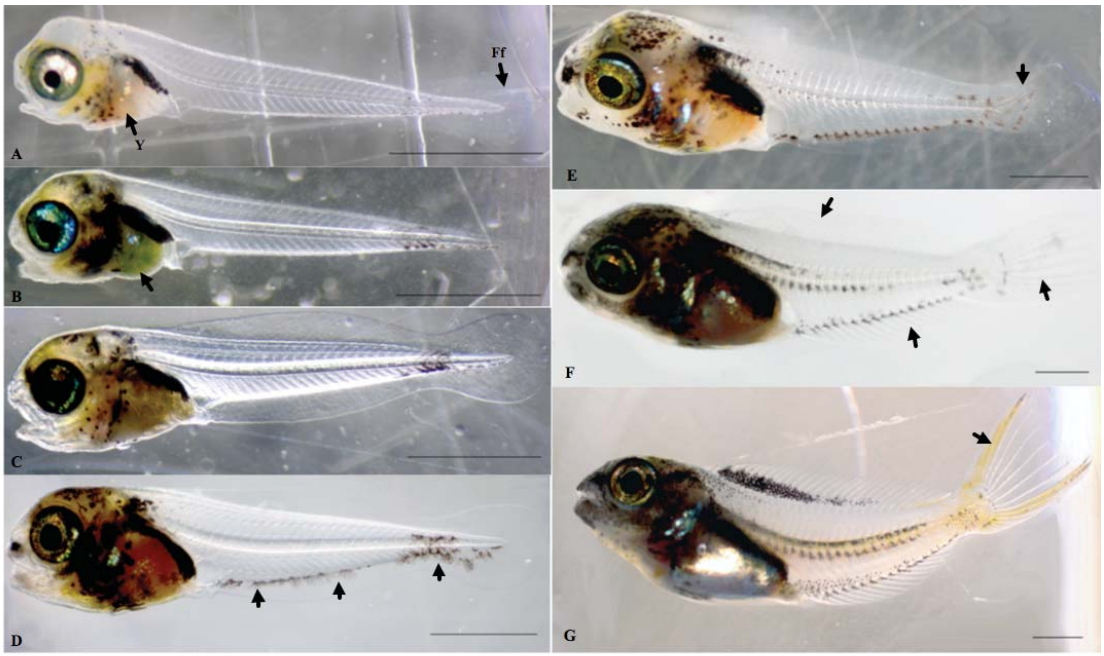

Figure 3.2: Larval development of *M. atrodorsalis* cultured using an established feeding protocol. Scale bars denote 1 mm. (***A*)**: A newly hatched larva approximately 181 h PF with a small yolk reserve (**Y)** and finfold (**Ff**). (***B***): 2 days post hatch (DPH) displaying a gut full of rotifers (arrow). (***C***): 5 DPH, showing anteroposterior elongation of the head. (***D***): 10 DPH showing increased stellate melanophores along the tail (arrows). (***E***): 15 DPH, showing flexion of the notochord (arrow). (***F***): 25 DPH, fin rays can clearly be seen on the dorsal, caudal and anal fins (arrows) (***G***): 35 DPH, post-settlement juvenile showing the beginnings of adult colouration and the distinct forktail (arrow).

Table 3.1: Standard length and body depth of *M. atrodorsalis* larvae when cultured under established standard feeding conditions.

Days post hatch	Standard length (mm) ± S.D.	Body depth (mm) ± S.D.
0	3.11 ± 0.15	0.63 ± 0.03
2	3.39 ± 0.11	0.68 ± 0.05
5	3.81 ± 0.12	0.84 ± 0.06
10	4.68 ± 0.40	1.08 ± 0.16
15	5.45 ± 0.48	1.44 ± 0.21
25	8.20 ± 0.81	2.44 ± 0.32
35	13.32 ± 1.88	3.73 ± 0.59

3.5 DISCUSSION

The forktail blenny *M. atrodorsalis* appeared to adapt well to relatively small culture tanks under captive conditions. Aggressive interactions among adult *M. atrodorsalis*, were observed but were not lethal. This parallels field observations of a related Red-Sea species, *M. nigrolineatus* which were reported to be largely solitary, aggressively chasing away conspecifics except reproductively receptive females (Fishelson, 1975). Similarly, a distinct hierarchy among males, with a dominant male defending a chosen territory, was also observed in aquarium held *M. nigrolineatus* (Fishelson, 1975). For *M. atrodorsalis*, the dominant male was observed to have almost exclusive breeding rights with the females. However, clutches of eggs were occasionally found inside shelters frequented by subordinate males, indicating some, albeit limited, reproductive success from other males.

Much of the courting behaviors observed by Fishelson (1975) for male *M. nigrolineatus*, including nodding, rearing-up and vertical hovering, were not seen in captive held *M. atrodorsalis.* The lack of obvious courting behaviors of the forktail blenny may indicate species differences or a modified behavior under captive conditions. In harem groups as in the present experiment, definite judgment on spawn frequency and fecundity of individual females was difficult to make. Although individual clutches could not be confidently differentiated, it was evident that some egg clutches of a similar developmental stage appeared to have a different colour to others, indicating possibly a different female had laid them. In the absence of a clear answer, egg clutch size for *M. atrodorsalis* in this study was estimated to be at least 300-500 eggs. Considering that the broodstock used in this study were maintained outdoors during a period spanning summer and winter, natural changes in photoperiod did not appear to affect their spawning periodicity. While monitoring single pairs of the species could provide more accurate data on fecundity and spawning

periodicity, in a commercial culture setting, it appears advantageous to hold spawning groups containing a high female to male ratio to maximize egg production.

Mouth gape measurements of newly hatched *M. atrodorsalis* larvae suggested that they were able to ingest small strain rotifers *B. rotudiformis* cultured in our laboratory, which had an average lorica width of 107 ± 15 μm and length of 165 ± 20 μm. Preliminary larval rearing confirmed that newly hatched larvae of *M. atrodorsalis* readily fed on the small strain rotifers. Additionally, unlike other marine ornamental fish, such as the Yellow-tailed Damsel, *Chrysiptera parasema*, and the Sunrise Dottyback, *Pseudochromis flavivertex* (Olivotto, et al., 2003; Olivotto, et al., 2006b), it appeared that larvae of *M. atrodorsalis* were able to grow and develop when fed rotifers cultured on *Nannochloropsis* sp.. Further, HUFA enrichment of rotifers was not required for achieving good survival and growth in the first 10 days of larval culture. However, HUFA enrichment of live prey may be required in later stages of larval development and may influence factors such as growth and time to metamorphosis, which will be assessed in subsequent experiments.

Overall, this chapter demonstrates that *M. atrodorsalis* adapt well to captive conditions, can be housed in groups of a few males to several females for conditioning and breeding, and larvae can be reared successfully on traditional live prey of rotifers and *Artemia*. These outcomes reinforce the view that this species is a good and viable candidate for captive culture to supply the industry of marine ornamental trade. Therefore, further investigation is warranted to develop this species for commercial and research-scale production.

Chapter 4: Establishing larval feeding regimens for M. atrodorsalis I: Rotifer density and time of prey switch

4.1 ABSTRACT

Initial stages of foraging and exogenous feeding represent the first major hurdle in hatchery culture of larval fish. This chapter investigates the survival and growth of first feeding *M. atrodorsalis* larvae when offered the standard live prey rotifers and later *Artemia* nauplii in two feeding trials. The first feeding trial tested the effect of rotifer density on survival and growth of newly hatched larvae. The results showed no significant difference in either survival or growth of the larvae when fed rotifers at 2, 5, 10 and 20 rotifers mL^{-1}, although the highest survival was achieved at 10 rotifers mL^{-1}. The second trial investigated the appropriate time to shift from feeding larvae with rotifers (10 mL^{-1}) to newly hatched GSL *Artemia* nauplii (3 mL^{-1}). Four treatments with prey shift occurring on 3, 6, 9 and 12 days post hatch (DPH), respectively, were set up. The results of these feeding trials suggest that to optimize survival and growth, newly hatched larvae of *M. atrodorsalis* should be fed rotifers at a density of 10 rotifers mL^{-1} for the first 7 days of life. The results also indicate that the majority of larvae appeared to display the ability to ingest *Artmia* nauplii between 6 to 10 DPH, however, co-feeding *Artemia* nauplii with rotifers earlier may help facilitate learning and hence optimize transition success.

4.2 INTRODUCTION

The period during which newly hatched larvae exhaust their endogenous yolk reserves and switch to exogenous feeding, presents itself as a major bottleneck for both foodfish aquaculture and MOA alike (Watanabe and Kiron, 1994; Rønnestad, et al., 1999; Holt,

2003; Olivotto, et al., 2005; Olivotto, et al., 2006a; Yúfera and Darias, 2007; Olivotto, et al., 2011). The onset of exogenous feeding marks a critical period in which food of appropriate size, attractiveness, palatability, density, and nutrition are required to satisfy the survival, and developmental needs of the larvae (Yúfera and Darias, 2007; Olivotto, et al., 2011). To meet the needs of a majority of first feeding marine fish larvae, provision of live prey is essential (Liao, et al., 2001; Divya, et al., 2011; Hamre, et al., 2013).

Though considered 'unnatural' prey for marine larvae, rotifers and *Artemia* can be cultured or sourced in large numbers globally, are accepted by a large range of commercially important fish and crustacean species, and are thus the live prey of choice for most commercial marine hatcheries (Sorgeloos, et al., 2001; Hagiwara, et al., 2007; Haché and Plante, 2011). Therefore, establishing reliable feeding regimes with rotifers and *Artemia* is often necessary in ensuring the commercial viability of culturing marine fish and crustaceans for both the food and ornamental markets. However, the production of live prey constitutes a significant financial, operational and infrastructure cost to hatchery economics (Le Ruyet, et al., 1993; Langdon, et al., 2007; Hamre, et al., 2013), making refining and optimizing live feed use a key directive in captive rearing of larval fish. Economical use of rotifers and *Artemia* relies on striking a balance between maximizing larval consumption rates, and limiting wastage, and the negative impact excess live prey has on the biotic and abiotic conditions in larval rearing tanks (Duray, et al., 1996).

It is recognized that prey density optima vary with species and larval age and principally the mobility and foraging capacity of larvae, in addition to a suite of other interacting variables and physical factors (MacKenzie and Kiørboe, 1995; Puvanendran and Brown, 1999a; Laurel, et al., 2001), making the broad application of prey density studies questionable. However, identifying potential prey density thresholds still holds significant value to improving larval culture practices and achieving balanced economics in a hatchery setting. At the same time, as fish larvae grow there are continuing considerations in

meeting their changing requirements for greater sized food of varying nutritional value. The type and timing of such food transitions is also an important consideration to defining balanced feeding protocols and minimizing waste.

Currently across the industry *M. atrodorsalis* has been successfully reared to a very limited extent at a research and commercial scale. In the context of this research (Chapter 3), *M. atrodorsalis* can be successfully captive reared from first hatching larvae to sizes suitable for the aquarium trade, using common hatchery methods, using live prey of rotifers and *Artemia*. This chapter presents the first steps in establishing a reliable larval feeding protocol for *M. atrodorsalis*, using rotifers and *Artemia*, based on rigorous scientific method. Two experiments were conducted with the aim of determining the most appropriate rotifer density to offer *M. atrodorsalis* at first feeding, and the most appropriate appropriate timing to transition from rotifers to newly hatched *Artemia* nauplii. These experiments sought to test the foraging capacity of *M. atrodorsalis*, quantify the effect of variable rotifer density on this species and determine a window in which this species is likely to best adapt to a transition from rotifers to newly hatched *Artemia* nauplii.

4.3 MATERIALS AND METHODS

4.3.1 Broodstock husbandry and live prey production

Broodstock source and husbandry, and live prey production techniques were described in Chapter 3, Section 3.3.1 and Chapter 3, Section 3.3.3, respecticvely.

4.3.2 Larval feeding experiments

4.3.2.1 General experimental setup and protocols

For each experiment, a shelter containing eggs was removed from the broodstock tank, and transferred into a 25-L hatching tank in the evening, just prior to predicted hatching. Gentle aeration was directed over the egg clutch with an air stone and the eggs left over night to hatch. On the morning of hatch (0 DPH), newly hatched larvae, displaying strong swimming behaviour and balance, were gently collected and distributed randomly into purposely designed 3-L replicate culture vessels described in detail in Chapter 2, Section 2.3.1. All culture vessels were placed within a temperature-controlled water bath. The water bath was connected to a recirculating system with a 500-L sump and 10-μm filtration, a biological trickle filter, foam fractionation, and UV sterilisation. Each replicate was maintained as an individual static system in the water bath. Water exchange in the vessels was carried out twice daily (at 0900 and 1700 h) with a gentle flow (250 mL min^{-1}) of filtered water from the recirculating system for 40-60 min to flush out uneaten prey and faeces before adding new live prey. The whole recirculating system was given a 30% water exchange twice daily with fresh seawater. In both experiments, temperature was maintained between 26.0 to 29.0 °C, salinity 34 to 38 ‰, and pH 8.0 to 8.2, NH_3 and NO_2 < 0.02 ppm and NO_3 < 0.5 ppm, respectively.

Daily visual counts of surviving larvae were taken throughout the experimental period. On the last day of experimentation, all remaining healthy larvae were anaesthetized and photographed under a dissecting microscope for SL and BD measurements.

4.3.2.2 Rotifer density experiment

To determine the optimal rotifer density for newly hatched larvae of *M. atrodorsalis*, survival and growth of larvae were evaluated under 5 density treatments: 0 (unfed control), 2, 5, 10 and 20 rotifers mL^{-1}. Twenty-five 3-L culture vessels were setup with 5 replicates per treatment; each replicate stocked with 20 larvae. To ensure rotifer density was maintained at designated levels, all replicates were checked visually post-flushing and

water samples taken and counted 1 h post-feeding to confirm the density daily. Densities not within ± 1 rotifers mL^{-1} for the 2 and 5 rotifers mL^{-1} treatments, and not within ± 2 rotifers mL^{-1} for the 10 and 20 rotifers mL^{-1} treatments were adjusted by adding more rotifers, or flushing excess rotifers out and then re-checked. Based on the outcomes of Chapter 3, which indicated *M. atrodorsalis* appear to take interest in *Artemia* nauplii from 5 DPH, the rotifer density experiment here was run until 7 DPH.

4.3.2.3 Prey switch experiment

Upon identifying the optimum rotifer density for early larvae, a separate experiment was undertaken in which *M. atrodorsalis* larvae were subjected to an abrupt prey switch, in order to test the best time to implement a prey transition from rotifers to *Artemia*. An abrupt prey switch from rotifers to *Artemia* was implemented on 3, 6, 9 and 12 DPH respectively.

For this experiment, newly hatched larvae from a single clutch were initially reared communally to 3 DPH in a 60-L tub. The tub was kept as a static system with 30% water exchanged twice daily. Rotifer density in the tub was maintained at 10 mL^{-1}. On the morning of 3 DPH, healthy larvae were transferred to each of 20 replicate culture vessels (3 L) with 25 larvae stocked into each vessel, i.e. 5 replicates per treatment. Apart from larvae in the 3 DPH prey shifting treatment, which were given *Artemia* nauplii at 3 mL^{-1} straight away, larvae in all other treatments were maintained on rotifers at 10 mL^{-1} until the day their respective prey change was due to occur. On the day of prey shifting, larvae were fed *Artemia* nauplii at 3 mL^{-1} after all rotifers had been flushed from tanks during routine water exchange in the morning. The abrupt change in diet type was designed to eliminate the possibility of larvae actively selecting a familiar prey (Cox and Pankhurst, 2000) over newly introduced *Artemia* nauplii. This strategy identified the time at which a majority of *M. atrodorsalis* larvae were capable of successfully capturing and ingesting *Artemia*

nauplii. No algae were added to culture vessels being fed *Artemia*. The experiment was terminated 15 DPH as beyond this point, *M. atrodorsalis* larvae are known to actively feed on *Artemia*.

4.3.3 Data analysis

All measurements are expressed as a mean ± standard error (SE). Body measurements for the feeding experiments were assessed to confirm normality (probability plots of residuals) and homogeneity of variance (Levene's test for homogeneity of variances), and then analysed with one-way ANOVA, using Statistica Version 8.0 (Statsoft^{TM}, Inc.). A multiple-sample survival analysis was employed to determine if significant differences existed among treatments. If a significant difference was found among survival data, two-sample survival analysis (Gehan's generalised Wilcoxon test) was used to determine specifically which treatments were different. A statistical probability of $p < 0.05$ was accepted as significant in all tests. It should be noted that during sampling of larvae, two fish specimens from the rotifer density experiment (one from the 5 rotifers mL^{-1}, and one from the 20 rotifers mL^{-1} treatment) were found moribund, characterised by severe emaciation. These fish were flagged *a priori* as suspicious and were not included in data analysis (Quinn and Keough, 2003).

4.4 RESULTS

4.4.1 Rotifer density experiment

Survival rate of larvae in the unfed control (0 rotifers mL^{-1}) decreased sharply during the first two days, and all were dead by 3 DPH (Fig. 4). Mean larval survival rate in all other treatments ranged between 62-74% at 7 DPH, with the 10 rotifers mL^{-1} treatment having the highest survival of 74 ± 12% (Fig. 4.1). No significant difference ($p > 0.05$) in survival

rate was detected among rotifer densities between 2 and 20 mL^{-1}, although the unfed control had significantly lower survival than all other treatments at 3DPH ($p < 0.001$). Similarly, no significant difference in standard length or body depth of larvae was found at the end of the experiment for rotifer densities between 2 and 20 mL^{-1} ($p > 0.05$; Table 4.1).

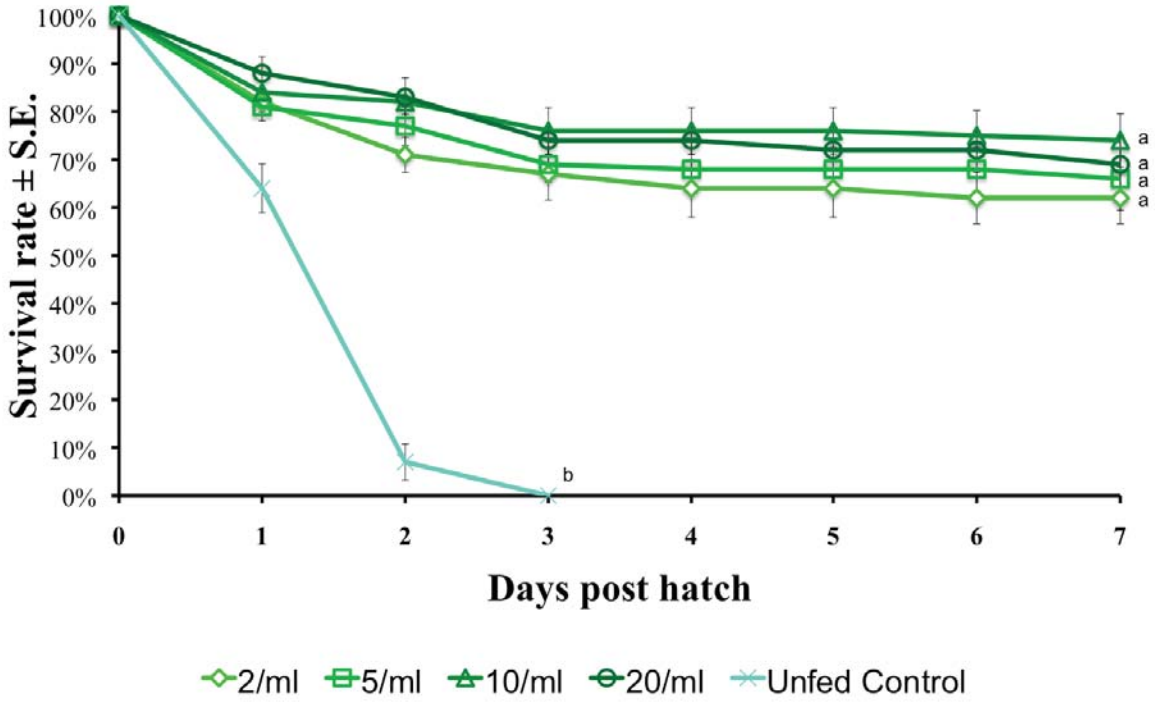

Figure 4.1: The effect of rotifer density on daily survival of newly hatched *M. atrodorsalis* larvae. Different letters denote a significant difference ($p < 0.05$).

