# Development of larval fish rearing techniques and nutrient requirement for the green mandarin, *Synchiropus splendidus*: a popular marine ornamental fish

Thesis submitted by

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# Declaration on Ethics

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

The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee.

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## Statement of contribution of others

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## Abstract

The green mandarin fish, *Synchiropus splendidus* (Herre, 1927) is a small, brilliantly coloured benthic marine fish distributed in the tropical Pacific and Indian Oceans. It is within the group of fish known as dragonets (Family: *Callionymidae*) and is among the most desirable species to marine aquarium hobbyists worldwide. Unfortunately, the current supply of *S. splendidus* for the aquarium trade comes solely from the wild. Moreover, it has been observed that most specimens on sale are males, suggesting probably selective harvesting from the wild populations. As a result, the captive breeding of *S. splendidus* is urgently required as an alternative to ensure a sustainable supply of this popular species for the aquarium trade to reduce the pressure on its natural populations.

*S. splendidus* is a pelagic-spawner and has been considered as a relatively hard-to-breed species due to its adults generally feeding on live small crustaceans, hence this species is difficult to maintain under captive condition. In addition, there is limited knowledge on its larval feeding habitat. Newly hatched larvae of *S. splendidus* is among the smallest larvae of marine fish, on average only 1.5 mm. The high vulnerability of pre-feeding larvae to handling stress and advent environmental conditions make their rearing even more difficult.

A series of experiments were conducted to improve captive breeding techniques for *S. splendidus*, which could also serve as a model species for ornamental marine pelagic-spawners. The present thesis consists of 9 chapters: following the first general introduction chapter (Chapter 1), the second chapter describes general materials and methods used (Chapter 2). The subsequent 6 data

chapters can be largely grouped into three main themes: 1) Chapter 3, 4 and 5 focused on the first feeding preys and culturing regimes for newly hatched and early larvae of *S. splendidus,* and investigated the underlying mechanisms for the superior performance of copepods as live prey from the perspectives of behavioral and lipid nutrition. 2) Chapter 6 then focused on the strategy and best timing for larval prey transition from copepods/rotifers to *Artemia* nauplii, and subsequently to larger enriched metanauplii during the critical metamorphosis period of *S. splendidus*. 3) Chapters 7 and 8 finally focused on optimizing the growth rate of post-settlement *S. splendidus* by their feeding with enriched *Artemia* metanauplii containing graded concentration of two key dietary LC-PUFAs, DHA and ARA. The final chapter (Chapter 9) summarizes the main results from all data chapters and posits implications for future research directions.

The experiments from Chapter 3 confirmed that *S. splendidus* newly hatched larvae fed on rotifers (10 $mL^{-1}$) alone had very low survival (3%) while the addition of the calanoid copepod *Parvocalanus crassirostris* at 2 $mL^{-1}$ for co-feeding with the rotifers dramatically improved both survival (>50%) and growth. A further rearing experiment feeding larvae with different copepod densities showed that the copepods provided at 1 $mL^{-1}$ for co-feeding with rotifers could produce compatible results to those at the higher density of 2 $mL^{-1}$. A subsequent experiment demonstrated that co-feeding rotifers with the copepods at different tested densities did not produce any clear beneficial effects, suggesting co-feeding rotifers is not necessary. The subsequent larval ingestion rate experiment showed that the copepods were always positively selected over the rotifers by *S. splendidus* larvae at all ages tested (4, 6, 8 and 10 DPH). Based on these results, an optimal feeding regime for *S. splendidus* early larvae by feeding

copepods solely at 1.0 $mL^{-1}$ was established.

Results presented in Chapter 4 showed that compared to the continuous copepod feeding control, larval prey shifted from copepods to rotifers on 4 or 8 DPH, leading to significantly lower survival and growth, whereas if the prey shift occurred later on 12 DPH, no significant difference in both survival and growth were detected at the end of the experiment on 15 DPH. The larval feeding behavior experiment showed that larvae of all ages tested (6, 8, 10 and 12 DPH) attacked copepods more frequently than rotifers, showing a strong preference toward copepods. Moreover, copepods were never rejected after being captured by larvae of all ages, whereas rejection following capture was commonly observed for rotifers. Furthermore, the feeding intervals (i.e. the time between a prey being ingested and the time of the next active foraging for food by a larva) on copepods by larvae of all ages were also significantly shorter than for rotifers.

The fatty acid analysis results (Chapter 5) demonstrated that the copepod *P. crassirostris* possessed a superior fatty acid profile compared to the rotifers, as *P. crassirostris* showed much better matched MUFA, PUFA and LC-PUFA profiles as well as DHA/EPA ratio to *S. splendidus* newly spawned eggs. It was also noted that of the most important essential fatty acids, DHA and ARA in the 2 DPH pre-feeding larvae were approximately only half that in the newly-released eggs ($p<0.001$), whereas EPA remained relatively stable ($p>0.05$), suggesting dietary supply of DHA and ARA are likely to be important for subsequent larval survival and development.

Larval feeding experiments described in Chapter 6 showed that larvae had

their prey switched from the copepods to *Artemia* nauplii on 18 and 21 DPH without a transitional rotifers feeding period, giving the highest larval survival compared to other feeding treatments. In particular, introducing a rotifer feeding period starting at 12 DPH led to significant lower larval survival when compared to the treatments in which larval prey switched from the copepods to *Artemia* nauplii directly on 15, 18 and 21 DPH, respectively ($p<0.01$); however the growth performance of larvae from different treatments were not significantly different. Nevertheless, for the larvae fed rotifers from 12 DPH, if rotifers were switched to *Artemia* nauplii on 18 or 21 DPH, larval survival were still reasonably high (>70%), which appears acceptable in the case of commercial production. Hence, in the case of limited copepod supply, which commonly occurs since copepod intensive culture techniques are still under development and generally very costly, rotifers might be used to substitute copepods for feeding larvae between 12 and 17 DPH.

It was further demonstrated that among different treatments of prey switched on 22, 25 and 28 DPH in both live and dead forms, introducing live enriched *Artemia* metanauplii to replace the *Artemia* nauplii on 25 DPH obtained the best survival and growth of post-settlement *S. splendidus* on 42 DPH. On the other hand, those fed dead enriched *Artemia* from 22, 25 and 28 DPH had significantly lower survival and growth compared to those fed live enriched *Artemia* on corresponding days.

Chapters 7 and 8 investigated the optimal dietary DHA and ARA for optimizing the growth of post-settlement *S. splendidus* from 25 DPH. The results showed that no single mortality was observed in any treatments feeding enriched

*Artemia* containing graduated levels of DHA or ARA, respectively, at the end of the experiments on 45 DPH. However, the growth performance in terms of standard length (SL), body width (BW) and dry weight (DW) were significantly affected by dietary DHA level; and BW, DW but not SL by the dietary ARA level. The regressions of SGR data with dietary DHA level suggested that dietary DHA level for the highest SL, BW and DW was 6.91, 6.68 and 6.48 mg DHA/g DW, respectively. Meanwhile, for the dietary ARA, it was 7.29, 7.06 and 7.34 mg ARA/g DW, obtaining the highest SL, BW and DW, respectively. It is unexpected that the analysis of fatty acids profiles of the 45 DPH old *S. splendidus* from different treatments indicated that they might possess limited capacity of biosynthesizing LC-PUFAs from LOA or ALA, which is not common among marine fish larvae.