Table 4.1: Standard length and body depth of *M. atrodorsalis* larvae measured at the end of the rotifer density and prey change experiments. No significant difference in standard length or body depth was found among treatments in both experiments.

Measurement ± S.E.	**Treatment**			
	Rotifer Density			
	2 mL^{-1}	5 mL^{-1}	10 mL^{-1}	20 mL^{-1}
Standard Length (mm)	4.49 ± 0.03	4.42 ± 0.03	4.51 ± 0.03	4.43 ± 0.02
Body Depth (mm)	0.95 ± 0.01	0.94 ± 0.01	0.94 ± 0.01	0.96 ± 0.01
	Day of prey change (Rotifers to *Artemia* nauplii)			
	3 DPH	6 DPH	9 DPH	12 DPH
Standard Length (mm)	5.06 ± 0.19	5.30 ± 0.09	5.28 ± 0.06	5.17 ± 0.06
Body Depth (mm)	1.12 ± 0.07	1.26 ± 0.04	1.26 ± 0.03	1.28 ± 0.03

4.4.2 Prey switch experiment

A negative response to an abrupt change in prey type was observed in all treatments, but appeared more pronounced on 3, 6 and 9 DPH (Fig. 4.2). At 15 DPH, the larvae subjected to a prey change on 3 DPH displayed the lowest survival rate of 4.8 ± 5.2%, which was significantly lower than all other treatments ($p < 0.001$). A prey change on 6 DPH also produced significantly poor survival (22 ± 6.1 %) compared to the treatments in which prey change took place on 9 and 12 DPH ($p < 0.001$). The later two treatments displayed the highest survival at 15 DPH (Fig. 4.2).

In contrast to survival, growth of the surviving larvae at the end of the trial, measured as standard length and body depth, was not significantly different ($p > 0.05$) among all treatments (Table 4.1).

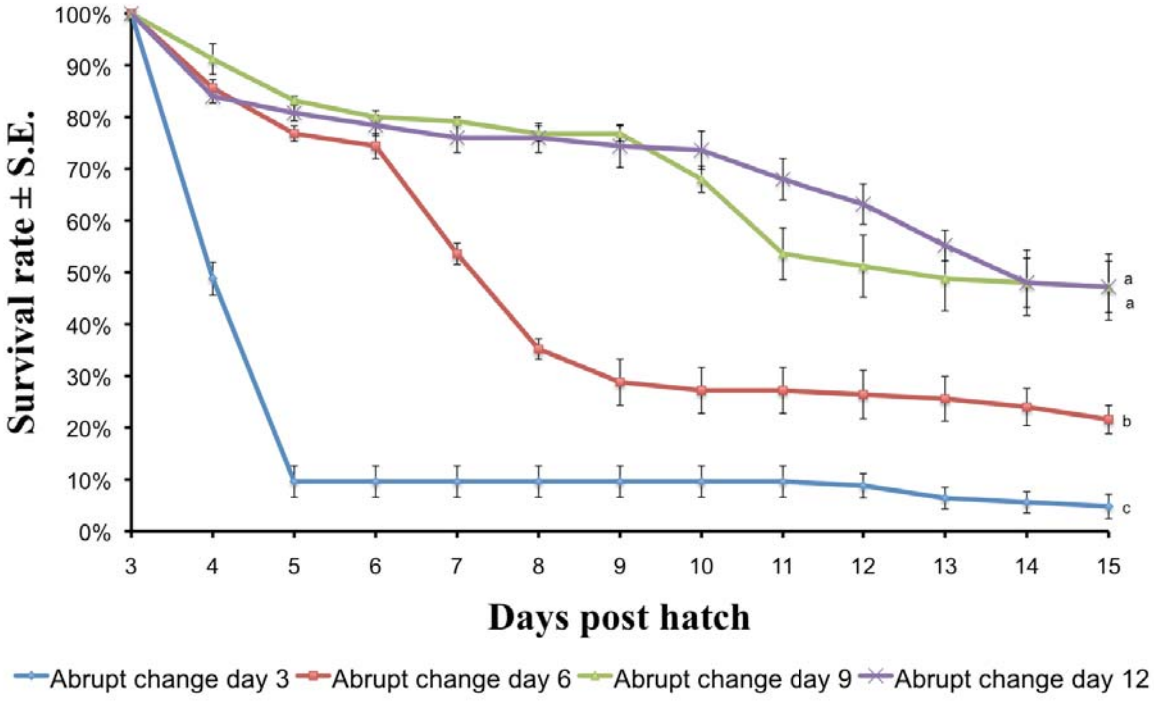

Figure 4.2: The effect of an abrupt prey change from rotifers to *Artemia* nauplii on daily survival of *M. atrodorsalis* larvae. Different letters denote a significant difference ($p < 0.05$).

4.5 DISCUSSION

The results of the experiments presented in this chapter make the first steps toward developing a reliable live feed protocol for larvae of *M. atrodorsalis* using the traditional live prey of rotifers and *Artemia*. While the outcomes of any prey density study must be treated with caution when replicating them at a commercial level, the first trial in this chapter demonstrated the flexibile and adaptable foraging behaviours in *M. atrodorsalis*, suggesting this species is highly robust and able to adapt to a broad range of prey density scenarios. The ability to tolerate a rotifer density as low as 2 mL^{-1} without any significant negative impact on survival or growth, suggests that the hunting and foraging capabilities of *M. atrodorsalis* are highly developed at first hatching; a characteristic that is desired by larviculturists and highly valuable to larger scale captive culture. However, while feeding *M. atrodorsalis* larvae at a rotifer density of 2 mL^{-1} is possible in a well mixed, closely monitored larval tank system, it is likely to be a risky practice in a commercial hatchery setting without extensive monitoring and precise control. Likewise a rotifer density as high

as 20 mL^{-1} may be adopted, however this density appears well beyond the requirements of *M. atrodorsalis* and unnecesarily risks poor water quality brought about by mortality and excreted metabolites, and competition for key resources, such as dissolved oxygen, from unutilised prey. The production of live prey also represents a key expense in hatchery culture making overfeeding an undesirable waste of hatchery resources. Therefore, a prey density of 10 mL^{-1} is recommended as a balanced approach for this species to avoid the potential negative impacts and risks of under-feeding, and the negative impact that over-feeding has on hatchery economics.

According to Yúfera and Darias (2007), survival of fish larvae can fall sharply when appropriate feeding and environmental conditions are not met. It is commonly accepted that in order to optimize feeding efficiency of larvae, an appropriate overlap in prey items is required by larvae when transitioning them to a sequentially larger prey item (Cox and Pankhurst, 2000). This allows the larvae to undergo a period of behavioral and sensory learning and to gain experience in capturing the new prey item (Godin, 1978; Cox and Pankhurst, 2000). Therefore, given the large size increment from the small strain rotifers (165 ± 20 μm lorica length by 107 ± 15 μm lorica width) to *Artemia* nauplii (499 ± 33 μm body length by 250 ± 42 μm body width) in the prey shifting trial, it is not surprising that a negative survival response was observed in all treatments. A more pronounced response was observed at younger larval stages, at 3 and 6 DPH, but reduced in severity at 9 and 12 DPH. This suggests that there was a limitation in the morphological ability, of larvae attempting to ingest the larger prey earlier on, regardless of their learning ability. Later-stage larvae were larger and thus were likely to be less limited by their size, however, given no prior experience with *Artemia* nauplii, it appeared they suffered some mortality in the days following exposure to the novel prey. In this case the ability to learn to identify and capture this novel prey was likely to be a critical factor for these older larvae (9-12 DPH). The results of the prey change experiment suggest that a majority of *M. atrodorsalis* larvae

were able to feed upon *Artemia* nauplii between 6-10 DPH, and therefore, this period is likely the ideal time to shift prey from rotifers to *Artemia*. However, according to Cox and Pankhurst (2000), capture competency and efficiency of larvae improves if the larvae have been exposed to the novel prey prior to a particular encounter. Therefore, it should be considered that a small percentage of *M. atrodorsalis* larvae in the second feeding trial were able to both undergo appropriate learning and were morphologically able to capture and ingest *Artemia* as early as 3 DPH. The second feeding trial also indicated that the SS rotifers from our laboratory were adequate to maintain good survival from hatching until 10 DPH. Beyond 10 DPH, low-level mortality was observed in the treatment still being fed rotifers only (prey shift implemented at 12 DPH), suggesting that SS rotifers had become an inadequate prey beyond 10 DPH.

Although the number of surviving larvae in the 3-DPH abrupt-prey-change treatment was low, it is interesting to note that no significant differences in growth were observed among treatments in both feeding trials, which was unexpected. This may suggest that early larvae of *M. atrodorsalis* are heavily geared for growth and that early switch to traditional *GSL* strains of *Artemia* has little advantage over extending the rotifer-feeding period.

In summary, the outcomes of this chapter demonstrated that early larvae of *M. atrodorsalis* are very efficient foragers and flexible candidates for larval culture, with a rotifer density as low as 2 mL^{-1} being sufficient to support good survival and growth, to at least 7 DPH. While a rotifer density ranging between 2 mL^{-1} and 20 mL^{-1} was appropriate for *M. atrodorsalis* larvae from first feeding until 7 DPH, to strike a good balance between providing adequate predator-prey encounters and minimizing excessive use of rotifers, a prey density of 10 rotifers mL^{-1} is recommended and will be adopted for subsequent experiments. Some larvae of *M. atrodorsalis* also displayed the ability to begin ingesting newly hatched GSL *Artemia* nauplii as early as 3 DPH, but did not appear to require the larger prey until 10 DPH or beyond. While this outcome wille be useful for developing a

reliable prey feeding protocol for this species, further work will be done in subsequent experiments to optimise prey transition timing and technique and confirm how long *M. atrodorsalis* tolerate a rotifer only diet before a prey change is required.

Chapter 5: Establishing larval feeding regimens for M. atrodorsalis II: effects of co-feeding and Artemia strain on time of prey change

5.1 ABSTRACT

This study aimed to establish feeding strategies covering the whole larval period of the forktail blenny, *Meiacanthus atrodorsalis*, based on the standard hatchery feeds of rotifers and *Artemia*. Three purposely designed experiments were conducted to determine the appropriate times and techniques to transition larvae from rotifers to *Artemia* nauplii of a Great Salt Lake (GSL) strain, and a specialty AF strain, as well as subsequent transition to enriched metanauplii of GSL *Artemia*. With a 3-day co-feeding period, larvae adapted well to a transition from rotifers to newly hatched GSL *Artemia* nauplii as early as 5 days-post-hatch (DPH), and as early as 3 DPH when fed the smaller AF *Artemia* nauplii. However, prolonging the rotifer feeding period up to 11 DPH did not negatively affect survival. Larvae fed *Artemia* nauplii of the AF strain showed 17-21% higher survival, 24-33 % greater standard length and body depth, and 91-200% greater dry weight, after 20 days relative to those fed nauplii of the GSL strain. Meanwhile, enriched *Artemia* metanauplii of the GSL strain were shown to be an acceptable alternative to AF *Artemia* nauplii for later larvae, producing similar survival and growth when introduced from 8 DPH.

Based on the results of these experiments *M. atrodorsalis* larvae should be fed rotifers between 0-2 DPH, introducing AF *Artemia* nauplii from 3 DPH, followed by enriched GSL *Artemia* metanauplii from 8 DPH onward, with a 3 day co-feeding period between each prey change.

5.2 INTRODUCTION

Improving culture protocols for popular marine ornamental fish is a key step in building sustainability into the marine aquarium industry, and lies largely in overcoming challenges in larviculture, in particular larval feeds and feeding (Ostrowski and Laidley, 2001; Olivotto, et al., 2011).

Multiple factors can significantly affect the successful use of rotifers and *Artemia* in marine fish larviculture. Among them are species and/or strain choice, which is closely associated with the size and nutritional value of the prey, the feeding history of the live prey, and the timing and method of prey transitions, all of which influence larval survival and growth (Fukusho and Iwamoto, 1981; Watanabe, et al., 1983; Watanabe and Kiron, 1994; Sorgeloos, et al., 2001).

Rotifers are available in multiple morphotypes, based on average adult size, that match the mouth-gape-size of many first feeding fish larvae (Hagiwara, et al., 2007). They exhibit a narrow size range in their lifecycle, swim in a slow whorling pattern, and can be nutritionally improved at all stages via enrichment (Hagiwara, et al., 2007). *Artemia* are not only different taxonomically, but also exhibit significant differences in body shape, swimming pattern and speed, and more substantial and rapid size and mobility changes over their life cycle, relative to rotifers. The two most commonly utilized stages of *Artemia* in marine hatcheries; newly hatched Instar I nauplii and Instar II metanuaplii, also differ substantially from one-another in size and swimming strength (Samocha, et al., 1989). Therefore, timing and method of transition from rotifers to *Artemia*, and from one *Artemia* stage to another, are important points-of-control in the feeding regime of marine fish larvae, and must match the morphological, physiological and foraging competency of fish larvae to allow them to cope with changes in prey type (Samocha, et al., 1989; Sorgeloos, et al., 2001).

At the same time the nutritional value of *Artemia* offered to fish larvae can have a significant affect on larval growth and survival. The nutritional profile of *Artemia* can vary based on species, strain, source of cysts, the development stage of the *Artemia* itself, and the diet offered to enrich the feeding stages (Sorgeloos, et al., 2001). Instar I *Artemia* nauplii are lecithotrophic and so their nutritional quality is directly related to their endogenous reserves, and cannot be artificially improved (Sorgeloos, et al., 2001). The extent of control over the nutritional value of Instar I nauplii is hence limited to the choice of species, the strain type, and source of cysts, with levels and ratios of highly unsaturated fatty acids (HUFAs) considered key quality criteria (Watanabe, et al., 1983; Sorgeloos, et al., 2001). However, the nutritional value of heterotrophic instar II *Artemia* metanauplii can be manipulated and controlled via enrichment, using freeze-dried powders or emulsions, so improvements to the nutritional quality of inferior strains of *Artemia* can reduce the impact of variable strain and batch quality (Arulvasu and Munuswamy, 2009).

Ultimately, the establishment of appropriate and detailed feeding regimes through properly designed experiments that take into consideration major influencing factors, is fundamental to successful marine larviculture (Watanabe, et al., 1983; Samocha, et al., 1989; Holt, et al., 2007). However, such an approach is generally lacking for numerous marine ornamental species, even for species belonging to the family Blenniidae, a taxonomic group considered relatively better studied (Chapter 1). Species within this genus accept rotifers at hatching (Olivotto, et al., 2010a)(Chapter 1 & 3), and demonstrate the capacity to transition from rotifers to newly hatched GSL *Artemia* nauplii between 6-10 DPH. However, significant gaps still exist in optimizing the time of introduction and use of *Artemia* as prey in larval culture.

The overall objective of the research presented in this chapter was to establish a complete feeding regime for *Meiacanthus atrodorsalis* until settlement using standard hatchery live prey. To achieve this, three experiments were designed to comprehensively investigate the

use of *Artemia* in the larval feeding regime of the forktail blenny, *Meiacanthus atrodorsalis*; a model species for the *Meiacanthus* genus. It was firstly sought to quantify the effect of a co-feeding transition period on the change of the larval diet from rotifers to Instar I *Artemia* nauplii, and the effect of *Artemia* nauplii of different strains on larval survival and growth (Fig. 5.1). To complete the experiment series and establish a reliable live prey feeding regime to weaning ages, the effect of timing the introduction of larger Instar II *Artemia* metanauplii to the larval feeding regime of *M. atrodorsalis* was also tested (Fig 5.1).

5.3 MATERIALS AND METHODS

5.3.1 Broodstock husbandry

Broodstock source and husbandry were described in Chapter 3, Section 3.3.1. However, for this and subsequent chapters the broodstock were separated into three groups consisting of one male and three females. Hides found with deposited eggs were allowed to remain in the broodstock tank until a few hours before hatching when they were moved indoors and incubated artificially until hatching following protocols described in Chapter 4, Section 4.3.3.1.

5.3.2 Live prey production

SS-type rotifer *Brachionus rotundiformis,* were batch cultured in 100 L tanks at 25-29 °C, and were fed concentrated microalgae paste, *Nannochloropsis* sp. (Reed Mariculture, California, USA). Rotifers were fed twice daily to the larvae at a density of 10 ± 2 rotifers mL^{-1}. *Nannochloropsis* paste was also added and maintained at a density of ~200,000 cells mL^{-1} in the larval rearing tanks while rotifers were fed.

Two strains of *Artemia franciscana*, a commonly available Great Salt Lake (GSL) strain and a Vietnamese specialty 'AF' strain, both sourced from INVE technologies, Belgium, were used. These two strains are hereafter referred to as GSL and AF *Artemia*, respectively. Compared to the GSL strain, the AF strain has smaller sized nauplii as well as containing higher levels of n-3 highly unsaturated fatty acid (HUFA). The mean length of newly hatched nauplii of GSL and AF strain used in our experiments was 499 ± 5 µm and 434 ± 4 µm respectively. According to the manufacturers specifications, the concentration of n-3 HUFA was tenfold higher in the AF *Artemia* (10 mg/g) relative to the GSL *Artemia* (1 mg/g dry weight) used in our experiments. *Artemia* were fed to larvae twice daily at a density of 3 ± 0.5 individuals mL^{-1}. In larval tanks the density of rotifers, algae and *Artemia* were maintained by performing twice-daily density checks at 9 am and 5 pm respectively with adjustments made as needed.

To improve the poor HUFA content of the GSL *Artemia* strain compared to the AF strain, metanauplii were enriched with a commercially available enrichment powder, Algamac 3050 (Aquafauna Bio-Marine, Inc.). Following the manufacturers directions, GSL metanauplii were placed into 20 L of heavily aerated seawater at a density of 15-20 nauplii mL^{-1} and enriched with 0.2 g L^{-1} of blended Algamac 3050 for 12 h. Enriched metanauplii were then harvested and rinsed for 5 min using freshwater before being fed to the larvae. The average size of the enriched metanauplii used in the experiments was 709 ± 8 µm at the time of feeding.

5.3.3 Larval feeding experiments

5.3.3.1 Communal larval rearing and general experimental procedures

For each larval feeding experiment, newly hatched *M. atrodorsalis* larvae, hatched on the same day, were firstly reared communally under the same feeding protocol before being

transferred to multiple 3-L replicate culture vessels to start different treatments. The tanks used for communal rearing were 100 L fiberglass cylindrico-conical tanks described in Chapter 2, lit overhead by fluorescent ceiling lights. The tanks were connected to a recirculating system with 10-μm filtration, a biological filter with foam fractionation and UV sterilization. The in-flow of filtered water from the recirculating system to the larval tank was maintained at approximately 4.2 L h^{-1}, giving the tank a complete water exchange every 24 h. Temperature in the communal tank was maintained between 27 to 29.5 °C, salinity 35 to 37 gL^{-1}, pH 8.0 to 8.2, NH_3, NO_2 < 0.02 mg L^{-1}, and NO_3 < 5 mg L^{-1}.

To start different treatments in an experiment, based on respective experimental design (Fig. 5.1), actively swimming larvae on a designated day of development, defined as days-post-hatching (DPH), were transferred (5 larvae at a time) from a communal rearing system to the 3-L replicate culture vessels. The vessels were placed in a temperature-controlled water-bath connected to a recirculating system with 10 μm filtration, a biological trickle filter, foam fractionation and UV sterilization. Each replicate vessel received water exchange from the water bath recirculating system twice daily (8 am and 4 pm) by directing gentle flow (250 mL min^{-1}) into each replicate for 40-60 min to flush out uneaten prey and detritus before new live prey were added. For all experiments, temperature in the water-bath system was maintained between 27 to 30 °C, salinity 33 to 36.5 gL^{-1}, pH 8.0 to 8.2, NH_3 and NO_2 < 0.02, and NO_3 < 5 mg L^{-1}, respectively.

In all experiments, larval survival and growth in terms of standard length, body depth and dry weight, were measured and compared. Each morning, the number of surviving larvae in each replicate were counted *in-situ*, while on the final day of the experiment, all larvae were anesthetized (AQUIS® at 0.08 mL L^{-1}) and photographed under a dissecting microscope for standard length and body depth measurements. Larvae were then dried individually at 60 °C for 48 h before being weighed to the nearest 0.1 mg (Adam equipment AAA 250LE) to determine dry weight.

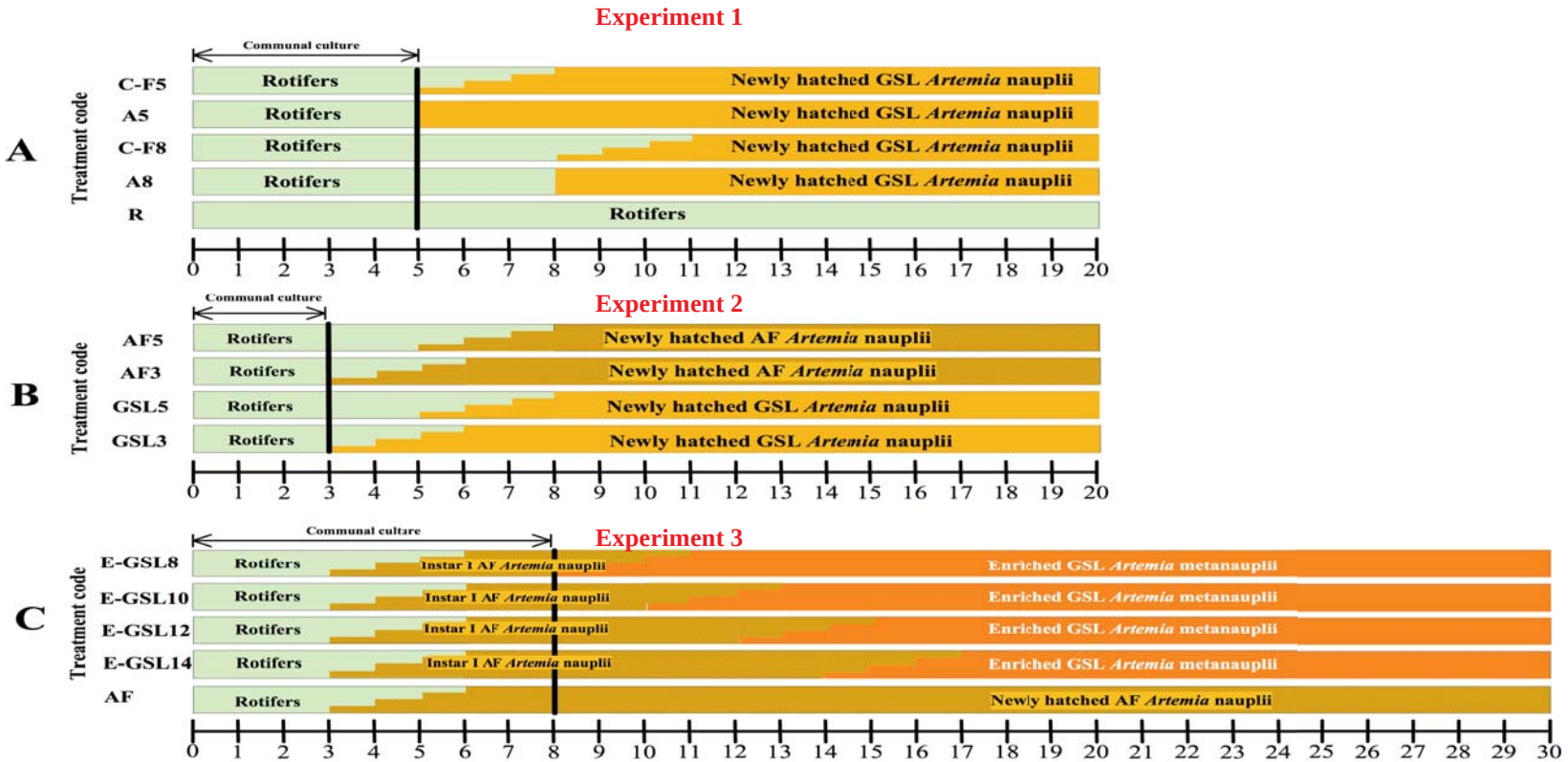

Figure 5.1: Experiment designs showing feeding regimes of experiment 1, 2, and 3. Black vertical lines represent the time (days-post-hatching – DPH) larvae where transferred from the communal culture tank to replicate experimental vessels to start each experiment. (**A)** Experiment 1; C-F5: larvae fed newly hatched GSL *Artemia* nauplii starting 5 DPH with a 3 day rotifer co-feeding period; A5: the larval diet was abruptly changed to *Artemia* nauplii on 5 DPH without a rotifer co-feeding period; C-F8: larvae fed *Artemia* nauplii starting 8 DPH with a 3 day rotifer co-feeding period; A8: larvae diet was abruptly changed to *Artemia* nauplii on 8 DPH without a rotifer co-feeding period; R: larvae fed rotifers alone throughout. **(B)** Experiment 2; AF5: larvae fed newly hatched AF *Artemia* nauplii starting 5 DPH with a 3 day rotifer co-feeding period; AF3: larvae fed newly hatched AF *Artemia* nauplii starting 3 DPH with a 3 day rotifer co-feeding period; GSL5: larvae fed newly hatched GSL *Artemia* nauplii starting 5 DPH with a 3 day rotifer co-feeding period; GSL3: larvae fed newly hatched GSL *Artemia* nauplii starting 3 DPH with a 3 day rotifer co-feeding period. **(C)** Experiment 3; E-GSL8; larvae fed newly hatched AF *Artemia* nauplii starting 3 DPH with a 3 day rotifer co-feeding period, then fed enriched GSL *Artemia* metanauplii starting 8 DPH with a 3 day AF *Artemia* nauplii co-feeding period; E-GSL10: larvae fed newly hatched AF *Artemia* nauplii starting 3 DPH with a 3 day rotifer co-feeding period, then fed enriched GSL *Artemia* metanauplii starting 10 DPH with a 3 day AF *Artemia* nauplii co-feeding period; E-GSL12: larvae fed newly hatched AF *Artemia* nauplii starting 3 DPH with a 3 day rotifer co-feeding period, then fed enriched GSL *Artemia* metanauplii starting 12 DPH with a 3 day AF *Artemia* nauplii co-feeding period; E-GSL14: larvae fed newly hatched AF *Artemia* nauplii starting 3 DPH with a 3 day rotifer co-feeding period, then fed enriched GSL *Artemia* metanauplii starting 14 DPH with a 3 day AF *Artemia* nauplii co-feeding period; AF: larvae fed newly hatched AF *Artemia* nauplii throughout, after a 3 day rotifer co-feeding period starting 3 DPH.

5.3.3.2 Experiment 1: Determining the time for introducing Artemia nauplii and effects of a rotifer co-feeding period

This experiment was conducted to determine the best time to introduce newly hatched GSL *Artemia* nauplii as well as the effects of a rotifer co-feeding period on survival and development of larvae of *M. atrodorsalis*. All larvae for this experiment were reared communally and fed 10 rotifers mL^{-1} between 0-4 DPH inclusive (Section 5.3.3.1), before being transferred to replicate vessels on the morning of 5 DPH (Fig. 5.1A). The change from rotifers to newly hatched GSL *Artemia* nauplii was tested on larvae at 5 and 8 DPH, respectively, with an abrupt switch of diet from 10 rotifers mL^{-1} to 3 *Artemia* nauplii mL^{-1} or with a 3 day co-feeding transition period (Fig. 5.1A). During the 3 day co-feeding period, the initial density of rotifers (10 rotifers mL^{-1}) was decreased at a rate of 2.5 rotifers mL^{-1} day^{-1} with concurrent increase of *Artemia* nauplii density by 0.75 nauplii mL^{-1} day^{-1}, reaching a final density of 3 *Artemia* nauplii mL^{-1} on day 4. In both abrupt change and co-feeding treatments, the larvae were then offered *Artemia* nauplii at 3 nauplii mL^{-1} until 20 DPH. A fifth treatment in which larvae were fed rotifers only was used as a negative control (Fig. 5.1A). There were four replicates for each treatment, and each replicate tank stocked with 15 larvae each.

5.3.3.3 Experiment 2: Comparing effects of feeding larvae on GSL and AF Artemia nauplii

This experiment compared the use of newly hatched nauplii of two *Artemia* strains, GSL and AF, on survival and growth of *M. atrodorsalis*. In particular, it was sought to investigate whether the small size and high quality AF strain might enable an earlier feeding transition from rotifers to newly hatched *Artemia* nauplii. All larvae used in this experiment were reared communally and fed 10 rotifers mL^{-1} between 0-2 DPH inclusive

before being transferred to replicate vessels on the morning of 3 DPH (Fig. 5.1B). Each treatment was replicated 4 times, and each replicate tank was stocked with 20 larvae. To enable a comparison with experiment 1, an identical treatment of co-feeding rotifers between 5-7 DPH for GSL *Artemia* nauplii was adopted and the experiment was run until 20 DPH (Fig. 5.1A and 5.1B).

5.3.3.4 Experiment 3: Determining the time to introduce enriched GSL Artemia metanauplii

The aim of this experiment was to determine the best time to introduce enriched Instar II *Artemia* metanauplii to *M. atrodorsalis* larvae. In this experiment, *M. atrodorsalis* larvae were reared communally for the first 7 DPH before being randomly allocated to 3 L replicate vessels on 8 DPH. During the communal rearing phase, larvae were fed 10 rotifers mL^{-1} between 0-2 DPH inclusive before a co-feeding transition period with newly hatched AF *Artemia* nauplii between 3-5 DPH inclusive, and then fed AF nauplii at 3 nauplii mL^{-1} solely between 6-7 DPH inclusive; note this feeding regime achieved the best larval survival in experiment 2 (Fig. 5.1C). Enriched GSL *Artemia* metanauplii were then introduced to the larvae at 4 different times on 8, 10, 12 and 14 DPH with a 3 day co-feeding period using newly hatched AF *Artemia* nauplii. To act as a benchmark, a treatment with larvae fed AF nauplii throughout was also setup (Fig. 5.1C). All treatments consisted of 4 replicates stocked with 20 larvae. Experiment 3 was run until 30 DPH to encompass the larval settling period (20-30 DPH) during which larvae adopt a benthic habit and begin to accept inert diets.

5.3.4 Data analysis

All measurements are expressed as a mean ± standard error (SE). Data were assessed and found to conform to normality (probability plots of residuals) and homogeneity of variance

(Levene's test for homogeneity of variances) assumptions. A two-way analysis of variance (ANOVA) was used to test the interaction of introduction time (fixed) and feeding strategy (fixed) in experiment 1, and of introduction time (fixed) and food type (*Artemia* strain used; fixed) in experiment 2. Main effects were identified for each factor, and if a significant interaction was found multiple comparisons were performed with a Bonferroni adjustment to determine which factor combinations were significant from each other. Plots of marginal means for each factor were constructed as a visual aid to describe the interaction.

Data from experiment 3 was analyzed with one-way ANOVA, where feeding regime was considered to be a single fixed factor. A statistical probability of $p < 0.05$ was accepted as significant. If a significant difference was found, a post-hoc Tukey's analysis was performed to determine which treatments were different. All statistical analyses were performed with IBM SPSS statistics version 23.

5.4 RESULTS

5.4.1 Experiment 1: Determining the time for introducing Artemia nauplii and effects of a rotifer co-feeding period

Both the timing of prey transition from rotifers to *Artemia* nauplii and a rotifer co-feeding period had a significant effect on the survival of *M. atrodorsalis* larvae ($p = 0.003$). While there was no significant interaction between the effects of timing and feeding strategy on survival ($p = 0.082$), the use of a co-feeding period was the main factor ($p = 0.001$) that determined how larvae responded at each introduction time (5 or 8 DPH). The highest survival occurred in treatments where larvae were co-fed rotifers with newly hatched GSL *Artemia* nauplii between 5-7 DPH and 8-10 DPH inclusive, with an average survival of 45 ± 4% and 45 ± 7% respectively at the end of experiment on 20 DPH (Fig. 5.2). The treatment fed rotifers alone had comparable daily survival to other treatments until 11 DPH

(except the treatment with an abrupt diet change on 5 DPH) before a sharp decrease in survival after 11 DPH resulting in total mortality by 19 DPH (Fig. 5.2). Larvae that were switched diet abruptly on 5 DPH sustained mass mortality of 84 ± 2% between 5 to 9 DPH, resulting in a significantly ($p < 0.05$) lower final survival (5 ± 3%) than all other *Artemia* feeding treatments (Fig. 5.2). Larvae that were abruptly switched to newly hatched *Artemia* nauplii on 8 DPH fared better, but still responded with a lower survival (30 ± 9%) compared to treatments with a rotifer co-feeding period, although the differences were not significant ($p > 0.05$)(Fig. 5.2).

The average standard length, body depth and dry weight of the surviving larvae at the end of experiment 1 did not differ significantly between *Artemia* feeding treatments ($p = 0.303$, $p = 0.401$, $p = 0.154$ respectively). No main effects or interactions between timing and feeding strategy were found for any growth parameter. Average standard length ranged from 5.59 ± 0.08 to 5.85 ± 0.11 mm, body depth 1.63 ± 0.03 to 1.70 ± 0.07 mm and dry weight 1.32 ± 0.2 to 1.82 ± 0.1 mg in all treatments (Table 5.1).

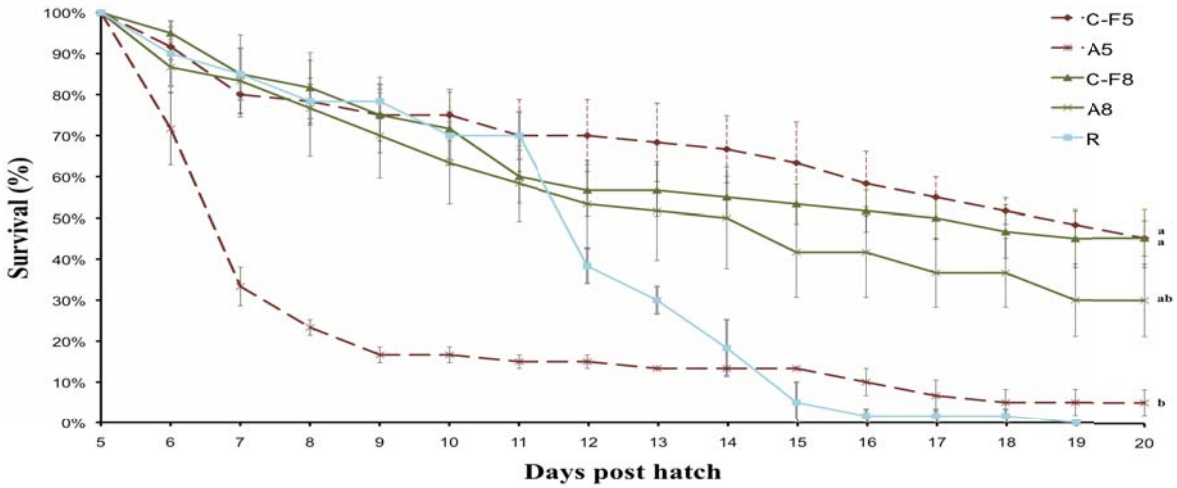

Figure 5.2: Daily survival of *M. atrodorsalis* larvae from experiment 1, showing the effect of timing of introducing newly hatched *Artemia* nauplii and with or without a rotifer co-feeding period. C-F5(—•—): larvae fed *Artemia* nauplii starting 5 DPH with a 3 day rotifer co-feeding period; A5 (—*—): larval diet abruptly changed to *Artemia* nauplii on 5 DPH without a rotifer co-feeding period; C-F8(—▲—): larvae fed *Artemia* nauplii starting 8 DPH with a 3 day rotifer co-feeding period; A8(—×—): larval diet abruptly changed to *Artemia* nauplii on 8 DPH without a rotifer co-feeding period; R(—■—): larvae fed rotifers alone throughout. Different letters denote significant differences ($p < 0.05$). Refer to Fig. 1A for treatment descriptions.

Table 5.1:Growth parameters of surviving *M. atrodorsalis* larvae on 20 DPH from experiment 1. No significant difference was found between any of the treatments ($p > 0.05$)

Growth Parameters	**Treatment**			
	C-F5	A5	C-F8	A8
Standard Length (mm)	5.8 ± 0.1	5.9 ± 0.1	5.6 ± 0.1	5.6 ± 0.1
Body Depth (mm)	1.7 ± 0.0	1.7 ± 0.1	1.6 ± 0.0	1.6 ± 0.0
Dry Weight (mg)	1.8 ± 0.1	1.3 ± 0.2	1.7 ± 0.1	1.6 ± 0.1

For treatment descriptions please refer to Fig. 5.1A

5.4.2 Experiment 2: Comparing the effects of feeding larvae on GSL and AF Artemia nauplii

Both the timing of co-feeding newly hatched *Artemia* nauplii and *Artemia* strain had a significant effect on the survival of *M. atrodorsalis* larvae ($p = 0.002$). While there was no significant interaction between the effects of introduction time and *Artemia* strain on survival ($p = 0.323$), food type was the main factor that drove the survival response in larvae ($p < 0.001$), with the use of AF *Artemia* nauplii improving larval survival. The

highest survival on 20 DPH occurred in the treatments fed newly hatched AF *Artemia* nauplii introduced on 3 (41 ± 7%) and 5 DPH (40 ± 4%) respectively, both with a 3 day rotifer co-feeding period (Fig. 5.3). In contrast, the lowest survival (10 ± 5%) occurred in the treatment fed newly hatched GSL *Artemia* nauplii introduced on 3 DPH with a 3 day rotifer co-feeding period, which was significantly lower than all treatments where AF nauplii were used ($p < 0.05$; Fig. 5.3). However, this treatment was not significantly different to the treatment fed newly hatched GSL *Artemia* nauplii introduced on 5 DPH, also with a 3 day co-feeding period. The latter treatment had a survival of 23 ± 6% and was not significantly different to any other treatments (Fig. 5.3).

Significant differences were detected in growth parameters of surviving larvae from different treatments ($p < 0.001$), which largely related to the *Artemia* strain used rather than the time that newly hatched *Artemia* nauplii of either strain were introduced (Table 5.2). While no significant interaction ($p = 0.180$ for standard length and $p = 0.434$ for body depth) was found for standard length and body depth parameters between introduction time and food type, food type was the main effect ($p < 0.001$) that drove a significantly better response in larvae. For instance, larvae fed AF *Artemia* nauplii from both 3 DPH and 5 DPH had a 24-29 % longer standard length, and 28-33 % larger body depth as compared to those larvae fed newly hatched GSL nauplii (Fig. 5.4 & Table 5.2). A significant interaction was found between introduction time and food type for dry weight ($F(1,114) = 7.616$, $p = 0.007$). Again, food type was the main effect ($p < 0.001$) that drove this interaction. Specifically, feeding AF *Artemia* nauplii earlier resulted in significantly heavier ($p < 0.001$) larvae at the end of experiment 2 (Table 5.2). Overall larvae fed AF *Artemia* nauplii from both 3 DPH and 5 DPH had a 91-200% greater dry weight than those larvae fed newly hatched GSL nauplii (Table 5.2). The highest dry weight (2.67 ± 0.15 mg) was found in larvae fed newly hatched AF nauplii on 3 DPH with a 3 day rotifer co-feeding period, followed by larvae fed AF nauplii on 5 DPH with the same co-feeding period length

(2.1 ± 0.12 mg), which was significantly lower (Table 5.2). Larvae fed newly hatched GSL nauplii on 3 DPH (0.89 ± 0.19 mg) and 5 DPH (1.10 ± 0.11 mg) with the same 3 day rotifer co-feeding period had significantly lower dry weights than the treatments fed AF nauplii, but no significant difference was detected between themselves (Table 5.2).