# Table of Contents

# List of Tables

# List of Figures

# Chapter 1

## General introduction

### 1.1 General background

#### *1.1.1 The current marine ornamental industry*

The increasingly popular aquarium hobby is fuelling the rapid growth of the aquatic ornamental industry (AOI), which is an important global industry worth nearly 15 billion US dollars (Prang, 2007; Wittington and Chong, 2007). As the rapid development of captive care and life support system technologies has made keeping marine aquaria much easier over the past decades, the demands for marine ornamental fish, particularly coral-reef species, have increased exponentially (Sales and Janssens, 2003; Shuman et al., 2004; Calado, 2006; Livengood and Chapman, 2007). Over one billion ornamental fish comprising more than 4000 freshwater and 1400 marine species are traded internationally each year (FAO, 2012). Although marine ornamental species only account for approximately 25% of the total ornamental fish trade, the proportion is much higher when estimated as a percentage of world trade by value (Olivier, 2003; Livengood and Chapman, 2007; Wittington and Chong, 2007). Moreover, in contrast to freshwater ornamental fish that are mostly captive bred, it was estimated that around 95% of marine ornamentals for the aquarium trade are collected directly from the wild (FAO, 2012). Such heavy reliance on wild caught marine ornamentals to satisfy consumer demand is considered to cause direct negative repercussions on coral reefs and marine ecosystems (Olivotto et al., 2016).

As the world's largest exporters of ornamental marine species for the private aquarium trade, the Philippines and Indonesia often employ destructive fishing techniques, such as the use of cyanide or explosives (Mak et al., 2005). The use of this poison is known to harm both targeted and non-targeted reef fishes, threatening the food sources of the local population, while the use of explosives destroys reef structure and inflicts severe or permanent collateral damage to coral reef ecosystems (Rubec et al., 2001; Wood, 2001, Calado, 2006). Furthermore, due to delayed and chronic chemical toxicity, poor handling practices, diseases and transport stress, it is estimated that from ocean to aquaria, the post-collection mortality is nearly 80% (Vincent, 2002; Wabnitz et al., 2003). With such poor survival of collected specimens through the chain of custody, along with the current dependence of the marine aquarium industry on wild sources, there is increasing heightened concern that the marine ornamental industry may not be sustainable in the near future unless steps are taken to limit destructive exploitation and seek alternatives. In order to promote the stabilization of coral reef ecosystems and ensure the sustainability of the marine ornamental industry, an obvious and vital action would be the development of captive breeding techniques for marine ornamental species, also known as marine ornamental aquaculture (MOA) (Olivotto et al., 2007, 2016). The captive production for MOA could supplement or even completely replace the supply of wild caught specimens for the marine ornamental trade, hence contributes to the reduction of pressure on their natural population. Meanwhile, the establishment of an economically viable MOA industry is also likely to generate significant economic benefits for both developed and developing countries (Moorhead and Zeng, 2010).

*1.1.2 The current marine ornamental aquaculture*

In many respects, MOA is still at a very early stage, receiving limited research attention and experiencing very slow development compared to the technical and industrial advances made in foodfish aquaculture (Shei et al., 2010). To date, captive larval rearing successes have been largely limited to small, experimental, or hobbyist scales (Holt, 2003; Wittenrich et al., 2007). Of the over 2000 commonly traded species, less than 100 species have been successfully cultured, and among them, most are still at the experimental scale with low, not commercially viable survival rates (Moorhead and Zeng, 2010). This is mainly because of the limited knowledge of their biology and feeding habit, hence a poor understanding of captive breeding techniques. Commercial MOA cannot succeed without the concerted scientific research effort, which develops the techniques to yield a high quantity of and quality eggs, and to successfully culture a large quantity of larvae through to a marketable end point (Tlusy, 2002; Holt, 2003).

However, very few scientific publications exist documenting aspects pertinent to captive culture techniques for marine ornamental fish (Shei et al., 2010). Additionally, multiple bottlenecks have severely limited the progress of MOA, among them, two key bottlenecks have been identified: 1) problems with maturation and spawning under captive conditions; and 2) probably the most important bottleneck, is the high mortality associated with inappropriate live food items for larval first feeding (Ostrowski and Laidley, 2001; Olivotto et al., 2006; Avella et al., 2007). Concurrent with limited knowledge on the complex reproductive strategies and dietary nutritional needs of first feeding larvae, there

is an obvious, and urgent need to address these key bottlenecks that cripple the progress of MOA. Since the bottlenecks are similar to those of more challenging foodfish species, it is believed that through careful appraisal and adaptation of these recent foodfish achievements, advances could be made more rapidly for the MOA industry (Ostrowski and Laidley, 2001).

## 1.2 First feeding for marine ornamental fish

### *1.2.1 The influencing factors for first feeding*

The first feeding in fish larvae can be considered as the period from which the ingestion is possible up to the moment when larval growth is detected (Yúfera and Darias., 2007). The main characteristic of this phase is that the source of nutrient and energy necessary to continue the larval development changes from the yolk reserves to the ingested food. The first feeding period is widely considered as highly critical for successful larval culture as mass or total larval mortality often occurs after the exhaustion of the yolk-sac reserve if larvae were unable to hunt and ingest diets with appropriate nutritional quality (Mæhre et al., 2013). During larval ontogeny, the first difficulty in raising fish larvae arises due to bioenergetic constraints at first feeding. High larval growth rates require high weight-specific metabolism, but the larval digestive system is still rudimentary, with poorly developed digestive enzyme activity (Takeuchi et al., 2001; Chesney et al., 2005; McKinnon et al., 2003; Conceicao et al., 2010). Such digestive systems are in most cases incapable of processing formulated diets and consequently a vast majority of marine larvae require live prey items during their early life stages (Garcia-Ortega et al., 1998; Shields et al., 1999).

The failures of many larvae to survive when using live prey for first feeding could be attributed to biological and environmental factors, including shape and size of prey (Gordon and Hecht, 2002; Ignatius et al., 2001), visibility of prey (Pena et al, 2004), swimming behavior and speed of prey (Sarma et al., 2003; Ostrowski and Laidley, 2001; Wittenrich et al., 2007), nutritional quality of prey (Vega-Orellana et al., 2006), larval digestive abilities (Vega-Orellana et al., 2006; Watanabe and Kiron, 1994), resistance to starvation of larvae, and sub-optimal environmental conditions for culture (Watanabe and Kiron, 1994; Pena et al., 2004). Among these factors, the size of prey compared to the mouth gape of larvae should be the most important one. Some marine fish larvae have relatively small mouth, and only the smallest of rotifer or copepod nauplii can be ingested. The second key factor is the nutrition profile of the prey. Enrichment of traditional prey provides higher survival of larvae compared to un-enriched prey. In addition, certain swimming behaviors of prey items may be required to solicit a feeding response. Therefore, identifying a suitable live prey that is easily ingested, digested and absorbed is the key to overcome the bottleneck of first feeding of marine ornamental fish (Vega-Orellana et al., 2006).