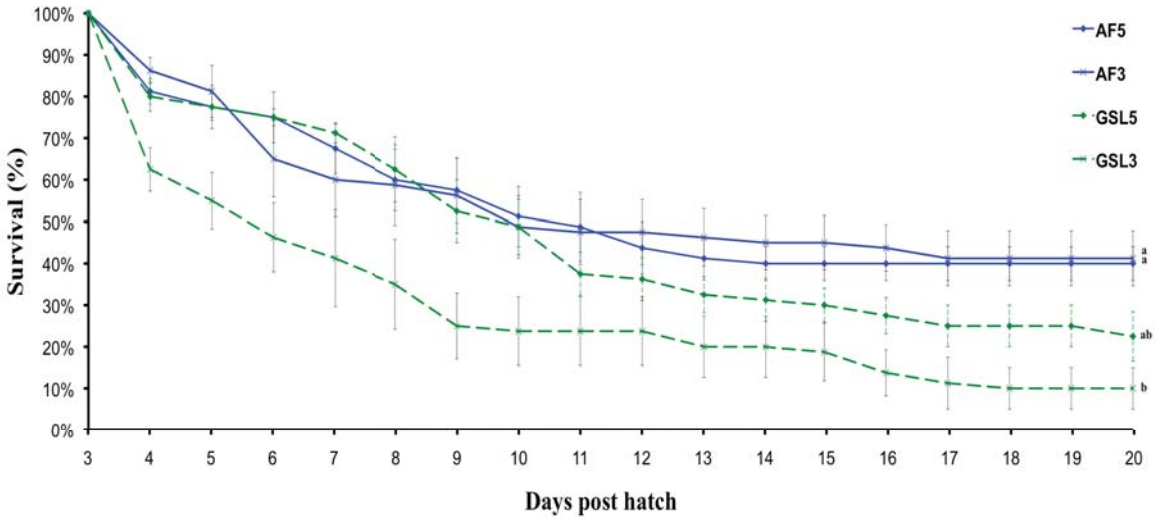

Figure 5.3: Daily survival of *M. atrodorsalis* larvae from experiment 2, showing the effect of feeding larvae on GSL or AF *Artemia* nauplii. AF5 (—●—): larvae fed newly hatched AF *Artemia* nauplii starting 5 DPH with a 3 day rotifer co-feeding period; AF3 (—✕—): larvae fed newly hatched AF *Artemia* nauplii starting 3 DPH with a 3 day rotifer co-feeding period; GSL5 (—●···): larvae fed newly hatched GSL *Artemia* nauplii starting 5 DPH with a 3 day rotifer co-feeding period; GSL3 (—✕···): larvae fed newly hatched AF *Artemia* nauplii starting 3 DPH with a 3 day rotifer co-feeding period. Different letters denote a significant difference ($p < 0.05$). Refer to Fig. 1B for treatment descriptions.

Table 5.2: Growth parameters of surviving *M. atrodorsalis* larvae on 20 DPH from experiment 2. Values in the same row with different superscripts are significantly different ($p < 0.05$).

Measurement ± S.E.	**Treatment**			
	AF5-7	AF3-5	GSL5-7	GSL3-5
Standard Length (mm)	7.5 ± 0.2^{a}	7.3 ± 0.1^{a}	5.8 ± 0.1^{b}	5.9 ± 0.2^{b}
Body Depth (mm)	2.4 ± 0.1^{a}	2.3 ± 0.1^{a}	1.8 ± 0.0^{b}	1.8 ± 0.1^{b}
Dry Weight (mg)	2.1 ± 0.1^{a}	2.7 ± 0.2^{b}	1.1 ± 0.1^{c}	0.9 ± 0.2^{c}

For treatment descriptions refer to Fig. 1B

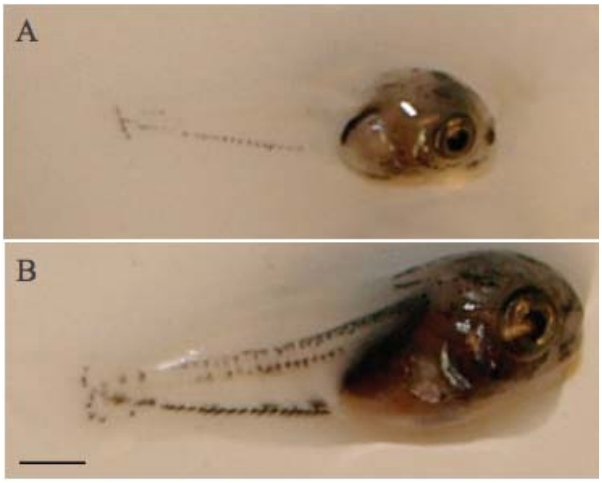

Figure 5.4: Larvae sampled at the end of experiment 2 (20 DPH) demonstrating the typical difference in size of larvae fed GSL *Artemia* (**A**) vs AF *Artemia* (**B**). Scale bar = 1mm

5.4.3 Experiment 3: Determining the time to introduce enriched GSL Artemia metanauplii

The times at which enriched GSL *Artemia* metanauplii were introduced had no significant effect on the survival of *M. atrodorsalis* larvae (p = 0.515). Larval survival on 30 DPH ranged from 58 ± 10%, for the treatment introduced to enriched GSL *Artemia* metanauplii on 10 DPH, to 76 ± 3%, for the treatment introduced to enriched GSL *Artemia* metanauplii on 8 DPH respectively (Fig. 5.5)

Likewise the times at which enriched metanauplii were introduced did not significantly effect any growth parameter of surviving larvae (p = 0.129 for standard length, p = 0.199 for body depth, and p = 0.154 for dry weight). Overall standard length ranged from 9.10 ± 0.23 to 9.73 ± 0.26 mm, body depth from 2.84 ± 0.23 to 3.03 ± 0.07 mm, and dry weight from 4.88 ± 0.37 to 6.21 ± 0.45 mg respectively (Table 5.3).

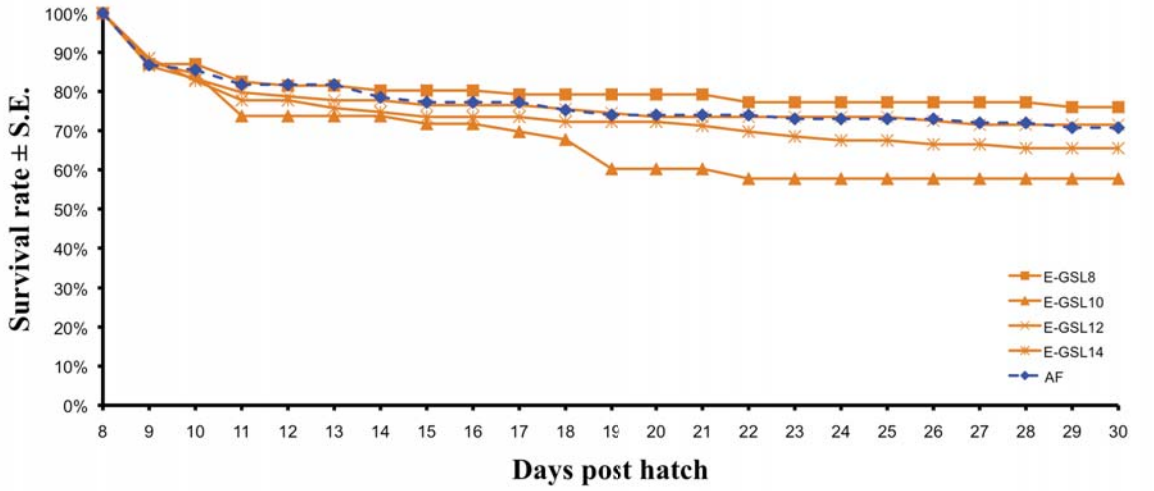

Figure 5.5: Daily survival of *M. atrodorsalis* larvae from experiment 3, showing the effect of timing of feeding larvae on enriched GSL *Artemia* metanauplii compared to feeding AF *Artemia* nauplii only. E-GSL8 (■): larvae fed enriched GSL *Artemia* metanauplii starting 8 DPH with a 3 day AF *Artemia* nauplii co-feeding period; E-GSL10 (▲): larvae fed enriched GSL *Artemia* metanauplii starting 10 DPH with a 3 day AF *Artemia* nauplii co-feeding period; E-GSL12 (✕): larvae fed enriched GSL *Artemia* metanauplii starting 12 DPH with a 3 day AF *Artemia* nauplii co-feeding period; E-GSL14 (✳): larvae fed enriched GSL *Artemia* metanauplii starting 14 DPH with a 3 day AF *Artemia* nauplii co-feeding period; AF (◆): larvae fed newly hatched AF *Artemia* nauplii throughout. Error bars have been omitted for clarity. No significant difference in survival on the final day was found among treatments ($p > 0.05$). Refer to Fig. 1C for treatment descriptions.

Table 5.3: Growth parameters of surviving *M. atrodorsalis* larvae on 30 DPH from experiment 3. No significant difference was found between any of the treatments ($p > 0.05$).

Measurement ± S.E.	**Treatment**				
	E-GSL8	E-GSL10	E-GSL12	E-GSL14	AF
Standard Length (mm)	9.4 ± 0.2	9.1 ± 0.3	9.7 ± 0.3	9.2 ± 0.3	9.7 ± 0.2
Body Depth (mm)	3.0 ± 0.1	2.9 ± 0.1	3.0 ± 0.1	2.8 ± 0.2	3.0 ± 0.2
Dry Weight (mg)	5.8 ± 0.4	5.3 ± 0.4	6.2 ± 0.5	4.9 ± 0.4	5.6 ± 0.3

For treatment descriptions refer to Fig. 1C

5.5 DISCUSSION

The experiments in this study, together with the results of Chapter 3 and 4, collectively establish a successful and complete larval feeding regime for *M. atrodorsalis* based on traditional hatchery prey of rotifers and *Artemia*. Our findings show the importance of introducing newly hatched *Artemia* nauplii to the diet of *M. atrodorsalis* larvae prior to 11

DPH and the benefits of a co-feeding period with rotifers when *Artemia* nauplii were introduced for the first time. The use of smaller AF *Artemia* substantially reduced the time at which newly hatched nauplii could be fed to the larvae, and substantially improved survival and growth of *M. atrodorsalis* larvae. It was also demonstrated that with a co-feeding period, larger enriched GSL *Artemia* metanauplii could be introduced to the feeding regime as early as 8 DPH without affecting larval survival or growth.

The high cost of *Artemia* cysts is often the principle concern determining the extent to which *Artemia* is used in a marine hatchery. In some instances, rotifer cultures are routinely maintained and readily available in a hatchery, making prolonged rotifer use more favorable in order to reduce *Artemia* use (Sorgeloos, et al., 2001; Callan, et al., 2003; Conceição, et al., 2010). In such a scenario, this study demonstrates that a rotifer-feeding period can be extended up to 11 DPH in culturing *M. atrodorsalis* larvae without adversely affecting survival. On the other hand, the production of rotifers specifically for feeding ornamental fish larvae could constitute a significant additional cost owing to infrastructure, space requirements and expenses associated with feeding rotifers and skilled labour. This may prompt the early elimination of rotifers in favour of *Artemia*, which offer off-the-shelf convenience (Samocha, et al., 1989; Ruscoe, et al., 2004; Nhu, et al., 2009). In the latter scenario, our study shows that newly hatched commonly available GSL *Artemia* nauplii could be introduced to the feeding regime as early as 5 DPH, or newly hatched AF *Artemia* nauplii as early as 3 DPH, to minimize rotifer use. The practical implications of these findings are important at a commercial hatchery level as they identify flexibility in the feeding regime of *M. atrodorsalis* larvae using traditional prey of rotifers and *Artemia*. Therefore reliable production of this species is robust to changes in supply and availability of rotifers and *Artemia* experienced in a hatchery.

When introducing *Artemia* nauplii to the diet of larval *M. atrodorsalis* for the first time, co-feeding with rotifers for a short period facilitated an earlier and smoother transition (as

early as 5 DPH for the common GSL strain and 3 DPH for the smaller AF strain). An abrupt switch simplified the feeding regime, and while it had no significant effect on growth parameters, it did have an obvious age-dependent effect on larval survival (Chapter 3). In this study, the greater mortality observed in larvae under 8 DPH appeared to be linked to their inability to ingest *Artemia* nauplii as larger prey. This was supported by the results of the subsequent experiment (experiment 2), which demonstrated that the smaller AF *Artemia* nauplii could be fed to *M. atrodorsalis* larvae earlier than the GSL strain. The ability of young fish larvae to competently handle and ingest *Artemia* nauplii has also been reported to relate strongly to prior exposure to this prey (Cox and Pankhurst, 2000). It is well documented that live prey identification and capture success varies with the age and previous experience of fish larvae, and can be improved through learning (Godin, 1978; Reiriz, et al., 1998; Brown and Laland, 2001). In a hatchery setting, co-feeding has been identified as a useful method for facilitating learning and thus improving feeding success and efficiency on both live and inert diets (Steingrund and Fernö, 1997; Aristizábal and Suárez, 2006; Ramesh, et al., 2014). This study validates these findings, demonstrating the effectiveness of a co-feeding period in helping smoother diet transition for early larvae. Even for older (8 DPH) *M. atrodorsalis* larvae, that appeared to posses the capacity to adapt quickly to an abrupt switch to *Artemia* nauplii, co-feeding rotifers still contributed to their ability to cope with change, possibly by allowing underdeveloped and/or smaller larvae within cohorts to ‘catch up’ before cessation of rotifer feeding.

An important finding of this study was that significant improvements in larval survival and growth resulted from *M. atrodorsalis* larvae being fed newly hatched *Artemia* nauplii of the high quality AF strain relative to the common GSL strain. Similar outcomes have been noted for early larval rearing of the cobia, *Rachycentron canadum*, showing the nutritional benefits of AF *Artemia* (Faulk and Holt, 2003; Benetti, et al., 2008; Nhu, et al., 2009). Much like *M. atrodorsalis* larvae, cobia larvae could be reared on common strains of

Artemia nauplii (Faulk and Holt, 2003), however, feeding of AF nauplii, even for a short period, had positive effects on both survival and growth (Nhu, et al., 2009; Nguyen, et al., 2011). Therefore, if available and affordable, AF *Artemia* nauplii should be used preferentially over GSL strain for larval rearing of *M. atrodorsalis*, as this specialty strain clearly benefited the larvae.

The introduction of larger Instar II *Artemia* metanuaplii has two important implications in fish larviculture. Firstly, they serve as a practical 'size step-up' in the larval feeding regime, improving the potential energetic gain for each successful capture attempt by larvae, relative to smaller Instar I nauplii (Polo, et al., 1992; Cox and Pankhurst, 2000; Scharf, et al., 2000). Secondly, Instar II *Artemia* metanauplii are the first stage in the life cycle of *Artemia* to exhibit non-selective filter feeding, allowing greater control over their nutritional content through enrichment (Watanabe, et al., 1983; Van Ballaer, et al., 1985; Woods, 2003b; Figueiredo, et al., 2009). When fish larvae are able to accept the larger heterotrophic metanauplii, nutritional enrichment can be performed to facilitate the use of cheaper, more readily available *Artemia* strains to replace expensive specialty strains, such as the AF strain (Conceição, et al., 2010), and benefit hatchery economics. This study demonstrated that enriched GSL *Artemia* metanauplii could be introduced to the diet of *M. atrodorsalis* larvae on 8 DPH with a 3-day co-feeding period before being fed as the sole prey beyond 11 DPH. Additionally, it was shown that relative to newly hatched AF *Artemia* nauplii, similar survival and growth can be achieved using enriched GSL *Artemia* metanauplii in older larvae, demonstrating that commercially available enrichments can improve nutritional values of common GSL strains of *Artemia* and achieve similar results. In a study on the effect of *Artemia* strains and enrichment on larviculture of sea bass, *Dicentrarchus labrax*, Van Ballaer, et al. (1985) reported similar outcomes: while better survival and growth was achieved using a San Pablo Bay, California strain as compared to

a Great Salt Lake, Utah strain, enriching the GSL *Artemia* significantly improved its value to sea bass larvae.

This study demonstrated several key outcomes in the larviculture of *M. atrodorsalis* using standard hatchery prey of rotifers and *Artemia*. Firstly, there is inherent flexibility in the feeding regime in that different types of prey or strains can be used. Secondly, while more expensive and high quality AF *Artemia* improved survival and growth of *M. atrodorsalis* early larvae, a commercially available enrichment could effectively improve the value of the common GSL *Artemia* strain, matching the survival and growth of larvae fed AF *Artemia*, aged 8 DPH and beyond, when they could effectively ingest larger metanauplii. On this basis, we recommend a larval feeding regime for *M. atrodorsalis* with rotifers fed solely between 0-2 DPH inclusive, introduction of newly hatched AF *Artemia* nauplii from 3 DPH, and introduction of enriched GSL *Artemia* metanauplii from 8 DPH, ideally with a 3 day co-feeding period between each prey change (Fig. 5.6A). However, if AF *Artemia* are unavailable or considered too expensive, an alternative feeding regime in which rotifers are fed solely between 0-4 DPH inclusive, newly hatched GSL *Artemia* nauplii are introduced from 5 DPH, and enriched GSL *Artemia* metanauplii introduced from 8 DPH, also with 3 day co-feeding periods between each prey change, could be adopted (Fig 5.6B). Both regimes will yield high survival and growth in *M. atrodorsalis* larvae.

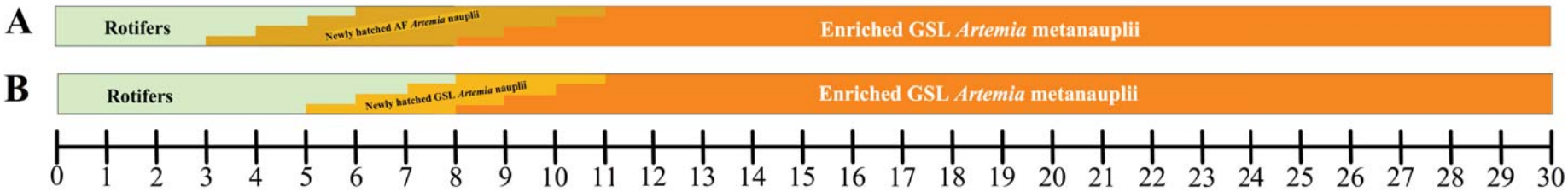

Figure 5.6: Recommended live prey feeding regimes for *Meiacanthus atrodorsalis* larvae to settlement (20-30 DPH). **(A)** Using newly hatched AF *Artemia* nauplii; feed rotifers from 0-2 DPH inclusive, followed by newly hatched AF *Artemia* nauplii starting 3 DPH with a 3 day rotifer co-feeding period, then enriched GSL *Artemia* metanauplii starting 8 DPH with a 3 day AF *Artemia* nauplii co-feeding period. **(B)** When AF *Artemia* are unavailable or cost prohibitive; feed rotifers from 0-4 DPH inclusive, newly hatched GSL *Artemia* nauplii starting 5 DPH with a 3 day rotifer co-feeding period, followed immediately by feeding enriched GSL *Artemia* metanauplii starting 8 DPH with a 3 day co-feeding period with newly hatched GSL *Artemia* nauplii.

Chapter 6: Weaning captive bred M. atrodorsalis to a commercial formulated diet: optimizing timing, feeding frequency and ration

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6.1 ABSTRACT

This study aimed to establish a benchmark regime for weaning the forktail blenny, *Meiacanthus atrodorsalis*, a popular marine ornamental fish, to a commercial marine fish hatchery diet. Three experiments were conducted to investigate the best age for weaning, and the optimal feeding frequency and ration for this species. Weaning *M. atrodorsalis* from live prey to the formulated diet was successful at all ages tested between 16 and 30 days post hatch (DPH), with survival ranging from 71-95% on 40 DPH. However, fish weaned starting 19 DPH and beyond showed 35-54% greater standard length, 28-56% greater body depth and 121-291% greater dry weight, as well as a 30-49% reduction in deformity relative to those weaned starting 16 DPH. The greatest standard length, body depth and dry weight of 17.9 ± 0.2 mm, 5.0 ± 0.1 mm and 30.9 ± 1.2 mg respectively, and the lowest rate of deformity of 6 ± 3 % was recorded in fish weaned latest, starting 30 DPH. Of the feeding frequencies tested (from 1 feed every 3 days to 4 feeds per day) high survival on 49 DPH of between 94-97% was achieved when *M. atrodorsalis* were fed once every 2 days or more frequently from 32 DPH. However, fish fed once per day or more achieved 10-26% greater standard length, 8-29% greater body depth and 54-300% greater dry weight relative to those fed once every 2 days and once every 3 days. Within the feeding rations tested (2-15% body weight per day), survival was the highest when fish were fed a ration of 5-15% body weight per day, ranging between 86-93% on 49 DPH. However, there was a clear growth advantage of 18-29% greater standard length, 9-20%

greater body depth and 41-160% greater dry weight when *M. atrodorsalis* were fed a ration of 8% body weight per day or more, relative to a ration of 2 and 5%. Above an 8% daily ration, growth gains were negligible. Based on the findings, it is recommended that weaning *M. atrodorsalis* onto a commercial hatchery diet commence between 22-25 DPH, followed by feeding at least once daily at a ration of 8% body weight per day.

6.2 INTRODUCTION

While live feeds are often instrumental in rearing early fish larvae, their use stands as a major cost to hatcheries in the form of infrastructure, floor space and skilled labour (Ruscoe, et al., 2004; Curnow, et al., 2006a). Live feeds are also prone to culture crashes, adding risk to hatchery operations, and they are a factor in inconsistent survival and growth of fish, due to high variability in nutritional quality associated with culture and enrichment conditions (Curnow, et al., 2006b; Engrola, et al., 2009a; Moorhead and Zeng, 2010). The use of formulated diets allows for precise control over the delivery of nutrients to fish (Cahu and Zambonino Infante, 2001; Bonaldo, et al., 2011; Hamre, et al., 2013), a field of research largely untouched in marine ornamental aquaculture (Sales and Janssens, 2003; Moorhead and Zeng, 2010; Olivotto, et al., 2011). In fact, successful weaning to formulated feeds normally represents a key final step in food-fish hatchery operations and hence major research efforts have been devoted to this goal with the objective of reducing the use of live prey organisms in favor of formulated diets to the largest extent (Sorgeloos, et al., 2001; Zohar and Mylonas, 2001; Nguyen, et al., 2011; Ouraji, et al., 2011; Takeuchi and Haga, 2015).

While research to optimize weaning techniques has been documented in various food fishes (Rosenlund, et al., 1997; Kanazawa, 2003; Engrola, et al., 2005; Nguyen, et al., 2011; Hoestenberghe, et al., 2015), there is a paucity of such information for marine ornamental fish (Gordon, et al., 1998; Moorhead and Zeng, 2010; Olivotto, et al., 2011). In

general, three key considerations in establishing the best weaning protocol to formulated diets are: 1) identification of appropriate timing for weaning; 2) optimizing feeding frequency, and 3) optimizing feeding ration in order to achieve the best fish survival and growth while minimizing labor and feed wastage. Obviously, identifying the earliest possible time to wean cultured fish to a formulated diet will not only substantially simplify larval rearing procedures and significantly decrease labour and live-feed production costs (Muguet, et al., 2011), but also improve control over nutrient delivery to fish via diet formulation. Once fish are weaned onto a formulated diet, feeding frequency and ration are two important considerations as they dictate feed intake and utilization by fish, and therefore growth (Grayton and Beamish, 1977; Johnston, et al., 2003). Optimized weaning procedures can positively impact hatchery economics, bringing about savings in labor and feed usage, maintaining water quality, and improving key industry metrics such as growth, size uniformity, and reduced rates of deformity (Olivier and Kaiser, 1997; Johnston, et al., 2003). However, except for the clownfish, *Amphiprion* spp., and a seahorse, *Hippocampus abdominalis* (Gordon, et al., 1998; Gordon and Hecht, 2002; Johnston, et al., 2003; Woods, 2003a), no other studies exist documenting effective protocols for weaning to inert or formulated diets in ornamental fish species. Clearly such protocols are important to commercial hatchery production of popular marine ornamental fish for the aquarium trade (Gordon, et al., 1998; Johnston, et al., 2003; Sales and Janssens, 2003; Woods, 2003a).

The objective of this chapter was to investigate and establish benchmark-feeding protocols to successfully wean *M. atrodorsalis* onto a commercial formulated diet. This chapter presents a series of 3 experiments that examine the effect of timing for weaning, feeding frequency and ration on survival, growth, and rate of deformities in *M. atrodorsalis*. In combination with previous chapters a reliable and complete hatchery feeding protocol for the culture of *M. atrodorsalis* larvae from first feeding to 49 DPH is successfully established.

6.3 MATERIALS AND METHODS

6.3.1 Larval culture

Newly hatched larvae were sourced from breeding groups of *M. atrodorsalis* maintained at James Cook University (JCU). Eggs were taken from the guarding males and hatched artificially. The newly hatched larvae were stocked and reared communally until they reached the designated ages required for the experiments. The tank used for communal rearing was a 100-L fiberglass cylindrico-conical tank described in Chapter 2, lit overhead by flourscent ceiling lights (twin Sylvania 36W cool-white lighting fixture). The tank was connected to a recirculating system with 10-μm filtration, a biological filter with foam fractionation and UV sterilization.

Newly hatched larvae were initially fed the SS-type rotifer *Brachionus rotundaformis* at 10 individuals mL^{-1} from 0-2 DPH (days-post-hatching), and then transitioned to newly hatched nauplii of AF strain *Artemia* (INVE technologies, Belgium) from 3 DPH and fed at 3 individuals mL^{-1} until 7 DPH. Instar II metanauplii of the GSL strain of *Artemia* (INVE technologies, Belgium), enriched with algamac 3050 (Aquafauna Bio-Marine, Inc.), were then introduced from 8 DPH, with a co-feeding period with AF nauplii for 3 days, and fed at 3 individuals mL^{-1} until the larvae reached the designated ages for the trials.

From 0-10 DPH the in-flow of filtered water from the recirculating system to the communal tank was maintained at approximately 4.2 L h^{-1}, giving the tank a 100% water exchange every 24 h. From 11 DPH onward, water inflow rate increased to 12.6 L h^{-1} and the tank was topped up 4 times daily (at 8:30 am, 12:00 pm, 3:30 pm and 6:00 pm, respectively) with newly enriched GSL *Artemia* metanauplii to maintain a density of 3 individuals mL^{-1} throughout the light phase (8:00 am - 10 pm). Temperature in the communal tank was maintained between 26 to 29 °C, salinity 34 to 37 ‰, pH 8.2 to 8.3,

NH_3 and NO_2 < 0.5, and NO_3 < 5 ppm, respectively. PAR (photosynthetically active radiation) readings measured at the surface of the communal tank averaged 5.19 µmol m^{-2} s^{-1} (LI-COR, LI-250A light meter).

A standardized weaning procedure over 3 days was adopted for all 3 experiments.

That is, on the first day of weaning, the density of enriched *Artemia* metanauplii was reduced to 2 individuals mL^{-1} and formulated diet fed at a rate of 1 particle mL^{-1} at each of the 4 top-up feeds per day. On the second day the density of enriched *Artemia* metanauplii was reduced to 1.5 individuals mL^{-1} and formulated diet fed at a rate of 1.5 particles mL^{-1}, and on the third day enriched *Artemia* metanauplii density was reduced to 1 individual mL^{-1} and formulated diet fed at a rate of 2 particles mL^{-1}. By the fourth day, larvae were fed completely on formulated diet. Settled solids, uneaten food and any mortalities were siphoned out of the communal rearing tank each morning (8:00 am) when the lights first turned on.

6.3.2 Experimental design and setup

To start an experiment, healthy fish (actively swimming and making regular strikes at food) were transferred from the 100 L communal rearing tank to 9 L replicate culture vessels, described by Moorhead (2015). Larvae were transferred 2 days prior to the date treatments were imposed and the experiment commenced to allow acclimation to the new environment. Each of the replicate culture vessels was connected to a shared recirculating system with 10-µm filtration, a biological filter, a foam fractionator and UV sterilizer, and received filtered seawater from the recirculating system at approximately 130-150 mL min^{-1}. Automated fluorescent lights (twin Sylvania 36W cool-white lighting fixtures) were hung 500 mm above the 9 L culture vessels, providing a 14h light period. Each tank was also supplied with gentle aeration from an air stone. For all experiments, temperature was maintained between 26 to 30 °C, salinity 35 to 38 ‰, pH 8.2 to 8.4, dissolved oxygen 6.5

to 7.9 mgL^{-1}, NH_3 and NO_2 < 1.0, and NO_3 < 10 ppm, respectively. PAR readings measured at the surface of the replicate tanks averaged 56.5 µmol m^{-2} s^{-1}. Each morning, the number of surviving fish in each replicate were counted *in-situ*, then at the end of each experiment, all surviving fish were anaesthetized and then individually photographed under a dissecting microscope (Leica MZ125 fitted with an Olympus DB25 camera). They were then dried at 60 °C for 48 h for the measurement of dry weight to the nearest 0.1 mg on a balance (Adam equipment AAA 250LE). Photos of fish were imported to Adobe Photoshop (CS3 version 10.0) to measure standard length and body depth.

The formulated diet used for all experiments was the 'NRD' larval diet range manufactured by INVE (Thailand) Ltd., which was a sinking crumble designed for marine fish species. Two size ranges were used for experiments; NRD 2/4 (particle size range of 200-400 µm, and composition of 55% Protein, 9% Lipid, 1.9% Fiber and 8% moisture respectively, according to the manufacturer) was used to wean and feed *M. atrodorsalis* to 35 DPH old as the particle size range of this formulated diet matched the average body width (226 ± 4 µm) of the enriched *Artemia* metanauplii the larvae were feeding on. NRD 5/8 (particle size range: 500-800 µm) was fed from 35 to 49 DPH. Settled solids, uneaten food and any mortalities were siphoned out of each replicate every morning (8:00 am) when the lights first turned on.

6.3.2.1 Timing of weaning onto formulated diet experiment

To determine the best timing for weaning onto formulated diet and to quantify the effects of such timing on the survival and growth of *M. atrodorsalis*, 14 DPH larvae were transferred from the communal tank to twenty 9 L experimental tanks with 20 larvae per tank. On 16 DPH, 4 tanks were randomly assigned to each of 5 weaning treatments introducing formulated diet starting on 16, 19, 22, 25 and 30 DPH, respectively, with a 3 day transitional period as described in 2.2. These times were chosen to encompass the

period in which larvae underwent flexion and settlement and showed surface foraging behaviours. The experiment was run until 40 DPH at which all surviving fish were sampled and measured. In some treatments, surviving fish showed a high occurrence of spinal deformities. As a result, rate of spinal deformities was also visually recorded for each treatment.

6.3.2.2 Feeding frequency experiment

Based on the results of timing of weaning to formulated feed experiment (Section 6.3.2.1), experimental fish were weaned between 25-27 DPH, after which they were fed the commercial formulated diet to excess (judged by the presence of uneaten food 1 hour after feeding) twice daily in the communal tank for another 3 days to allow the fish to fully acclimate to the formulated diet. On 30 DPH, fully weaned fish were transferred from the communal tank to twenty-four 9 L experimental tanks with 20 fish per tank. Another 2-day acclimation period to the experimental conditions was imposed in which fish were fed to satiation twice daily. During each feeding session, fish in each tank were fed 0.5 g increments of formulated diet until satiation was reached. Satiation was judged when fish stopped feeding and further addition of formulated diet was ignored. On 32 DPH, 4 tanks were randomly assigned to one of 6 feeding frequency treatments feeding fish 4, 3, 2 and 1 time daily, once every 2 days, and once every 3 days. The experiment terminated on 49 DPH.

6.3.2.3 Feeding ration experiment

The feeding ration experiment aimed to determine the optimal feeding ration of the formulated feed for young *M. atrodorsalis*. Pre-experiment procedures followed that of the feeding frequency experiment, however once weaned, fish were fed the formulated diet to satiation once daily for all treatments, based on the results from the feeding frequency

experiment. On 30 DPH, weaned fish were transferred from the communal tank to twenty 9 L experimental tanks with 20 fish per tank. On 32 DPH, 4 tanks were randomly assigned each of the 5 feeding ration treatments of 2%, 5%, 8%, 11%, and 15% of fish biomass per day. During the experiment, fish wet weight was estimated every 7 days by sampling 3-5 fish from each replicate tank and average weight was calculated by pooling the weight data for each ration treatment. The daily amount of formulated diet fed to each tank was adjusted every 7 days accordingly by calculating the total biomass of fish in each replicate (from a random sample of 5 fish) multiplied by the imposed feeding ration. The experiment terminated on 49 DPH.

6.3.3 Animal ethics

All animal maintenance and experimentation was conducted in accordance with the guidelines, stipulations and restrictions set out by the animal ethics committee of James Cook University, Townsville, Queensland, under animal ethics approval #A1686.

6.3.4 Data analysis

All measurements are expressed as a mean ± standard error (SE). Survival and growth biometrics were assessed to confirm normality (probability plots of residuals) and homogeneity of variance (Levene's test for homogeneity of variances), before being analyzed with one-way ANOVA. A statistical probability of $p < 0.05$ was accepted as significant. If a significant difference was found a post-hoc Tukey's analysis was performed to determine which treatments were different. To meet the assumptions of one-way ANOVA in experiment 2, dry weight data were Log10 transformed. All statistical analyses were performed using Statistica, Version 8.0 software (Statsoft™, Inc.)

6.4 RESULTS

6.4.1 Timing for weaning onto formulated diet experiment

The timing of weaning had a significant effect on the survival of *M. atrodorsalis* ($p < 0.05$). At 40 DPH, the highest survival was found in the treatment in which weaning started on 22 DPH (95 ± 3%), followed by treatments which commenced weaning on 25 DPH (90 ± 5%) and 30 DPH (88 ± 3%; Fig. 6.1), with no significant difference detected among them ($p > 0.05$). The lowest survival occurred in treatments where weaning took place early, i.e. starting 16 DPH (71 ± 8%) and 19 DPH (75 ± 3%), both were significantly lower than that of treatments where weaning started later on 22 DPH and 25 DPH ($p < 0.05$) (Fig. 6.1). The treatment in which weaning started 30 DPH also had a significantly higher survival than the treatment where weaning started on 16 DPH ($p < 0.05$) but not the treatment where weaning started 19 DPH ($p > 0.05$).

Mean growth (SL, BD and DW) response was higher the later larvae were weaned, from 16 DPH to 30 DPH, with significant differences being detected among some of the treatments ($p < 0.05$) (Table 6.1). The treatment in which weaning started earliest (16 DPH) had significantly lower standard length (11.6 ± 0.3 mm), body depth (3.2 ± 0.1 mm) and dry weight (7.9 ± 0.5 mg) than all other treatments ($p < 0.05$). Although the treatment in which weaning commenced on 22 DPH had higher growth compared to the treatment where weaning started on 19 DPH, no significant differences in any growth parameters were detected between these two treatments ($p > 0.05$). The treatment in which weaning started 25 DPH had significantly higher body depth and dry weight than treatments weaned starting 19 DPH and 22 DPH, but the differences were not significant for standard length (Table 6.1). The treatment weaned commencing 30 DPH had significantly higher standard

length and body depth than all other treatments (p <0.05) but dry weight was not significantly heavier than the treatment weaned starting 25 DPH (Table 6.1).

The timing of weaning also had a significant effect on the rate of spinal deformities of surviving *M. atrodorsalis* at the end of the experiment ($p < 0.05$) and a general trend of decreasing deformity rate with deferred wean starting time was found (Fig. 6.2). The treatment in which weaning started the earliest on 16 DPH showed the highest proportion of deformed individuals with over half of fish (55 ± 5%) showing spinal deformities, which was significantly higher ($p < 0.05$) than all other treatments (Fig. 6.2). The lowest rate of spinal deformities of 6 ± 3% was found in the treatment with weaning that started latest on 30 DPH (Fig. 6.2), which was significantly lower ($p < 0.05$) than all other treatments except for the treatment where weaning started 25 DPH (13 ± 6%)(Fig. 6.2). Treatments weaned commencing 19 and 22 DPH had an average rate of spinal deformities of 25 ± 8% and 25 ± 7% respectively, which was not significantly different ($p > 0.05$) from the treatment weaned starting 25 DPH (Fig. 6.2).

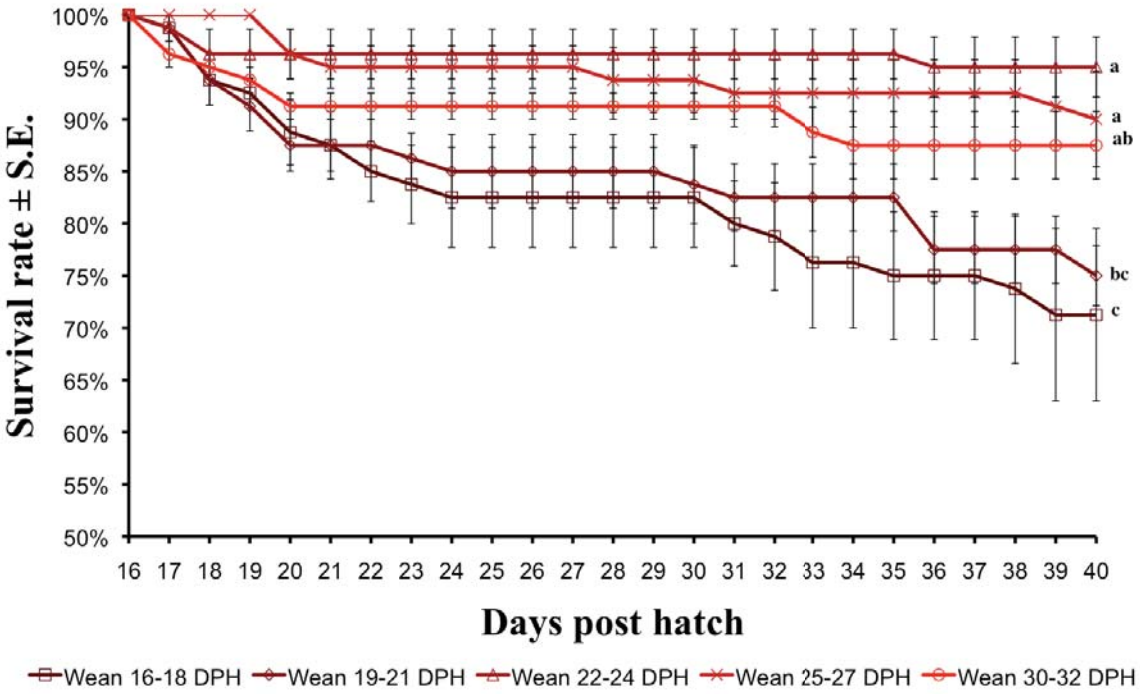

Figure 6.1: Daily survival of *M. atrodorsalis* under five different weaning regimes, from live prey to a formulated feed. Different superscripts denote significant differences in survival at the end of the experiment ($p < 0.05$).

Table 6.1: Standard length, body depth, and dry weight of 40 DPH *M. atrodorsalis* under five different weaning regimes from live prey to a formulated feed

Measurement ± S.E.	**Treatment – Weaning Time**				
	16-18 DPH	19-21 DPH	22-24 DPH	25-27 DPH	30-32 DPH
Standard Length (mm)	11.6 ± 0.3^{a}	15.7 ± 0.4^{b}	15.9 ± 0.3^{b}	16.6 ± 0.3^{b}	17.9 ± 0.2^{c}
Body Depth (mm)	3.2 ± 0.1^{a}	4.1 ± 0.1^{b}	4.3 ± 0.1^{b}	4.6 ± 0.1^{c}	5.0 ± 0.1^{d}
Dry Weight (mg)	7.9 ± 0.5^{a}	17.5 ± 1.2^{b}	20.0 ± 1.0^{b}	26.3 ± 1.4^{c}	30.9 ± 1.2^{c}

Values in rows with different superscripts are significantly different ($p < 0.05$).