### *1.2.2 Traditional live prey for first feeding*

Captive larval rearing of marine fish has relied mainly on two traditional live prey items: the rotifer, *Brachionus* spp., and the nauplii of brine shrimp, *Artemia* spp (Holt, 2003). The advantages of these two traditional live preys were high culture density, ease of mass production, off-the-shelf convenience, and the option to manipulate the nutritional profile through enrichment (Nhu et al., 2009; Van et al., 2014). However, gut analyses of wild caught marine fish larvae have

revealed the most commonly consumed natural prey consists of copepods and their eggs, protozoans, dinoflagellates, and mollusc larvae (Watanabe and Kiron, 1994; Holt, 2003; Mckinnon et al., 2003). *Artemia* are rarely part of the natural diet of marine species, while rotifers are only found in the larvae of a minority of estuarine and coastal species (Van Stappen, 2002). Hence, the larval culture of some species, particularly marine ornamental fish, has been hindered by the inadequacy of these two live feeds (Rodriguez and Hirayama, 1997; Schipp et al., 1999; Holt, 2003; McKinnon et al., 2003; Olivotto et al., 2008a, 2008b). Another major drawback of these conventional live prey is that their sizes are generally greater than the optimum prey size of marine ornamentals (Ostrowski and Laidley, 2000; Olivotto et al., 2006, 2008a, 2016; Gopakumar and Santhosi, 2009; Job, 2011). Most larvae of marine ornamental fish generally have smaller mouth gape (Holt, 2003; Job et al., 2006), which could not successfully capture and ingest the traditional live preys (Job, 2011). In some cases, the larvae still refuse to ingest them even when the mouth gapes were large enough (Schipp et al., 1999; McKinnon et al., 2003), suggesting the importance of prey movement as a feeding initiative (Young, 1994; Vega-Orellana et al., 2006; Cassiano and Ohs, 2011; Olivotto et al., 2006). When traditional preys are successfully ingested by the larvae, the digestibility is still a cause for concern, as it is believed to be insufficient to promote proper digestive system growth and development in most marine larvae (Schipp et al., 1999; Luizi et al., 1999). In addition to these biological limitations, both rotifers and *Artemia* nauplii are naturally nutritionally deficient for the growth and survival of marine fish larvae, because of the lack of sufficient amounts of n-3 LC-PUFAs, in particular DHA and EPA (Avella et al., 2007; Olivotto et al., 2008a; Liu and Xu, 2009). As a result, the nutritional value of cultivated live feeds has to be manipulated through enrichment (Hamre et al.,

2008). In foodfish, it has been clearly proved that enriched preys provided a significantly better performance for fish larvae (Tuncer et al., 1993). Similar results were also found in marine ornamental fish, for example, the yellowtail damselfish could only survive and successfully complete the metamorphosis when fed on enriched rotifers (Gopakumar et al., 2009). Despite the successful manipulation of fatty acid composition in rotifers and *Artemia* nauplii, the lipids will mainly be incorporated in the tracylglycerides (energy-yielding fatty acids), while leaving the phospholipids-fraction less affected (Shield et al., 1999; Sargent et al., 1999). Moreover, enrichments are often insufficient to achieve an optimal essential fatty acid (EFA) profile (Helland et al., 2003) as *Artemia* naturally tend to convert DHA to EPA, making it difficult for enrichment preparations to provide a DHA to EPA ratio greater than one (Navarro et al., 1999; Bell et al., 2003) and rotifer enrichment seems promising only with respect to a few selected micronutrients and vitamins (Hamre et al., 2008; Srivastava et al., 2011). Lastly, the species-specific nutritional requirements make the enrichment even more difficult to achieve the optimal nutritional profiles for individual fish species (Velu and Munuswamy, 2003; Hamre et al., 2008). As a result, there is a need for identification of alternative feeds that do not have these inadequacies and can promote adequate survival and growth of first feeding larvae.

### *1.2.3 Copepods as alternative live prey for first feeding*

#### 1.2.3.1 Utilization of copepods as live prey in larviculture

Although some marine ornamental species are cultured successfully with traditional or enriched live feeds, there is an increasing list of species which could not be reared successfully through these methods (Moorhead and Zeng, 2011).

Clearly, the most suitable live feed organisms to be considered candidates for MOA would be of those types and sizes found in the gut of wild larvae (Holt, 2003; Olivotto et al., 2006; Baensch and Tamaru, 2009a, 2009b). Among various options, copepods appear to have better potential to supplement or replace rotifers and *Artemia* for marine ornamental larval culture, as numerous field studies have demonstrated, copepod nauplii are the most important prey for marine fish larvae, typically making up 50% or more of their stomach content (Munk and Nielson, 1994; Chesney, 2005; Kleppel et al., 2005). Additionally, many culturing studies have shown that the use of copepods has generally led to considerably improved survival, growth, pigmentation, and digestive tract development, as well as lower frequencies of skeletal deformities, and higher stress tolerance when compared with rotifers or *Artemia* (Shields et al., 1999; Hamre et al., 2002; Rajkumar, 2006; van der Meeren et al., 2008). Olivotto et al. (2008) have found that yellowtail clownfish larvae, *Amphiprion clarkia* had better survival and growth when they were fed on the copepod *C. typicus* compared to those fed traditional preys. More cases of superior performances of copepods as the first feeds were also found in seahorses (Koldewey and Martin-Smith, 2010; Job et al., 2006; Celino et al., 2012), pipefish (Payne and Rippingale, 1998) angelfish (Laidley et al., 2008; Olivotto et al., 2006a) and blennies (Olivotto et al., 2010). Therefore, whenever available, it is a good strategy to use copepods as live feeds, in particular when developing larval rearing protocols for previously uncultured species.

#### 1.2.3.2 The advantages of using copepods as live prey

The superior performance of marine fish larvae using copepods as live prey should contribute to effectively addressing problems of size, digestibility,

attractiveness, and nutritional value in marine larvicultue (Ignatius et al., 2001; Olivotto et al., 2006a, 2008a, 2008b, 2010; Baensch and Tamaru, 2009; Drillet et al., 2011). Firstly, copepod nauplii are generally smaller than the smallest strain of rotifers or *Artemia*, making them ideal prey for many marine larvae with small mouth-gape at first feeding (Olivotto et al., 2006a; Gopakumar and Santhosi, 2009; Drillet et al., 2011), while still providing a wide range of body sizes from nauplii stages, to copedites stages, to sexually mature adults (O'Bryen and Lee, 2005; Gemmell and Buskey, 2011). Secondly, copepods contain high levels of digestive enzymes, ensuring that they are better digested, which may collectively contribute to satisfying larval nutritional requirements when compared to traditional live feeds (Luizi et al., 1999; Ignatius et al., 2001; Olivotto et al., 2010b). Thirdly, the characteristic stop-and-go swimming pattern of copepods is believed to stimulate a strong feeding response in early larvae, resulting in a substantially increased ingestion rate (Buskey et al., 1993; Støttrup and Norsker, 1997; Laidley et al., 2008). Finally, copepods generally have a better profile of essential fatty acids and free amino acids, as well as micronutrients including vitamins, minerals, and pigments (Rønnestad et al., 1999; Ignatius et al., 2001; Hamre et al., 2008). Specifically, copepods contain significantly higher levels of DHA and EPA when compared to enriched rotifers or *Artemia* (Toledo et al., 1999; Koedijk et al., 2010; Lindley et al., 2011) and they also exhibit DHA/EPA ratios that are substantially > 1 (Shields et al., 1999; Bell et al., 2003; Conceição et al., 2010). Moreover, it has been well established that an exogenous supply of free amino acids is necessary to support growth and survival in first feeding larvae (Helland et al., 2003; Lindley et al., 2011), and copepods contain higher levels of free amino acids compared to rotifers, and more than twice the amount per gram wet mass when compared to *Artemia* nauplii (Helland et al., 2003; van der

Meeren et al., 2008). Copepods' biochemical composition displays high stability over time despite large variations in environmental conditions (van der Meeren., 2008), which confer them another major advantage as live feeds for marine larvae.

1.2.3.3 Calanoid and harpacticoid copepods

With the increasing interest in copepods, many species have been used for larviculture, including calanoid copepods such as *Acartia* spp. (Schipp et al., 1999), *Eurytemora* spp. (Shields et al., 1999) and *Parvocalanus* spp. (Olivotto et al., 2006), and harpacticoid copepods such as *Euterpina acutifrons* (Kraul et al., 1992), *Tisbe* spp. (Stottrup and Norsker, 1997; Olivotto et al., 2008a, 2008b, 2008c), and *Trigriopus japonicus* (Fukusho, 1980). Harpacticoids have relatively higher tolerance to temperature and salinity, ability to feed on a wide range of live and inert diets, shorter generation time (8-29 days), faster population growth, the ability to be cultured at high densities, and higher reproductive potential (Støttrup and Norsker, 1997), hence they are often mentioned as the best candidate for mass production. However, they require a large surface area rather than volume, as they are predominantly found on tank walls rather than in the water column. Compared to benthic harpacticoids, calanoid copepods are pelagic throughout their life cycle, hence it is believed to be a better candidate for the pelagic fish larviculture. Additionally, the calanoid copepod possesses higher HUFA content, and is generally smaller in size (Olivotto et al., 2008). Within the families of larval fish that specialize on copepod prey, there is a clear preference for calanoid copepods, particularly small species (Sampey et al., 2007). However, the drawbacks of

calanoids are comparatively lower culture densities and more labor cost (Payne and Rippingale, 2001; Holt, 2003).

#### 1.2.3.4 Limitation of calanoid copepod utilization

Copepods can either be harvested from naturally occurring wild populations, cultivated from outdoor ponds, or they can be cultivated indoors. If harvested from nature, the availability of copepods and nauplii will be highly seasonal and variable, disease and parasites can be introduced in the rearing facility, and control of the nutritional value, species and stages of the copepods is limited. Indoor cultivated copepods eliminate these deficiencies, but have the drawback of limited mass production, since intensive culture of calanoid copepods is still difficult as they can only reach low culture densities (Ianora et al., 1996; Støttrup and Norsker, 1997; Olivotto et al., 2008). Culturing copepods is also labor intensive, requiring large tanks or mesocosms for culture that needs to be fed different algal combinations as diets (Holt, 2003). Despite these drawbacks, recent research has led to substantial improvements in calanoid copepod intensive culture techniques (McKinnon et al., 2003; Milione and Zeng, 2007, 2008; Camus and Zeng, 2008, 2009; Camus et al., 2009; Camus and Zeng, 2012). In particular, stable intensive culture and supply of the copepod, *Parvocalanus crassirostris*, has been achieved (Alajmi and Zeng, 2014, 2015; Alajmi et al., 2014, 2015; Kline and Laidley, 2015). Given that marine ornamentals generally produce far fewer larvae compared to most foodfish species, the utilization of copepods for commercial larval culture is more feasible.

**Table 1.1** Summary of using copepods as the first feeding prey for marine ornamental fish

| Common name | Species | Copepods | References |
|---|---|---|---|
| Lemonpeel angelfish | *Centropyge flavissimus* | Calanoid - *Parvocalanus* spp. | Olivotto et al., 2006 |
| Flame angelfish | *Centropyge loriculus* | Calanoid - *Parvocalanus crassirostris*. | Laidley et al., 2008 |
| Yellow tang | *Zebrasoma flavescens* | Calanoid - *Parvocalanus crassirostris* | Laidley et al., 2009 |
| Peacock hind | *Cephalopholis argus* | Calanoid - *Parvocalanus crassirostris* | Ajiboye et al., 2011 |
| Small-mouthed pipefish | *Stigmatopora argus* | Calanoid - *Gladioferens imparipes* | Payne et al., 1998 |
| Yellowtail clownfish | *Amphiprion clarkia* | Calanoid - *Centropage typicus* | Olivotto et al., 2008 |
| Striped blenny | *Meiacanthus grammistes* | Harpacticoid - *Tisbe* spp. | Olivotto et al., 2010 |
| Threespot damselfish | *Dascyllus trimaculatus* | Harpacticoid - *Euterpina acutifrons* and Calanoid - *Pseudodiaptomus serricaudatus* | Gopakumar and Santhosi, 2007; Gopakumar et al., 2007 |
| Humbug damselfish | *Dasyllus aruanus* | Harpacticoid - *Euterpina acutifrons* and Calanoid - *Pseudodiaptomus serricaudatus* | Gopakumar and Santhosi, 2007; Gopakumar et al., 2007 |
| Blue damselfish | *Pomacentrus caeruleus* | Harpacticoid - *Euterpina acutifrons* and Calanoid - *Pseudodiaptomus serricaudatus* | Gopakumar and Santhosi, 2007; Gopakumar et al., 2007 |
| Sapphire devil damselfish | *Chrysiptera cyanea* | Harpacticoid - *Euterpina acutifrons* and Calanoid - *Pseudodiaptomus serricaudatus* | Gopakumar et al., 2009 |
| Blue Mauritius angelfish | *Centropyge debelius* | Wild copepods, primarily as Calanoid - *Clausocalanus* sp. and Cyclopoid - *Oithona* sp. | Baensch and Tamaru, 2009 |
| Green mandarinfish | *Synchiropus splendidus* | Combination of rotifers and wild copepods | Wittenrich et al., 2010 |

## 1.3 Live prey selection for first feeding larvae

The prey selection of fish larvae is affected by a number of factors, related both to the prey and larvae (Planas and Cunha, 1999; Olivotto et al., 2010). Larval foraging ability; visual acuity (Neave, 1984); prey contrast (Dendrinos et al., 1984); prey density, shape and mobility (Holmes and Gibson, 1986); and the relationship between mouth size and prey size are of most importance in determining food selection (Shirota, 1970).