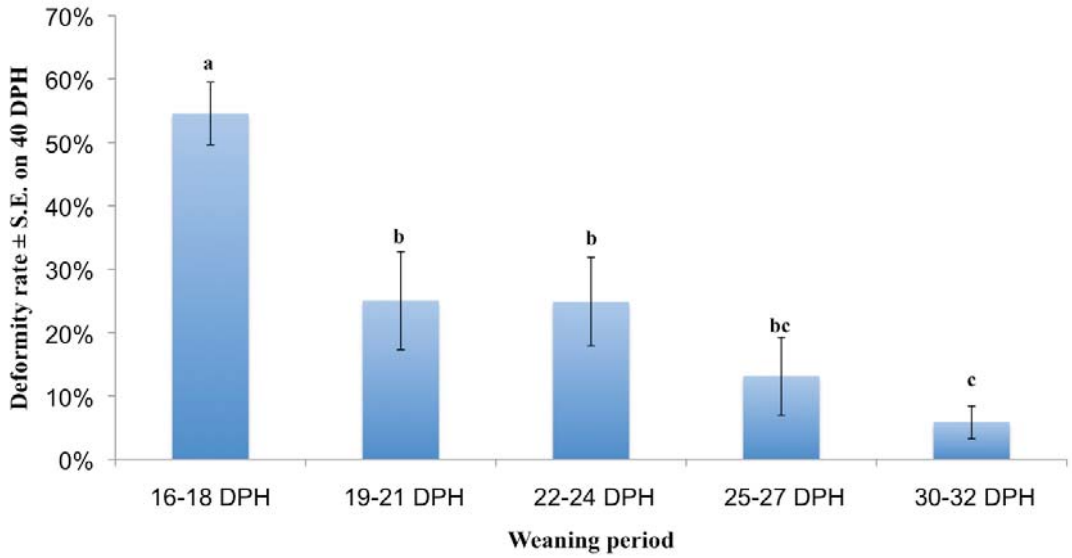

Figure 6.2: Average rate of spinal deformities of 40 DPH *M. atrodorsalis* subjected to five different weaning periods. Treatments with different superscripts indicate a significant difference ($p < 0.05$)

6.4.2 Feeding frequency experiment

Feeding frequency had a significant effect on survival of *M. atrodorsalis* juveniles ($p < 0.05$). The survival of the treatments in which fish were fed 1, 2, 3 and 4 times daily and once every 2 days was not different, ranging between 94 % to 97 % (95 ± 3%, 97 ± 2%, 97 ± 2%, 94 ± 4% and 94 ± 4%, respectively) at 49 DPH (Fig. 6.3). Juveniles fed once every 3 days had the lowest survival of 76 ± 3%, which was significantly lower than all other treatments ($p < 0.05$) (Fig. 6.3).

Significant differences were detected in growth parameters in response to different feeding frequencies ($p < 0.05$). Average standard length, body depth and dry weight generally increased in juveniles that were fed more frequently (Table. 6.2). However, juveniles fed once or more per day were not significantly different ($p > 0.05$) from each other, with average standard length ranging between 18.8 ± 0.3 to 19.1 ± 0.4 mm, body depth between 5.1 ± 0.1 to 5.3 ± 0.1 mm, and dry weight between 30.2 ± 1.3 to 35.5 ± 1.8 mg (Table. 6.2). Growth performance of the juveniles fed once every 2 days and every 3 days

(standard length: 17.0 ± 0.2 and 15.1 ± 0.2 mm, body depth: 4.7 ± 0.1 and 4.1 ± 0.1 mm, and dry weight: 19.6 ± 0.9 and 8.3 ± 0.6 mg, respectively) was significantly ($p < 0.05$) inferior than those fed once or more per day (Table 6.2). Juveniles fed once every two days had significantly superior growth performance than those fed once every three days ($p < 0.05$) (Table 6.2).

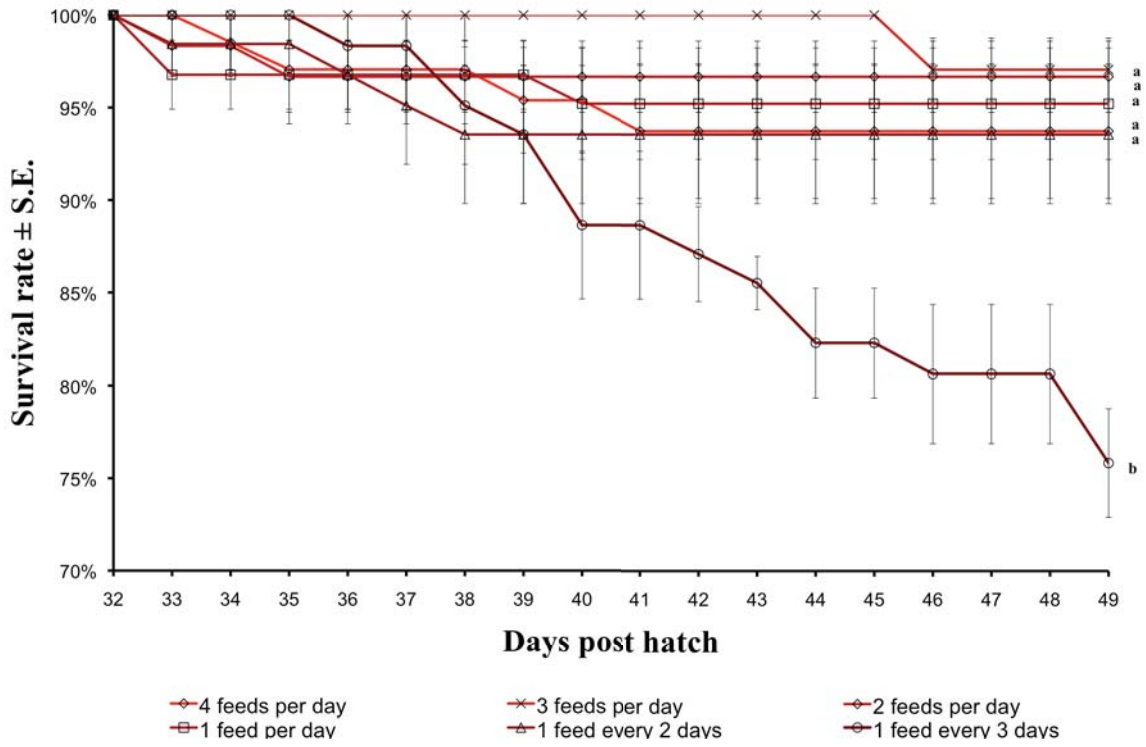

Figure 6.3: Daily survival of *M. atrodorsalis* under six feeding frequency treatments. Different superscripts denote significant differences at the end of the experiment ($p < 0.05$)

Table 6.2: Standard length, body depth, and dry weight of 49 DPH *M. atrodorsalis* under the six different feeding frequency treatments. 4F/D, 3F/D, 2F/d and 1F/D: 4, 3, 2 and 1 feed per day; 1F/2D: 1 feed every 2 days; and 1F/3D: 1 feed every 3 days.

Measurement ± S.E.	**Treatment – Feeding Frequency**					
	4F/D	**3F/D**	**2F/D**	**1F/D**	**1F/2D**	**1F/3D**
Standard Length (mm)	19.1 ± 0.4^{a}	18.8 ± 0.3^{a}	19.0 ± 0.3^{a}	18.9 ± 0.3^{a}	17.0 ± 0.2^{b}	15.1 ± 0.2^{c}
Body Depth (mm)	5.3 ± 0.1^{a}	5.2 ± 0.1^{a}	5.2 ± 0.1^{a}	5.1 ± 0.1^{a}	4.7 ± 0.1^{b}	4.1 ± 0.1^{c}
Dry Weight (mg)	35.5 ± 1.8^{a}	30.7 ± 1.5^{a}	30.2 ± 1.3^{a}	32.5 ± 1.2^{a}	19.6 ± 0.9^{b}	8.3 ± 0.6^{c}

Values in a same row with different superscripts are significantly different ($p < 0.05$).

6.4.3 Feeding ration experiment

Feeding ration had a significant effect on survival of *M. atrodorsalis* juveniles ($p < 0.05$). Juveniles fed rations of 8%, 11% and 15% body weight once per day had similar survival of between 90-93% on 49 DPH (90 ± 4%, 93 ± 2% and 92 ± 2%, respectively) and were not significantly different ($p > 0.05$) from each other (Fig. 6.4). Juveniles fed a low ration of 2% body weight once daily had the lowest survival of 80 ± 4%, which was significantly ($p < 0.05$) lower than the above 3 treatments (Fig. 6.4). While juveniles fed a ration of 5% body weight once per day (86 ± 2%) had a higher survival than those fed 2% body weight per day but lower survival than those fed 8%, 11% and 15% body weight once per day, however, the differences were not statistically significant ($p > 0.05$) (Fig. 6.4).

Feeding ration did have a significant effect on the growth performance of the juveniles ($p < 0.05$)(Table 6.3). Juveniles fed a daily ration of 8% or more had very similar average standard length, body depth and dry weight (ranging between 18.6 ± 0.4 to 18.9 ± 0.4 mm, 4.8 ± 0.1 to 4.9 ± 0.1mm and 28.9 ± 1.3 to 32.5 ± 1.5 mg respectively), which were significantly higher ($p > 0.05$) than those fed 2% and 5% body weight once per day (Table 6.3). Standard length and body depth of juveniles fed 5% and 2% ration were not significantly different, but dry weight of the former was significantly higher than that of the latter (Table 6.3).

Food conversion ratio (FCR) and specific growth rate (SGR) generally increased with increasing feeding ration. SGR (based on weight gain) was the highest but FCR was the poorest in juveniles fed a ration of 15% body weight once per day; in contrast, the lowest SGR was found in juveniles fed the ration of 2% but the best (lowest) FCR was found in the 5% body weight per day treatment (Table 6.3).

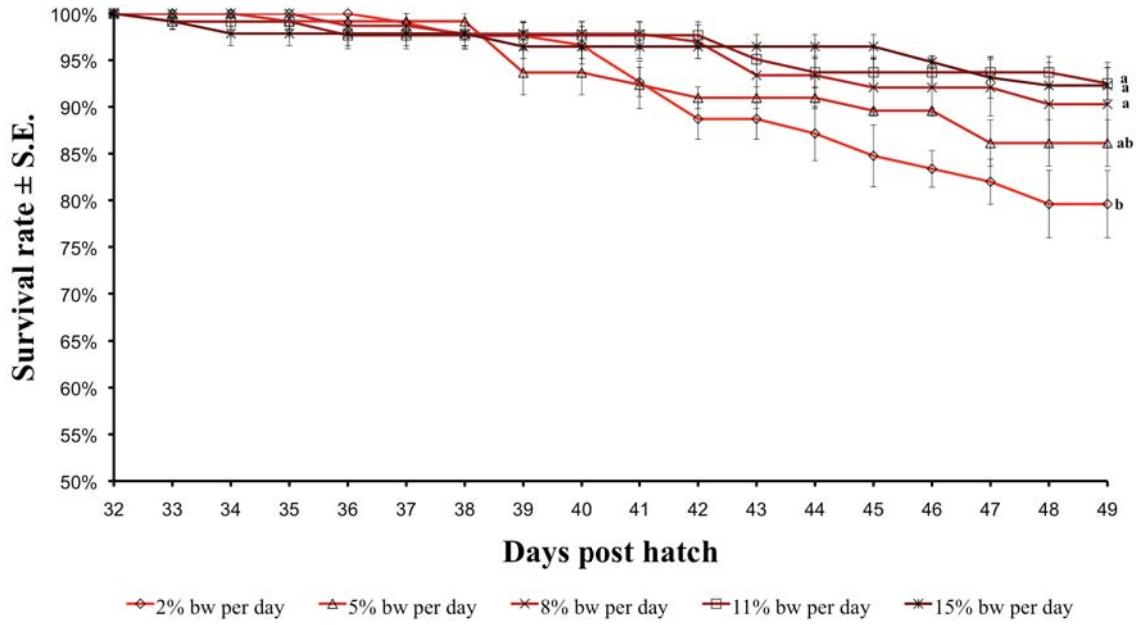

Figure 6.4: Daily survival of *M. atrodorsalis* from different feeding ration treatments. Different superscripts denote significant differences ($p < 0.05$)

Table 6.3: Standard length, body depth and dry weight of 49 DPH *M. atrodorsalis* from the five feeding ration treatments

Measurement ± S.E.	**Treatment – Feeding Ration (% BW day^{-1})**				
	2% day^{-1}	5% day^{-1}	8% day^{-1}	11% day^{-1}	15% day^{-1}
Standard Length (mm)	14.7 ± 0.3^{a}	15.7 ± 0.4^{a}	18.6 ± 0.3^{b}	18.9 ± 0.4^{b}	18.6 ± 0.4^{b}
Body Depth (mm)	4.1 ± 0.1^{a}	4.4 ± 0.1^{a}	4.8 ± 0.1^{b}	4.8 ± 0.1^{b}	4.9 ± 0.1^{b}
Dry Weight (mg)	12.4 ± 0.6^{a}	20.4 ± 1.1^{b}	28.9 ± 1.3^{c}	32.5 ± 1.5^{c}	32.5 ± 1.7^{c}

Values in a same row with different superscripts are significantly different ($p < 0.05$).

6.5 DISCUSSION

Reducing dependence on live prey through early weaning, and reducing labor costs and feed wastage are critical factors to profitability of any commercial fish production (Le Ruyet, et al., 1993; Cahu and Zambonino Infante, 2001; Curnow, et al., 2006a; Bonaldo, et al., 2011). Key marketing and production criteria in ornamental fish aquaculture include

survival, growth, size uniformity, finnage, pigmentation and absence of deformity (Watson and Shireman, 1996; Olivier and Kaiser, 1997; Tamaru, et al., 2001; Chambel, et al., 2015). This study shows that the ornamental fish *M. atrodorsalis* can be weaned successfully from live prey to INVE NRD as early as 16 DPH, however, under this strategy survival and growth were significantly reduced and spinal deformities were high. The highest survival in *M. atrodorsalis* was obtained when transitioned from live prey to the INVE NRD from 22 DPH, which coincides with the time at which they are known to begin to metamorphose and settle (Chapter 3). On the other hand, weaning delayed to 30 DPH achieved the best growth and the lowest rate of deformities. These findings are similar to that of the clownfish, *Amphiprion percula* that showed better growth and survival when weaned to an inert diet beyond its settlement age of 7 DPH (Gordon, et al., 1998; Gordon and Hecht, 2002). This parallel is likely related to both developmental and behavioural factors.

Gordon, et al. (1998) and Gordon and Hecht (2002) reported that gut development of *A. percula* showed significant changes around the period of metamorphosis and settlement, which likely allowed them to more effectively digest formulated feeds from 9 DPH. In our study, a significant drop in the rate of spinal deformities, which is considered an indicator of nutritional deficiency (Le Ruyet, et al., 1993; Cahu, et al., 2003; Faulk and Holt, 2009), occurred when weaning started after 19 DPH, suggesting that *M. atrodorsalis* may also undergo significant gut developmental changes that improved its capacity to digest and assimilate the formulated diet around settlement age. Similar conclusions have been made for food fish species, such as sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), turbot (*Scophthalmus maximus*), Atlantic halibut (*Hippoglossus hippoglossus*), Senegalese sole (*Solea senegalensis*), and flounder (*Paralichthys lethostigma*) (Le Ruyet, et al., 1993; Rosenlund, et al., 1997; Cahu and Zambonino Infante, 2001; Cahu, et al., 2003; Engrola, et al., 2009a; Faulk and Holt, 2009; Mai, et al., 2009), emphasizing the importance

of weaning timed around and after metamorphosis in reducing rates of deformities and malformations.

Alongside gastrointestinal development, significant behavioral and habit changes also occur in many marine ornamental fish during metamorphosis and settlement (McCormick, 1993; Hoey and McCormick, 2004; Olivotto, et al., 2005; 2006b; 2010a; Shei, et al., 2010; Wittenrich, et al., 2010; Madhu, et al., 2016). Much like other reef fish, settlement in *M. atrodorsalis* is marked by a change in habit from free swimming in the water column to benthic life and increased utilization of tank surfaces and other structures for hiding and foraging (Chapter 3). Since the INVE NRD diet used in this study was a sinking crumble that eventually settled on the bottom of the culture tanks, settling and settled *M. atrodorsalis* post-larvae had better access to the crumble settled on tank surfaces while still having access to live prey in the water column. This could have greatly increased ingestion rates and hence improved survival and growth.

Based on the results of this study, it is recommended that *M. atrodorsalis* be weaned from live food to INVE NRD between 22 and 30 DPH to reduce live food use while still maintaining good survival, growth and low deformity rate. The present results showed that weaning started on 22 DPH had the best survival rate, however, growth and deformity rate were somewhat inferior to weaning started a few days later. Although, by optimizing the nutritional profile, improving the digestibility and optimizing buoyancy of inert diet fed to *M. atrodorsalis*, better growth and reduction in spinal deformities may be possible when weaning at or before 22 DPH. In the absence of a specific diet formulated for *M. atrodorsalis*, a balance needs to be struck between the economic and operational savings brought about by weaning early, compared to the increased growth and decreased rate of spinal deformities that comes with weaning later, and such a decision can be made by a hatchery manager based on specific hatchery conditions.

Determining feeding frequency and ration based on formulated diets can provide a valuable guideline for hatcheries to reduce wastage while maximizing productivity and profitability. The feeding frequency and ration experiments conducted in this study lead to the conclusion that juvenile *M. atrodorsalis* be fed INVE NRD diet once per day, at a ration of 8% body weight per day. Such a recommendation is based on examining the potential payoff in growth and survival, relative to culture costs, by feeding the fish more heavily and frequently (Woods, 2005). Gains in survival, growth, and food conversion ratio beyond a certain level are often negligible, wasteful and can significantly affect hatchery economics (Tsevis, et al., 1992; Johnston, et al., 2003). The results of the current study show that when the INVE NRD diet was used, beyond a frequency of once per day at a ration of 8% body weight per day, gains in growth were not significant. Measures of food conversion ratio and specific growth rate also showed that a ration of 8% body weight fed once per day returned the best balance between maintaining good growth rate in *M. atrodorsalis* juveniles while reducing feed wastage through efficient assimilation.

Other feeding studies conducted on juveniles of other marine ornamental fish species showed that generally a daily feeding ration of 5-10% body weight is adequate for good survival and growth (Johnston, et al., 2003; Woods, 2005), which matches these findings. The findings of this study also compare with the lower end of recommended feeding frequencies of 1-4 times daily for juveniles of other marine ornamental fish (Johnston, et al., 2003; Woods, 2005). While Losey (1972) and Pratchett, et al. (2001) suggested that *M. atrodorsalis* is predominantly a planktivore, observation of their feeding behaviour in this study suggest that post-settlement juveniles regularly grazed on tank surfaces, with strikes at tank walls and bottom commonly observed. The NRD diet used in this study was observed to be water stable for many hours (>10 h), which meant that *M. atrodorsalis* were able to feed on the diet throughout the day. This may explain why the feeding frequency experiment showed that feeding once per day was adequate for this species.

The results of this study demonstrate that *M. atrodorsalis* is able to tolerate weaning onto a specifc commercial hatchery diet (INVE NRD) as early as 16 DPH. Using the commercial marine hatchery diet INVE NRD, superior survival can be achieved by weaning at or after metamorphosis, from 22 DPH, with maximum growth and minimal deformities realized when weaning is started from 30 DPH. However, by optimizing the nutritional profile, improving the digestibility and optimizing buoyancy of inert diets fed to *M. atrodorsalis*, better growth and reduction in spinal deformities may be possible when weaning at or before 22 DPH. Since *M. atrodorsalis* displayed a better response to the inert diet around settlement, reducing metamorphosis and settlement times by optimizing pre-weaning diets may also contribute to a greater capacity of this species to accept inert diets at an earlier age. It has been demonstrated that copepods offered to marine fish larvae during early life vastly improves survival and growth, and accelerates development (Leu, et al., 2010; Saravanan, et al., 2010; Busch, et al., 2011; Olivotto, et al., 2011; Cassiano, et al., 2012). Olivotto, et al. (2010a) in particular demonstrated that improved survival and growth, and reduced metamorphosis times of the closely related *Meiacanthus grammistes* can be achieved with supplemental feeding of copepods from first feeding. This suggests that accelerated gut development and thus opportunity for earlier weaning times may also be accomplished in *M. atrodorsalis* by optimising feeding history prior to weaning.

Our study demonstrates that juveniles of this species can also survive on as little as one feed of NRD every 2 days and a daily ration of 2% during early juvenile stages between 32-49 DPH. However, they grew significantly better when fed at least once daily at a ration of 8% body weight per day. Therefore, when rearing larvae of *M. atrodorsalis* on standard hatchery live feeds of rotifers and *Artemia*, followed by weaning onto the commercially available NRD diet, it is recommended that weaning take place between 22-25 DPH, after which the diet be fed once daily at a rate of 8% body weight per day. This regime will

minimize live feed usage and achieve a good balance between maintaining good survival and growth, and reducing labor intensity and food wastage.

Chapter 7: General Discussion

A comprehensive line of research conducted in this thesis, covering broodstock managmenet, novel larval experimental system designs, larval feeding regimes and weaning juveniles to formulated feeds, successfully establishes reliable techniques for the captive breeding and culture of the forktail blenny, *Meiacanthus atrodorsalis*. The practical and economical use of standard hatchery live prey and formulated feeds were the prime focus of this study to allow immediate adoption into existing commercial hatchery operations and provide a model species from the Blenniidae family for broader coral reef fish research. The major outcomes of this research have been outlined and synthesized below with discusion in the broader context of developing captive breeding techniques for marine ornamental fish.

7.1 CHAPTER 2: Research-scale tank designs for the larval culture of marine ornamental species, with emphasis on fish

Four tank designs, a 3-L, 5-L, 9-L and 100-L design, were developed to address the primary challenge of accommodating the fragile pelagic larvae of marine ornamental fish, such as *M. atrodorsalis*, whilst allowing for adequate replication, behavioral observation and systematic investigation. All four tank designs successfully supported larval survival and growth in pilot studies on several marine ornamental fish, and full-scale experiments on *M. atrodorsalis*. The 3-L and 9-L designs in particular showed value in achieving the aims of this thesis and other scientific studies focused on low fecundity marine fish. Both

designs displayed great functionality and versatility in performing replicated experimental designs and met key criteria for experimentation by providing environmental uniformity, allowing non-invasive water exchange and visual inspection, and additionally, they were both simple and cost effective to construct. The MPK design was equally functional, usable and versatile. While it was more labor intensive and less cost effective to construct relative to the 3-L and 9-L designs, it displayed potential in maintaining more sensitive pelagic larvae owing to its gentle circular flow, and allowed *in-situ* visual inspection of larval behaviors and feeding patterns. The 100-L design bridged the gap between research and commercial scales of ornamental aquaculture. In research it was shown to be effective for communal rearing batches of larval fish prior to conducting replicated experiments. This design also showed potential to be adapted to commercial scale culture of marine ornamental species to meet the demand for low volumes of a diverse range of species, which is a characterisetic of the marine ornamental trade. Prior to the development of these designs, the scientific community has struggled to conduct well replicated experiments that support the environmental needs of larval fish, often adopting a range of tank types and volumes that are cost, resource and/or labor intensive (Watson and Hill, 2006; Wittenrich, et al., 2012), are poorly described, or are impractical or too large for adoption in a commercial setting for low fecundity species (Gopakumar, et al., 2009a; Gopakumar and Santhosi, 2009; Madhu, et al., 2016). The implications, application and value of the tank designs described in this thesis is significant and broad reaching, both in the scientific and commercial marine ornamental aquaculture. This chapter presents two notable applied outcomes. Firstly, the designs described here provide a versatile, scalable and repeatable tool to perform tasks ranging from experimental studies requiring high replication to larger ‘industry’ scales. Secondly, the designs satisfy the environmental requirements of sensitive pelagic larvae of a range of marine ornamental fish, including those that are yet to be studied in detail.

Since publishing the descriptions of these tank designs in Aquaculture Engineering, one or more of the designs have been used to conduct research in the field of marine biology (McLeod, et al., 2013; McLeod and Clark, 2016) and aquaculture of marine ornamental species (Pereira-Davison and Callan, 2017; Zeng, et al., 2018) which is a testament to the impact these tank designs have had on the scientific community in facilitating research on sensitive coral reef fish species.

7.2 CHAPTER 3: Breeding of the Forktail Blenny, Meiacanthus atrodorsalis: Broodstock management, embryonic development and larval rearing

To gain a reliable and regular supply of larvae for various experiments, reproductive behaviours, courting, spawning and egg caring of wild-caught adult *M. atrodorsalis* were observed and recorded. Detailed descriptions of embryo development and a range of important metrics, including clutch sizes, egg incubation duration as well as size and mouth gape dimensions of newly hatched larvae, were also recorded. Adults adapted well to the captive conditions provided, began displaying courtship behavior, and spawned after 96 days in captivity, without the need for invasive spawning induction or manipulation as seen in many aquacultured fish. Egg counts from individual clutches were low (300-500 eggs per clutch) in *M. atrodorsalis* relative to other ornamental and foodfish species. However, spawning occurred regularly, spanning through both summer and winter periods; and males were observed to tend clutches of eggs from several females, over several spawning encounters, which greatly increased overall egg production of the broodstock. Eggs were robust and substrate attached, and embryos hatched after an average of 181 hr incubation period under the care of the male. Newly hatched larvae were well developed, measuring 3.05 ± 0.1 mm SL, with a mouth gape of 307 ± 0.1 µm height and 263 ± 17.4 µm width. Larvae began foraging for prey immediately after hatching and accepted rotifers, a common hatchery prey. As the larvae grew bigger, they also readily accepted *Artemia*

nauplii and metanauplii. Using these live preys, the pelagic larval stage of *M. atrodorsalis* lasted approximately 35 days before settlement was observed in a majority of fish. Newly settled juveniles measured 13.32 ± 1.9 mm and resembled adults in colouration, behaviours and body shape.

Lack of knowledge relating to broodstock husbandry, reproductive biology and egg development can severely limit reliable seed supply for larval research or commercial production (Olivotto, et al., 2011). The observations and data documented in this thesis holds significant value to both the research and commercial community looking to establish breeding groups of *M. atrodorsalis*. It also acts as a standard for troubleshooting and to gauge the effect of manipulations to environment, captive conditions and diet, aimed at improving aspects of broodstock, egg and larval health, and other hatchery and research metrics. Research on other species of marine ornamental fishes often lacks detail relating to reproduction and embryonic development, underestimating its value in establishing and maintening captive broodstock that produce a reliable supply of seedstock for larval research and commercial production. The prime application of this chapter includes informing commercial production and future research of *M. atrodorsalis* by providing a detailed reference of metrics relating to the captive conditioning, spawning, egg development and baseline larval growth and development to gauge success. Data and observations documented in this thesis are also likely to benefit research and commercial production of other valuable 'blennys' within the genus of *Meiacanthus* that are closely related to *M. atrodorsalis.*

7.3 CHAPTER 4: Establishing larval feeding regimes for M. atrodorsalis I: Rotifer density and time of prey switch

To gain an understanding of the foraging competency of *M. atrodorsalis* at first feeding and establish an optimal rotifer density during the first 7 days of larval life, prey densities

ranging from ultra-low (2 and 5 rotifers mL^{-1}) to those routinely adopted in marine hatcheries (10 and 20 rotifers mL^{-1}) were tested. To establish a best time at which *M. atrodorsalis* larvae could be switched from the initial prey Rotifers to newly hatched GSL *Artemia* nauplii, a simple abrupt switch between the preys was imposed at four times (3, 6, 9 and 12 DPH). The first trial demonstrated that early larval *M. atrodorsalis* were efficient foragers, capable of surviving and growing at a rotifer density as low as 2 mL^{-1}. The second trial clearly demonstrated that a majority of larval *M. atrodorsalis* younger than 6 DPH displayed limited capacity to accept newly hatched GSL *Artemia* nauplii while a window for successful transition between the two preys lies between the ages of 6 - 10 DPH.

This chapter presents two notable applied outcomes. Firstly, the capacity of early larval *M. atrodorsalis* to survive and grow well at low prey densities, suggests they are resilient to such conditions that might negatively affect larvae of other marine fish species. Since live prey production at both research and commercial levels is considered volatile and often vulnerable to culture crashes, species resilient to variable live prey availability hold significant value in hatcheries. Secondly, while larval *M. atrodorsalis* demonstrated a capacity to adapt to a prey change from rotifers to *Artemia* nauplii after 6 DPH, a change performed earlier did not appear to be imperative or advantageous, in terms of survival or growth, until they reached an age of 10 DPH. This outcome reinforces the view that *M. atrodorsalis* larvae are resilient and adaptable, tolerating variations in their feeding regime, and are therefore a good species for commercial hatchery production.

7.4 CHAPTER 5: Establishing larval feeding regimes for M. atrodorsalis II: Effects of co-feeding and *Artemia* strain on time of prey change

To complete a reliable larval rearing regime for *M. atrodorsalis* using the standard hatchery live prey of Rotifers and *Artemia*, two further trials were conducted to build on the results of Chapter 4. These trials tested the effect a 3-day co-feeding period had on the time of

prey shift from Rotifers to newly hatched GSL *Artemia* nauplii, and the effect a specialty AF strain of *Artemia* had on the time of prey shift. A third trial tested times at which *M. atrodorsalis* were capable of changeing from a diet of newly hatched AF *Artemia* nauplii to enriched metanauplii of the GSL strain. The first trial demonstrated that co-feeding vastly improved larval survival of *M. atrodorsalis* at a younger age (5 DPH). However, the positive effect of a co-feeding period was less evident in older larvae (8 DPH). The second trial demonstrated the superiority of the AF *Artemia* strain relative to the GSL strain in terms of survival and growth of *M. atrodorsalis*. Offering the smaller newly hatched AF *Artemia* nauplii (relative to GSL strain) also resulted in a successful switch from rotifers beginning as early as 3 DPH. The third trial demonstrated that the ability to substitute AF *Artemia* nauplli with enriched metanauplii of the GSL *Artemia* strain started as early as 8 DPH for the larvae.

The key applications of this chapter are three fold. Firstly, the capacity of *M. atrodorsalis* to tolerate longer periods of rotifer feeding (up to 11 DPH) or transition to GSL *Artemia* nauplii early in larval life (5 DPH) raise the profile of *M. atrodorsalis* as a viable species for captive culture, owing to its highly flexible and adaptable feeding behavior. Secondly, while AF *Artemia* are less commonly used in mainstream foodfish aquaculture (Nhu, et al., 2009; Nguyen, et al., 2011), the vast improvement in survival and growth of *M. atrodorslis* when offered AF *Artemia* nauplii in place of GSL *Artemia* nauplii, and the ability to switch larvae from rotifers earlier (starting 3 DPH) holds its value to marine ornamental fish aquaculture. Ornamental aquaculture is an industry characterized by high value, low volume products (Murray, et al., 2012) with short turnover periods, making the use of specialty *Artemia* strains a more viable option since the negative impact of higher costs can be relatively easily absorbed. Thirdly, the ability to substitute AF *Artemia* with enriched metanauplii of the readily available and economical GSL *Artemia* strain as early as 8 DPH in the feeding regime of *M. atrodorsalis* is signficant. It shows that the specialty AF

Artemia need only be used for a short period before transitioning to the economical GSL *Artemia* strain while maintaining the superior survival and growth benefits brought about, essentially limiting the use of this specialty prey as a bridging prey from rotifers to the larger nutritionally enriched GSL *Artemia* metanauplii. Since the change from AF *Artemia* nauplii to enriched GSL *Artemia* metanauplii were successful at the earliest age tested, there may be potential to limit the use of AF *Artemia* even further by changing to enriched *Artemia* metanauplii sooner in the larval life of *M. atrodorsalis*.

7.5 CHAPTER 6: Weaning captive bred M. atrodorsalis to a commercial formulated diet: Optimizing timing, feeding frequency and ration

To complete a full feeding protocol for *M. atrodorsalis* larvae through to early juvenile stages, timing of weaning (starting 16, 19, 22, 25 and 30 DPH) onto a single commercial marine fish hatchery diet was investigated. Furthermore, two fundamental aspects of formulated diet use, optimal feeding frequency and ration (Liu and Liao, 1999; Johnston, et al., 2003), were also investigated. While a majority of *M. atrodorsalis* larvae demonstrated the ability to be weaned to the formulated diet as early as 16 DPH, reduced survival and high rates of deformity were observed in those transitions that occurred prior to 25 DPH, which may reflect the shortcomings of the diet tested, relative to the nutrient requirements of *M. atrodorsalis*. Efficient and economical use of the formulated diet was achieved when *M. atrodorsalis* juveniles were fed once daily at a ration of 8% body weight per day. Considering the paucity of research directed at weaning and use of formulated diets in marine ornamental fish, the outcomes have significant application to research and commercial production of *M. atrodorsalis*, and related species, and form a benchmark for comparing future research and developments on this important area of hatchery operation. For example, while *M. atrodorsalis* responded with lower survival and high rates of deformity when weaned at a young age (16, 19 and 22 DPH) on the INVE NRD diet, the

results presented in this chapter suggest that investigating other commonly available hatchery diets, or producing a tailored diet formulation may reduce deformities, improve survival, and allow this species to be weaned at or earlier than 16 DPH. Therefore, while a safe age at which *M. atrodorsalis* can be weaned was identified for this particular commercial hatchery weaning diet, significant opportunity to refine the feeding protocol, shorten the live prey feeding duration, and investigate nutritional aspects of formulated diets that particularly suit *M. atrodorsalis* and related species has also been identified. That said, until more suitable alternatives are found or specific feeds are developed for *M. atrodorsalis*, this chapter provides important outcomes relating to the introduction and efficient use of a general marine hatchery weaning diet that is widely available for captive culture of *M. atrodorsalis*.

7.6 SYNTHESIS OF RESULTS AND OUTCOMES

This thesis has thoroughly investigated key areas relating to the larviculture of *M. atrodorsalis* using standard hatchery prey of rotifers and *Artemia* through, weaning, and the efficient and conservative use of a standard hatchery diet, INVE NRD through early juvenile stages. As such sysnthesis of the results and outcomes of the trials conducted in this thesis have led to the formulation of a reliable feeding protocol for this species from first hatching to grow-out summerised in Figure 6.5.

M. atrodorsalis should be fed rotifers solely between 0-2 DPH inclusive, followed by introduction of newly hatched AF *Artemia* nauplii from 3 DPH, and introduction of enriched GSL *Artemia* metanauplii from 8 DPH, ideally with a 3 day co-feeding period between each prey change (Fig. 6.5). Weaning onto INVE NRD diet should commence from 25 DPH, after which it should be fed once daily at a ration of 8% body weight per day (Fig. 6.5). This feeding protocol will reliably yield high survival and good growth in *M.*

atrodorsalis and can be adopted immediately by both commercial and research sectors. Ultimately, the detail with which the feeding protocol was developed in this thesis will contribute significantly to the development of a comprehensive manual for the reliable culture of this and related species.

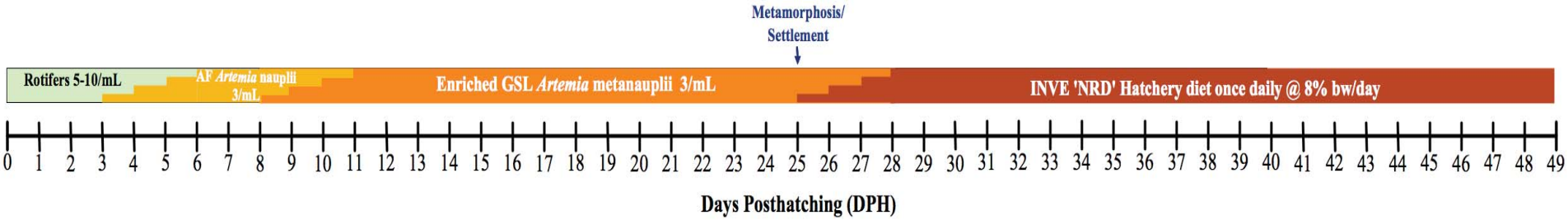

Figure 7.1: Feeding regime for *Meiacanthus atrodorsalis* to early juvenile stage (49 DPH). Feed rotifers from 0-2 DPH inclusive, followed by newly hatched AF *Artemia* nauplii starting 3 DPH with a 3 day rotifer co-feeding period, then enriched GSL *Artemia* metanauplii starting 8 DPH with a 3 day co-feeding period. Weaning onto INVE NRD diet should commence from 25 DPH and the inert diet fed once daily at a ration of 8% body weight per day

7.7 IMPLICATIONS AND FUTURE DIRECTION

This thesis has made a significant contribution to a small but growing body of literature related to captive breeding techniques for marine ornamental species. For the targeted species (*M. atrodorsalis)*, the outcomes presented here eliminate the empirical nature by which a vast majority of marine ornamental fish species are currently cultured. The outcomes of this thesis hold value to commercial hatchery production of *M. atrodorsalis* for the aquarium trade globally but also highlight its potential as a 'new' model for coral reef species, which can supplement existing model reef fish, such as clownfish (*Amphiprion* and *Premnas* sp.) and spiny damselfish (*Acanthochromis polyacanthus*), used in the broader research contexts of marine biology, ecology and climate change, owing to its flexible and achievable hatchery requirements.

While Australia boasts some of the healthiest and best managed coral reefs in the world, recent weather events leading to mass bleaching of large portions of Australian fringing reefs have led governing bodies such as the Great Barrier Marine Park Authority (GBRMPA) to consider management models that include active intervention through reef restoration and activities that build reef community resilience (such as the development of 'super corals'; resilient to heat stress events)(GBRMPA, 2017). In this context, the outcomes of this thesis are well placed to be adapted to future activities aimed at species-specific or reef-community restocking programs that include fish. Bodies of work such as this thesis are also poised to collectively benefit and build a MOA industry in Australia. While the MOA industry in Australia is currently restricted to small hobbyist operations, changes in legislation relating to wild collection from Australian reefs, and tightening ristrictions on translocation and importation exotic specimens is likely to accelerate the development of the industry in the near future to fill the void in the Australian marine

aquarium trade, currently filled by local collectors and importers of exotic marine ornamental species. Having a comprehensive feeding protocol already established for *M. atrodorsalis* will accelerate industrial-scale culture of this and related species in Australia as the MOA industry grows and navigates tougher wild collection and importation regulations.

This thesis has also identified a number of avenues for research specific to *M. atrodorsalis* and MOA to develop and improve. While the tank designs described in Chapter 2 present valuable tools to the research and commercial contexts of MOA, there is a serious need for detailed research in the area specific to tank and system desing in larviculture, as a key launching point for developing species-specific ornamental aquaculture protocols. Research success on species previously considered 'impossible' to culture in a captive setting rely primarily on creating an environment in which pelagic fish larvae display natural feeding and foraging behaviors, before focused investigation of feeding protocols and requirements can even be considered (Wittenrich, et al., 2012). Ornamental aquaculture in particular faces the greatest challenges in this respect as it relies heavily on providing a diverse range of species to meet market demand, with an equally diverse range of environmental requirements at the pelagic larval stage of each species. Future areas of research related to tank design can range from simple variations in tank background colour, to complex evaluation of the individual and interactive effects of tank shape, aspect ratios and flow dynamics.

Understanding aspects of broodstock conditioning, management, reproductive behaviours, spawning and egg care are also critical to obtain a reliable supply of seed stock for commercial and research-scale larviculture (Coward, et al., 2002; Mylonas, et al., 2010; Olivotto, et al., 2017). This thesis provides essential baseline observations and data to achieve this for *M. atrodorsalis* (Chapter 3). However, there is significant room to improve seedstock supply and quality through focused broodstock nutrition, more detailed

investigation of broodstock holding facilities with respect to tank and system design, substrate provisions and optimizing spawning groups, as well as improving incubation techniques to maximize hatch rates, and the quality of newly hatched larvae. Fortunately, given the demonstrated ability of *M. atrodorsalis* to adapt to small tank volumes, and adopt synthetic structures and substrates in which to reside and deposit eggs, much of this work could be done economically with minimal infrastructural costs.