### *1.3.1 Prey size and larval mouth gape*

The prey size relative to the mouth gape of fish larvae is crucial in determining whether the prey can be ingested by the larvae (Yúfera and Darias, 2007). Therefore, it is generally accepted that most first foods considered for culture of marine ornamental fish should range in size between 50 and 100 μm (Ostrowski and Laidley, 2000). At the beginning of feeding, prey is selected primarily by size rather than taste or other factors. Fish larvae are able to ingest prey similar in size to their mouth gape, but they prefer smaller prey. The most appropriate prey/gape ratio should be less than 50% (Shirota, 1970; Planas and Cunha, 1999; Østergaard et al., 2005). The age-dependent increase in size during the larval stages results in increased mouth size and swimming speed (Sih, 1993). Rapid increase of jaws and gape helps the larvae to increase size of prey ingestion (Shirota, 1970; Fernández-Díaz et al., 1994; Polo et al., 1992). The number of prey consumed also increases with age. Smaller prey should be easier to capture and therefore are consumed more than larger prey types during the early stages.

### *1.3.2 Prey swimming behavior and larval response*

Aside from size, other prey characteristics, including pattern of swimming motion and velocity, predator avoidance behavior, and relative availability, have all been found to affect prey selection by fish larvae (Checkly Jr, 1982; Meng and Orsi, 1991). In many cases, the inability of larvae to eat certain live food organisms may not be linked with size, but with swimming behavior of the prey items. For example, it has been shown that the larvae of some marine species (e.g. *Pomacanthus* spp.) simply refuse to eat rotifers even though the larvae are large enough to capture them (Young, 1994). In general, live zooplankton species such as rotifers and cladocerans have much lower rates of swimming velocity compared to copepods and do not show strong evasive response to predators, which means early larvae with limited swimming ability are likely to be more efficient in capturing them (Epp and Lewis, 1984). Furthermore, the characteristic irregular, zigzag fast swimming motion of the copepods as opposed to the consistent whirling movement of the rotifers may serve as a critical factor in triggering larval feeding response in early larvae, resulting in substantially increased ingestion rates (Buskey et al., 1993). For example, it has been reported that compared to the slow circular motion of rotifers, the characteristic jerking (i.e. zigzag) swimming motion of copepod nauplii stimulated predatory behavior in first feeding larvae of lemonpeel angelfish *Centropyge flavissimus* (Moe, 1997; Leu et al., 2009). Larval preference for copepod swimming motion has also been found in the orchid dottyback *Pseudochromis fridmani* larvae (Olivotto et al., 2006). This could be explained by the fact that copepods are the natural prey for most fish larvae, hence larvae have innate preference to their swimming motion.

Moreover, in a study on larval feeding of the perch *Perca fluviatilis*, Furnass (1979) concluded that the amplitude of prey movement was an important influencing factor in prey selection, since the smaller prey preferably selected by the larvae was substantially more active than the larger prey simultaneously available. Similarly, Utne-Palm (1999) reported that prey with higher activity increased the chances of prey detection and reaction distance of two-spotted goby, *Gobiusculus flavescens* larvae.

### *1.3.3 Ingestion rate of first feeding larvae*

In general, ingestion rate rises with higher prey density, which is attributed to the higher encounter rate. It has been demonstrated that increasing food or prey density increases ingestion rate in common carp, *Cyprinus carpio* (Jana and Chakrabarti, 1990), yellow perch, *Perca flavescens* (Graeb et al., 2004), and Mayan cichlids, *Cichlasoma urophthalmus* (Farhadian et al., 2012). Ingestion rate also increases with age, which contributes to the higher capture success of prey, as vision and attack skills in older fish larvae improve (Gerking, 1994). Moreover, the increased ingestion rate may be triggered by other species. For example, Moe (1997) argued that the stop-and-go motion of copepod nauplii was needed to initiate predatory behavior in the large angelfishes (*Pomacanthus* spp.), compared to the simple whirling motion of the rotifer. As a result, copepods may have an appetite stimulatory effect, which has been shown to increase ingestion rates of rotifers or cladocerans (Barroso et al., 2013; Farhadian et al., 2012). In the first several days of feeding, the larvae can ingest more than 100% of their own weight (Parra and Yúfera, 2000, 2001; Wuenschel and Werner, 2004). However, various studies showed the ingestion rates on marine fish larvae are highly variable.

Besides the variability, the ingestion estimations are strongly affected by methodology and experimental conditions. The difficulty in obtaining reliable data at the first feeding period is still high, during which not all larvae have begun eating yet, and a sharp increase in foraging can occur in the span of a few hours (Yúfera et al., 1993).

### *1.3.4 Feeding behavior of first feeding larvae*

Better understanding of larval feeding behavior not only provides insights into the predator-prey interaction, which is critical to the development of appropriate feeding regimes, but also helps in testing a fish model related to foraging capabilities (Juanes and Conover, 1994). Moreover, behavioral observations are useful in revealing underlying reasons for larval prey selection and foraging efficiency. For instance, cod larvae fed intensively cultivated copepods with a high lipid content have been shown to have a higher activity level and to be more effective predators than larvae fed rotifers of a lower quality in regard to lipid content (O'Brien-MacDonald et al., 2006). The increased efficiency in foraging prey will benefit net energy gain and consequently growth and survival (Dill, 1983; Wahl et al., 1995), and feeding behavior analysis can be used to assess this ability. Measurements of the larval quality can be based on swimming duration or prey capture success.

The sequential events required for successful prey capture are of paramount importance in determining feeding regimes. The events usually start with a passing encounter. Encounter opportunity depends on prey density, larval age, and aggressiveness. Next is targeting (larva turn and orient towards the prey),

followed by attack (larva move toward the prey), then capture (prey is inside the mouth of the larva), and finally either ingestion (larva consume prey) or rejection (larva eject prey from mouth)(Greene, 1985). In quite a few cases, prey can also escape after being captured by the fish larvae. Since each of these events can be easily quantified in a given period of feeding time, zooplankton of varying body sizes can be used to detect the most suitable prey for a given age group of larval fish. Finally, all the data can be used to calculate the attack rate (number of prey attacks as a percentage of targetings), capture rate (prey captures as a percentage of attacks), rejection rate (prey rejections as a percentage of captures), and the overall feeding efficiency (number of prey ingested as a percentage of targetings).

## 1.4 Lipid nutrition in marine fish larvae

### *1.4.1 Absolute requirements of essential fatty acids in marine fish larvae*

#### 1.4.1.1 n-3 long-chain polyunsaturated fatty acids: docosahexaenoic and eicosapentaenoic acid

Dietary lipids are important sources of energy and fatty acids are essential for normal growth and survival of fish. Of the various lipids in the diets of marine larvae, phospholipids (PL) and essential fatty acids (EFA) are two lipid components that appear to have received the highest attention in past research (Cahu and Infante, 2001; Kanazawa, 2003; Tocher, 2003; Glencross, 2009). Marine larvae require dietary provision of EFAs for normal growth and survival, particularly long chain polyunsaturated fatty acids (LC-PUFAs), including docosahexaenoic acid (22:6n-3; DHA), eicosapentaenoic acid (20:5n-3; EPA) of n-3 LC-PUFAs and arachidonic acid (20:4n-6; ARA) of n-6 LC-PUFAs (Rainuzzo et al., 1997; Bell et al., 2003). The n-3 LC-PUFAs have long been

identified as essential dietary components for marine fish, since marine fish can neither biosynthesize DHA not de novo or from shorter chain precursors such as linolenic acids (18:3n-3) (Sargent et al., 1997, 1999).

Several studies have demonstrated that a deficiency in n-3 LC-PUFAs will result in an overall health decrease of larvae, physical anomalies in the central nervous system, impaired visual development (Velu and Munuswamy, 2003), growth decrease, challenged metamorphosis (Watanabe and Kiron, 1994), reduced stress resistance (Vagelli, 2004), abnormal swimming and feeding activity (Olivotto et al., 2008; Olivotto et al., 2006a), and abnormal pigmentation (McEvoy et al., 1998). Based upon these effects, the optimal amounts of the correct EFA to satisfy the requirements for normal growth and development has become one of the most studied areas of lipid metabolism in marine fish larvae. The optimum dietary levels for n-3 LC-PUFAs have been determined for larvae of several species and they differ over a range between 0.3 and 39 mg/g dry weight (Izquierdo, 1996). For examples, the optimum n-3 LC-PUFA requirement level of 10-17 mg/g DM was reported for Asian seabass larvae, *Lates calcerifer* by Boonyaratpalin (1991), 10-15 mg/g DM for gilthead bream larvae and juvenile, *Sparus aurata* by Rodriguez et al (1998), 27.5 mg/g DM for silver pomfret early juvenile, *P. argenteus* by Hossain et al (2012). Moreover, DHA is known to have a higher efficiency as an essential fatty acid than EPA in improving growth, survival, and stress tolerance of many fish larvae (Watanabe et al., 1989; Watanabe and Kiron, 1994). The results showed that the optimum level of dietary DHA to support optimal growth and survival in silver pomfret, *Pampus argenteus*, is 15.2 mg DHA/g DM (Hossain et al., 2012), 20.8 mg DHA/g DM found for striped trumpeter, *Latris lineata*, by Bransden et al. (2005) and 17 mg DHA/g DM

for striped jack, *Pseudocaranx dentex*, by Takeuchi et al. (1992). However, Senegal sole, *Solea senegalensis*, have a low or negligible requirement for DHA, with 0.1-4.9 DHA mg/g DM reported by Morais et al. (2004). Similar results have also been obtained for some other nectobenthic fish, including Japanese flounder larvae, *Paralichthys olivaceus* (Izquierdo et al., 1992), plaice larvae, *Pleuronectes platessa* (Dickey-Collas and Geffen, 1992), and California halibut larvae, *Paralichthys californicus* (Vizcaino-Ochoa et al., 2010), which do not require DHA in the diet if adequate EPA is present or other n-3 LC-PUFAs are provided in the diet. These discrepancies also suggest that the difference between effects of DHA and EPA on fish is species-specific.

1.4.1.2 n-6 long-chain polyunsaturated fatty acids: arachidonic acid

Although the essentiality of n-3 LC-PUFAs for larval marine fish has been extensively studied, little research has focused on the requirement of ARA of n-6 LC-PUFAs, and only recent studies have demonstrated the importance of dietary ARA in the larvae of a few species. ARA may improve larval growth and pigmentation in several marine fish species since it provides precursors for eicosanoid production. The importance of ARA for normal growth and development has been demonstrated in turbot, *Psetta maxima* (Castell et al., 1994; Bell et al., 2003) and the efficacy of supplemental ARA in broodstock diets to improve egg and larval quality in Atlantic halibut, *Hippoglossus hippoglossus* (Mazorra et al., 2003). At a fixed dietary n-3 LC-PUFA level and DHA:EPA ratio, the elevation of ARA levels from 0.1 to 1.0% in microdiets for larval gilthead seabream, *Sparua aurata*, significantly improved larval survival, but only slightly enhanced growth (Bessonart et al., 1999). Meanwhile, ARA may act in a different way depending on the DHA concentration (Castell et al., 1994; Koven et al.,

2000). ARA seems to play an important role in flatfish pigmentation and eye migration, since in turbot, ARA levels in neural tissue lipids were negatively correlated with pigmentation (Estévez et al., 1997). Further studies showed that yellowtail flounder larvae required diets highly enriched in DHA whereas high dietary ARA inhibited growth, increased mortality and had negative effects on pigmentation (Ishizaki et al., 1998). Therefore, there is increasing evidence for the essentiality of dietary ARA for optimal growth and development of marine fish larvae, although excess can cause problems in some cases (Rodriguez et al., 1994; Ishizaki et al., 1998; Estévez et al., 1999; Hamre et al., 2007; Lund et al., 2007).

### *1.4.2 Optimal balance among essential fatty acids in marine fish larvae*

Evidence for the competitive interactions among EFAs (eg. the synthesis of eicosanoids between ARA and EPA, the synthesis of LC-PUFAs between n-3 and n-6 fatty acid series, DHA and EPA) suggest that there is a need to control not only EFAs absolute amounts in diets, but also the dietary proportions among the EFAs. Besides a minimum dietary requirement for each essential fatty acid, the relative proportions among the different PUFAs seems to be related to the best growth rates. Rodriguez et al. (1997) found high amounts of dietary EPA in relation to DHA may create structural imbalances in the composition of phospholipids and negatively affect growth and quality in cultured larvae, with significantly higher growth rates in sea bream larvae fed rotifers with a DHA/EPA ratio of 1.5 compared to those fed DHA/EPA ratios < 0.6. In addition, the egg lipid composition provides a valuable approximation of the optimal lipid composition of a larval diet, therefore, the ideal diet for fish larvae should have a lipid composition close to that of the fish egg (Sargent et al., 1999). Previous

studies on fish eggs have shown that most have a natural ratio of DHA:EPA of > 2 (Tocher and Sargent, 1984). The study conducted by Rainuzzo et al. (1994) showed that the malpigmentation was eliminated in turbot larvae when the DHA/EPA of the live prey was > 2. Further studies demonstrated the optimum DHA/EPA value for other marine fish larvae, including milkfish (DHA/EPA>1; Gapasin and Duray, 2001), starry flounder (DHA/EPA, 1.24; Ma et al., 2012) and grouper (DHA/EPA, 1.4; Wu et al., 2002). However, the question of what is the optimal ratio of DHA/EPA for marine fish larvae is strictly a question of what are the correct levels of DHA/EPA/ARA, this is because elevating the level of DHA simultaneously reduces the level of EPA, altering the ratio of ARA/EPA. As the competitive interaction of eicosanoid production is influenced by the dietary and cellular ratio of ARA/EPA, and imbalanced ratio of ARA/EPA appears to be as damaging in fish; therefore, the ratio of ARA/EPA in the diet seems important as well (Bell et al., 2003a). Unfortunately, little effort has focused on this area, possibly due to expensive and complicated experimental design (González-Félix et al., 2003).