This thesis investigated the larval rearing protocol of *M. atrodorsalis* to settlement in detail with a focus on standard hatchery live preys of Rotifers and *Artemia* (Chapters 4 and 5), which find the widest use at commercial hatcheries, hobbyist and research laboratories worldwide (Sorgeloos, et al., 2001; Hagiwara, et al., 2007; Haché and Plante, 2011). This focus on standard live preys facilitates a more immediate translation and application of culture techniques to a commercial aquaculture scale. However, there is a growing research base and demonstrated evidence supporting the benefits that natrual live preys, such as copepods, have on the survival, growth, and speed of development of marine fish larvae, relative to these traditional hatchery prey (Leu, et al., 2010; Olivotto, et al., 2010a; Busch, et al., 2011; Olivotto, et al., 2011; Cassiano, et al., 2012; Olivotto, et al., 2017). While Rotifers and *Artemia* are currently the most economical and technically simple prey to produce at large scales, they are rarely encountered by wild pelagic fish and do not poses the physical or nutritional qualities that make them as attractive, palatable and nutritionally complete relative to wild marine zooplankton species (Olivotto, et al., 2011; Cassiano, et al., 2012). Copepods in particular show the greatest potential to be used to evolve and improve commercial scale hatchery protocols reliant on Rotifers and *Artemia* (Leu, et al., 2010; Olivotto, et al., 2010a; Olivotto, et al., 2010b; Olivotto, et al., 2011; Cassiano, et al., 2012). Therefore, while this thesis focused on Rotifers and *Artemia* to develop a benchmark larval rearing protocol for *M. atrodorsalis*, future research focused on the use of

copepods will likely improve growth, survival and development of *M. atrodorsalis* and the reliability of the protocols presented here.

In line with the direction taken to develop a benchmark live prey protocol using commonly available live preys, this thesis also developed techniques to wean and efficiently raise juvenile *M. atrodorsalis* using a common marine hatchery formulated diet (Chapter 6). It meanwhile highlighted significant room to manipulate and enhance the nutritional value of the formulated diet to improve culture outcomes of this fish. An interesting outcome of this thesis for future research was that *M. atrodorsalis* showed tolerance to the formulated diet offered during late larval stages; rather than observing a catastrophic survival response to the formulated diet offered prior to metamorphosis and settlement age, *M. atrodorsalis* responded in a way that suggested they were capable of accepting the diet but suffered growth and development setbacks, indicative of the weaning diet lacking the correct digestibility and nutritional profile (Le Ruyet, et al., 1993; Engrola, et al., 2009b; Darias, et al., 2011). Only relatively few studies have successfully demonstrated weaning marine fish to formulated diets during their pelagic larval stages. Therefore, the results demonstrated here not only open an avenue to investigate alternative weaning diets, but also diet formulations specific to *M. atrodorsalis,* acting as model for the development of formulated diets that substitute or even eliminate live prey use for larvae of marine ornamental fish.

7.8 CONCLUSION AND KEY OUTCOMES

The key achievements of this thesis are 4 fold.

This thesis;

- Documented the establishment and conditioning of wild-caught *M. atrodorsalis* broodstock and recorded key aspects of captive reproduction and egg development.

- Developed and described in detail 4 research-scale tank designs for reliable and precise larviculture research
- Developed a detailed scientifically derived feeding protocol for *M. atrodorsalis* from first hatching to growout phase based on traditional live prey of rotifers and *Artemia* and a commercially available hatchery diet

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Appendix

APPENDIX A. Supplementary information to Chapter 2

A.1 CONSTRUCTION OF TANK DESIGNS

A1.1 3-L Tank Design

Assembly of these units involves cutting a 20 mm length of ø150 mm PVC pipe, to which nylon mesh of required pore size is glued (Fig A.1). For the purposes of our laboratory experiments, units with 300-600 μm mesh were constructed, allowing rotifers and *Artemia* nauplii to pass through the mesh while maintaining fish larvae within the culture chamber. The 20 mm length of ø150 mm PVC pipe and mesh is glued into the bottom of the ø150 mm PVC end cap (Fig A.1). A 170 mm length of ø150 mm PVC pipe is also glued into the end-cap effectively 'sandwiching' and securing the mesh into place (Fig A.1). The airlift is constructed of two ø13 mm 90° LDPE barbed irrigation elbow (Pope^{TM}) joined by ø13 mm LDPE pipe (Fig A.1). A ø4 mm elbow is installed as an air inlet in the LDPE pipe towards the bottom of the airlift assembly (Fig A.1). The standpipe is also made of a ø13 mm 90° LDPE barbed irrigation elbow and ø13 mm LDPE pipe (Fig A.1).

A1.2 9-L Tank design

Each culture unit consists of two 9-L nestable (250 mm height, 250 mm diameter at the top and 190 mm diameter at the base) plastic buckets, nylon mesh, a plastic spacer ring, and ø20 mm LDPE garden irrigation fittings (Holman^{TM}). For each unit the inner bucket is modified to form the culture chamber whereas the outer bucket is unmodified, forming the water holding reservoir (Fig. A.2). Several ø60 mm holes are drilled into the bottom of the

first bucket around a ø25 mm hole drilled into the centre of the bottom of the bucket (See Fig. 2A in main text). Nylon mesh of desired size covers all the larger holes (Fig. A.2), and into the centre hole is attached a ø20mm threaded, rigid LDPE irrigation riser with a ø4 mm 90° LDPE elbow installed as an air inlet, secured in place by a ø20mm LDPE nut from underneath (Fig. A.2). This assembly forms the central airlift. The first bucket is then placed inside the second bucket with a plastic spacer ring being placed between the buckets (Fig. A.2). The spacer ring supports the first bucket and creates a small draw-space between the two buckets, similar to that described for the 3-L tank design.

A1.3 Modified 'planktonkreisel' (MPK) design

The MPK design consists of two sheets of 4 mm acrylic that 'sandwiches' a halved cylinder, with a radius of approximately 108 mm, made from 3 mm acrylic, forming a unit that measures 400 mm by 135 mm by 250 mm (length by width by height respectively) with a volume of 5-L (Fig. A.3). At each end of the halved cylinder culture chamber are compartments, made of 3 mm acrylic, that facilitate stand-alone recirculation of the unit, and outflow of water when the unit is operated as flow-through (Fig. A.3). In compartment 'A' there is a standpipe made of a ø15 mm PVC that fits into a ø15 mm PVC coupling glued into the acrylic (Fig. A.3). In compartment 'A' there is also the inlet/suction side of an airlift assembly (Fig. A.3). The airlift assembly is constructed of 2 x ø15 mm PVC couplings, and 2 x ø15 mm 90º PVC elbows, joined by ø15 mm PVC pipe forming a 'loop', connecting compartment 'A' to compartment 'B'(Fig. A.3). A ø4 mm 90° LDPE elbow installed as an air inlet is glued into the vertical length of ø15 mm PVC pipe on the outlet side of the airlift loop (Fig. A.3).

The removable mesh inserts are made from a 3 mm acrylic frame on which mesh of desired size can be glued. A double groove is cut into each side of the 4 mm acrylic walls where the curved culture chamber meets compartment 'A'. This follows the double groove

feature in the ‘filter box’ design described by Kolkovski et al (2004), allowing a new mesh insert to be put in place before removing the old one during routine screen cleaning.

A1.4 100-L upwelling cylindricoconical design

The 100-L tank design is 600 mm high (including 430 mm wall height and 170 mm conical floor), has an internal diameter of 600 mm and has a 25° tapered conical bottom with a ø50 mm PVC valve socket fiberglassed into the tank, forming the central drain (Fig. A.4). It can be constructed with three legs made of ø80 mm PVC pipe fiberglassed into the sides of the tank, that can be cut to desired lengths to adjust the height of the tank.

In a similar manner to the tank design of Kolkovski et al. (2004) the 100-L upwelling tank has a hang-on gas exchange column on its side rim (Fig. A.4). The gas exchange column is made of a ø50 mm PVC pipe, ø50 mm x ø25mm PVC threaded reducing bush, and a ø25 mm polypropylene hose-tail, and is filled with plastic biological media (bioballs®) and heavily aerated with an air stone as described by Kolkovski et al. (2004)(Fig. A.4). A plastic bracket is glued to the gas exchange column to allow it to be hung on the rim of the tank (Fig. A.4). A ø25 mm tube connects the bottom outlet of the gas exchange column to an inlet-outlet assembly attached to the central drain of the tank (Fig. A.4). The inlet-outlet assembly integrates several PVC fittings including a ø50 mm x ø25 mm threaded PVC reducing tee, a ø50 mm 90° PVC elbow, a ø50 mm PVC valve, a ø50 mm PVC faucet take-off adapter, a ø50 mm x ø25 mm threaded PVC reducing bush, and a ø25 mm polypropylene hose-tail. This assembly attaches to the ø50 mm PVC valve socket integrated into the tank central drain.

The screen filter for this tank design consists of a length each of ø100 mm and ø90 mm PVC pipe, a ø90 mm and ø100 mm PVC end cap, a ø25 mm faucet socket, a ø25 mm valve socket, a length of ø25 mm PVC pipe, nylon mesh and a ø4 mm porous tube air ring (Fig. A.4). The ø90 mm and ø100 mm pipe lengths have multiple ø50 mm holes drilled

into them and are covered by nylon mesh of desired size to form two rigid interchangeable tube filter screens. These filter screens attach to the base assembly of the outlet screen filter made up of the remaining materials mentioned above (Fig. A.4).

The base assembly of the outlet screen filter consists of a ø90 mm PVC end-cap glued inside a ø100 mm end cap (Fig. A.4). A ø25 mm hole is drilled through the centre of the glued end-caps with the ø25 mm valve socket and faucet socket screwed together either side of the hole. A length of ø25 mm pipe is glued to the faucet socket (Fig. A.4). The screen filter then fits into the ø50 mm x ø25 mm threaded PVC reducing reducing bush in the inlet-outlet assembly of the tank (see Fig. 4B of the main text). The length of the ø25 mm PVC pipe must be such that the base assembly of the outlet screen filter is positioned just above the floor of the tank forming a small gap. Located inside the outlet screen filter is a removable length of ø25 mm PVC pipe that sets the water level in the tank (Fig. A.4).

A.2 MATERIALS COST AND BUILD TIME

Table A2.1: Approximate cost of materials and build time required for each of the tank designs

Tank Design	Approximate cost per tank (AU$)	Approximate build time per tank
3-L	$ 20	20-45 min
9-L	$ 20	10-30 min
MPK	$ 50	1-3 hrs
100-L	$ 300	3-5 hrs

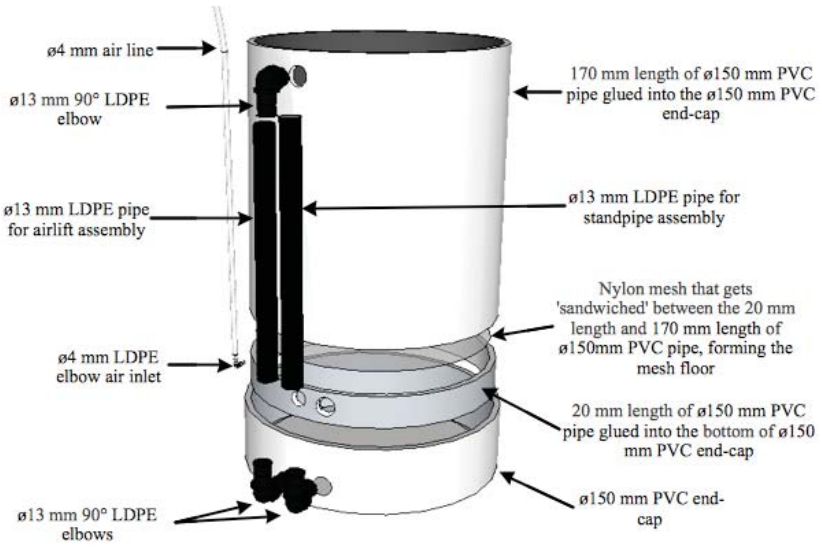

Fig. A.1 Exploded diagram of 3-L tank design showing the different components of the design

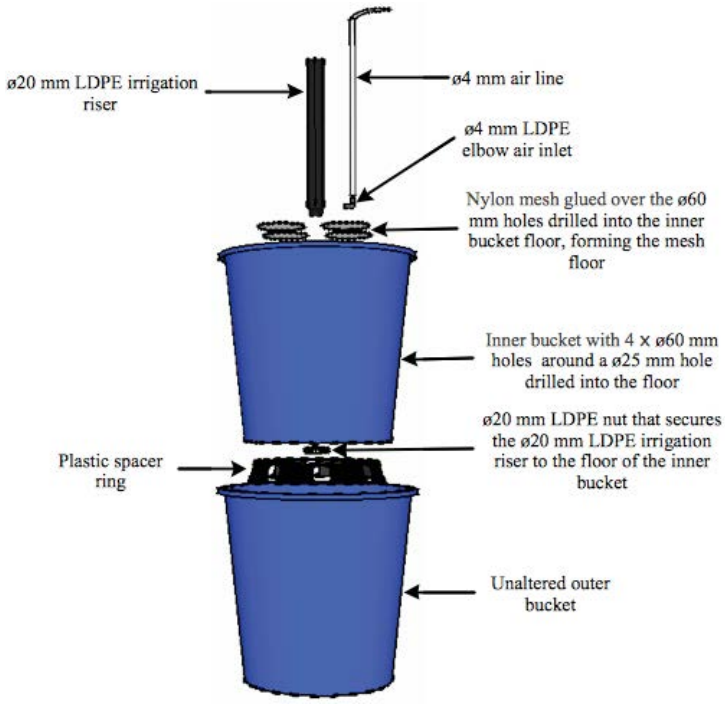

Fig. A.2 Exploded diagram of 9-L tank design showing the different components of the design

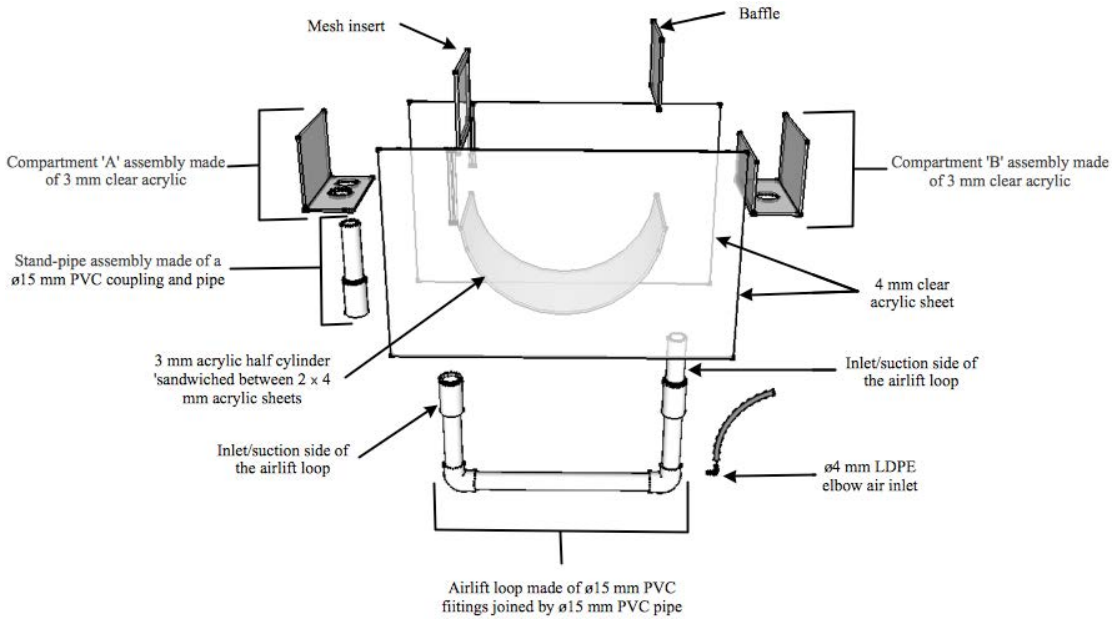

Fig. A.3 Exploded diagram of MPK tank design showing the different components of the design

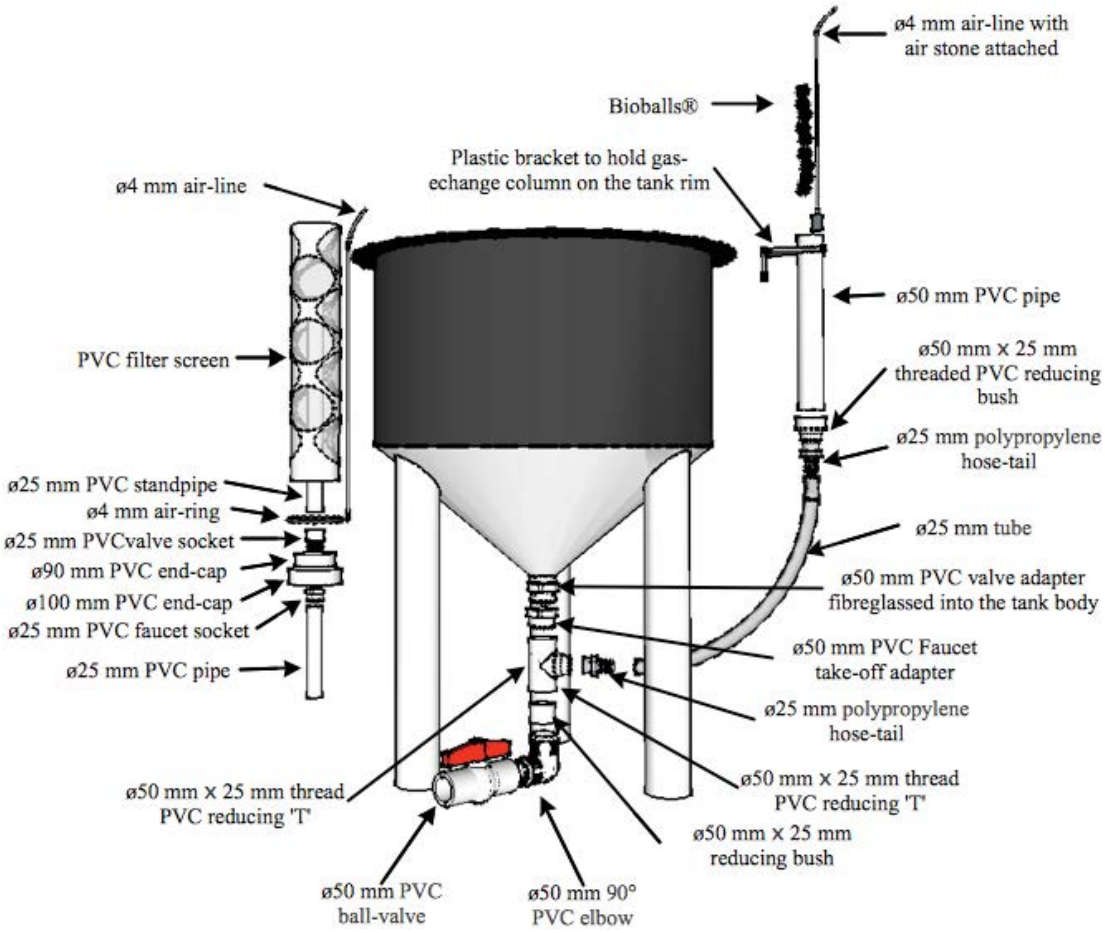

Fig. A.4 Exploded diagram of 100-L tank design showing the different components of the design

APPENDIX B. List of publications

Moorhead, J.A., 2015. Research-scale tank designs for the larval culture of marine ornamental species, with emphasis on fish. Aquacult. Eng. 64, 32-41.

Moorhead, J.A., Zeng, C., 2010. Development of captive breeding techniques for marine ornamental fish: A review. Rev. Fish. Sci. 18, 315-343.

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Moorhead, J. A., Zeng, C. 2017. Establishing larval feeding regimens for the Forktail Blenny *Meiacanthus atrodorsalis* (Günther, 1877): effects of *Artemia* strain, time of prey switch and co-feeding period. Aquac. Res. doi:10.1111/are.13254

Moorhead, J. A., Zeng, C. 2017. Weaning captive bred forktail blenny, *Meiacanthus atrodorsalis,* to a commercial formulated diet: Optimizing timing, feeding frequency and ration. Aquaculture 473, 259-265