**Table 1.2** Summary of dietary requirement of ARA, EPA or DHA for marine fish larvae

| Species | Common name | Fatty acids delivery | EPA | ARA | DHA | Value | Criteria | Feeding period | Reference |
|---|---|---|---|---|---|---|---|---|---|
| *Paralichthys californicus* | California halibut | *Artemia* enriched with different levels of DHA | -- | -- | 1.21-2.4% of TFA | DHA<EPA; DHA<ARA | S, G, BFA, P, WS | 32 days | Vizcaino-Ochoa et al., 2010 |
| *Paralichthys Olivaceus* | Japanese flounder | *Artemia* enriched with different levels of n-3 LC-PUFA | -- | -- | -- | DHA<EPA | S, G, BFA | 34 days | Izquierdo et al., 1992 |
| *Pleuronectes platessa* | Plaice | *Artemia* enriched with EPA and DHA | 1.9-10.1% of TFA | -- | 0-6.9% of TFA | DHA<EPA | S, G, | 50 days | Dickey-Collas and Geffen, 1992 |
| *Solea senegalensis* | Senegalese sole | *Artemia* enriched with LC-PUFA or sunflower oil or algal mixture | -- | -- | 0.1-4.9 mg/g DM | DHA<EPA | S, G, BFA | 12-28 days | Morais et al., 2004 |
| *Solea senegalensis* | Senegalese sole | Rotifers and *Artemia* enriched with different level of ARA | -- | -- | -- | DHA<ARA | S, G, BFA, P | 34 days | Villalta et al., 2005 |
| *Latris lineata* | Striped trumpeter | *Artemia* enriched with different levels of DHA | -- | -- | 20.8 mg/g DM | -- | S, G, BFA | 20 days | Bransden et al., 2005 |
| *Sparua aurata* | Gilthead seabream | Rotifers enriched with different ratios of EPA/DHA | -- | -- | -- | DHA>EPA | S, G BFA | 7 and 14 days | Rodriguez et al., 1997 |
| *Sparua aurata* | Gilthead seabream | Microdiets contained different levels of ARA | -- | 1.0-1.8% dry weight | -- | -- | S, G BFA | 21 days | Bessonart et al., 1999 |
| *Sparua aurata* | Gilthead seabream | Rotifers and *Artemia* enriched with different ratios of DHA/ARA | -- | -- | 8.9-23.0 mg/g DM | DHA>ARA | S,G, SR | 30 days | Koven et al., 2001 |
| *Seriola quinqueradiata* | Yellowtail flounder | *Artemia* enriched with DHA and ARA | -- | -- | -- | DHA>ARA | S, G | 13 days | Ishizaki et al., 1998 |

Notes: DM: dry matter; TFA: total fatty acids; S: survival; G: growth; BFA: body fatty acids; P: pigmentation; WS: weaning success; SR: stress resistance

### *1.4.3 Importance of essential fatty acids in marine ornamental larvae*

Like foodfish species, the importance of EFAs to marine ornamental larvae has been clearly demonstrated, achieving better survival and growth (Olivotto et al., 2003, 2005, 2006a, 2006b, 2008a; Avella et al., 2007), as well as decreasing anomalies in the development of the central nervous system (Avella et al., 2007). DHA must be present in the diet to maximize survival of larvae of the coral reef damselfish, *Acanthochromis polyacanthus* (Southgate and Kavanagh, 1999). Of additional interest to the marine ornamental industry are findings that suggest levels of DHA, EPA, and ARA in larval diets could affect 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 underlying mechanisms for such a correlation need further investigation.

### *1.4.4 Essential fatty acids delivery via live preys*

Compared to the juvenile and adult marine fish culture, formulated feeds are rarely used for the marine larvae culture (Cahu and Infante, 2001; Koven et al., 2001; Robin and Vincent, 2003; Kvåle et al., 2006). This is largely due to the small size and often poorly developed digestive system of marine fish larvae, thus having a major consequence with respect to defining their precise EFA requirements (Izquierdo et al., 2000; Conceição et al., 2010; Yúfera and Darias,

2007). Therefore, using live prey as the first feed become essential for successful marine larviculture. The LC-PUFA content of live prey is often considered to be the critical factor indicating their nutritional quality, and failure to provide required quantities of LC-PUFAs is a primary cause of the unsuccessful larval rearing of some marine species (Sargent et al., 1999; Glencross, 2009). There are three main methods of LC-PUFA delivery to first-feeding marine fish larvae, including via copepods, rotifers and *Artemia*. However, the latter two are nutritionally inadequate for marine fish, requiring supplementary enrichment to augment a natural paucity of n-3 LC-PUFAs. By using DHA-rich fish oil, such as emulsified tuna orbital oil, it is possible to achieve DHA/EPA ratio of > 2 in rotifers. However, the difficulty in enriching *Artemia* nauplii with DHA is a major drawback to their suitability as first-feeding prey for marine fish larvae, due to the marked propensity of *Artemia* to retroconvert DHA to EPA and therefore, contains significant amounts of EPA but negligible amounts of DHA (Navarro et al., 1999).

### *1.4.5 Saturated and monounsaturated fatty acids in marine fish larvae*

Most lipid nutrition studies in marine fish larvae have focused on supplying supplemental EFA to meet requirements for optimal larval growth and development. However, it should not be forgotten that other components of lipids, particularly saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), provide the “fuels” for fish growth. SFAs and MUFAs are considered preferred energy sources for some fish, although the preferred utilization and digestibility of those SFAs and MUFAs maybe vary from species to species (Tocher, 2003). Therefore, it is vitally important to strike a balance between

LC-PUFAs that are required for key functions, such as functional integrity of cell membranes, and other less unsaturated fatty acids required for energy production (Sargent et al., 2002). This may be particularly important for species with lower LC-PUFA requirements, such as nectobenthic Senegalese sole (Navarro-Guillen et al., 2014). In addition, providing sufficient amounts of SFAs and MUFAs in the diets is particularly important in reducing oxidation of the diets (Sargent et al., 1999). Clearly, more attention should be given to identifying the proper balance between LC-PUFAs and other energy-yielding fatty acids (the balance of dietary SFA:MUFA:PUFA ratios) in future fish larvae nutritional studies.

## 1.5 About this thesis

### *1.5.1 The selection of candidate species：Green mandarin fish*

The taxonomy of the green mandarin fish, *Synchiropus splendidus*, can be summarized as follows (Herre, 1927):

Phylum: Chordata

Class: Actinopterygii

Order: Perciformes

Suborder: Callionymoidei

Family: Callionymidae

Genus: *Synchiropus*

Species: *S. splendidus*

Common names: green mandarin, mandarinfish, mandarin dragonet

*S. splendidus* is a small, brilliantly colored, benthic marine fish distributed within the tropical Pacific and Indian Oceans. It is a very attractive species within

the group of fish known as dragonets (Family: *Callionymidae*), and is among the most desirable species to marine aquarium hobbyists worldwide (Sadovy et al., 2001). Unfortunately, the current supply of green mandarin fish for the aquarium trade is completely from the wild, and it is further observed that most specimens for sale are males (likely due to their relatively large size compared to females), suggesting selective harvesting from the wild populations. The selective fishing for males could negatively impact wild populations by resulting in female biased populations. Moreover, with larger males being selectively harvested, females might become increasingly reluctant to pair with the progressively smaller remaining males, leading to increased numbers of failed paring behavior of pre-spawning rises, and hence a longer time spent in the water column leading to higher possibility of predation (Sadovy et al., 2001). As a result, the captive breeding of *S. splendidus* is proposed as an alternative for a sustainable supply of this popular species for the aquarium trade. However, due to its strict diet on the live feeds, *S. splendidus* has been considered to be a hard-to-culture species. Moreover, as a pelagic-spawner, *S. splendidus* newly hatched larvae are very tiny, on average only 1.5 mm, and lack pigmented eyes, a digestive system, or a mouth (hatch as prolarvae). At this stage, they still have large yolk reserves that are used to undergo a second development phase of about 48 h in the water column. After this period, the prolarvae developed into active larvae with pigmented eyes and a rudimentary but functional digestive system. The extreme vulnerability of prolarvae to environmental conditions as well as the small mouth-gape makes rearing them even more difficult.

In recent years, the marine ornamental aquaculture research group at JCU has engaged in research on the development of captive breeding techniques for *S.*

*splendidus*. Previous research from this laboratory has shown that feeding rotifer alone, even enriched with enrichment medium characterized copepod nutrition profile, could not support decent survival of its early larvae (3-5% on 11 day post-hatching, DPH). However, when copepods were co-fed with rotifers at 2 copepods $mL^{-1}$, larval survival dramatically improved (up to 50% on 12 DPH) (Ricketts, 2012). Clearly, further investigations are urgently needed in order to establish a complete and reliable larval culture protocol for *S. splendidus* to ensure that it will become a reality that wild capture specimens can be replaced by the captive-bred ones.

(Adult male green mandarin fish, *Synchiropus splendidus*
Captive bred by Luchang Shao, photo by Stephen Zozaya)

*1.5.2 Aims and outlines of thesis chapter*

This PhD project focused on the first feeding of *S. splendidus* larvae, prey selection and feeding behavior, tissue fatty acids profile change with ontogenetic larval development, the optimal timing of diet transition, as well as effects of different LC-PUFAs levels on growth and functional development of post-settlement *S. splendidus*. All of these are largely unknown for this species in ornamental aquaculture. The overall aim is to expand our knowledge base of early life stage *S. splendidus*, and devise a reliable and cost-effective feeding protocol for larval culture. The results from this project could benefit the entire marine aquaculture community, and eventually help alleviate pressure on wild populations, which helping reef conservation while promoting an emerging marine ornamental aquaculture industry.

A series of experiments was designed and carried out using a quantitative approach in line with the following specific aims:

1. To review information available on marine ornamental industry and aquaculture, larval first feeding and prey selection and lipid nutrition in marine fish larvae, to provide knowledge basis for subsequent studies on early and late *S. splendidus* larvae (Chapter 1).
2. To provide general materials and methods on broodstock maintenance, live prey culture and experimental larvae rearing system (Chapter 2).
3. To assess the effects of copepod density and rotifer co-feeding on early *S. splendidus* larvae culture and to gain insight into the importance of

copepods as larvae prey through larval ingestion study (Chapter 3).

4. To evaluate the possibility of reducing the use of copepods by switching prey to rotifers for early *S. splendidus* larvae and to analyze the larval feeding behavior and efficiency (Chapter 4).
5. To assess the ontogenetic changes in the fatty acid composition of *S. splendidus* from the eggs, newly hatched and growing larvae as well as that of two live preys (Chapter 5).
6. To identify the optimal time for introducing *Artemia* nauplii and subsequently enriched metanauplii in either live or dead form for post-settlement *S. splendidus* (Chapter 6).
7. To determine the optimal level of dietary DHA for post-settlement *S. splendidus* (Chapter 7).
8. To develop a better understanding on the effect of dietary ARA on the survival and growth of post-settlement *S. splendidus* (Chapter 8).
9. To summarize all the results and posit future directions (Chapter 9).

# Chapter 2

## General materials and methods

### 2.1 Green mandarin fish, *Synchiropus splendidus* broodstock maintenance

*S. splendidus* broodstock fish were obtained from a commercial aquarium supplier in 2012. They were separated into four breeding pairs and each pair was kept in a 200 L cuboid tank. All the tanks were supplied with terracotta pot hides and the green seaweed *Caulerpa taxifolia*. The tanks were located in an outdoor, undercover area subjected to the natural photoperiod and connected to a temperature controlled recirculating system. During the study, water temperature of the recirculating system was maintained between 27.5-29.5 °C, salinity 29-37 ‰, pH 8.0-8.2, $NH_3$, $NO_2$ < 0.2 ppm, and $NO_3$ < 6 ppm. Feeding occurred twice daily in the early morning (7:00-8:00 am) and later afternoon (5:00-6:00 pm). The broodstock fish were fed a home-made gelatin bound diet developed for broodstock fish by this laboratory, which consisted of a mix of blended fresh fish, squid, mussel and prawn meat, with added multi-vitamin, dry spirulina and mineral supplements.

### 2.2 Rotifers, *Branchionus rotundiformis* culture and harvest

The rotifers, *B. rotundiformis* were cultured in 100 L conical tanks and fed *Nannochloropsis* sp. paste daily (Nanno 3600 Instant Algae) (Reed Mariculture Inc, California, USA). During the experiments, rotifers were harvested immediately before each feeding by sieving through a 53 µm mesh. The harvested rotifers were then quickly rinsed with clean seawater and concentrated into a beaker. The rotifer density in the beaker was estimated by averaging three 10

$mL^{-1}$ sub-samples taken from the beaker. The number of rotifers in each sub-sample was counted using a Bogorov's plate chamber under a microscope (Leica CME).

### 2.3 Copepods, *Parvocalanus crassirostris* culture and harvest

The copepods, *P. crassirostris* have been domesticated and maintained in this laboratory for more than 4 years. *P. crassirostris* were also cultured in a series of 250 L conical tanks. The copepods were fed daily on a mixed algal diet of *Isochrysis* spp. and *Chaeroceros muelleri* (at a 1:1 ratio) based on culture method developed in this laboratory (Alajmi et al., 2015). Similar to rotifers, harvesting was conducted prior to each feeding by siphoning water from the tanks through firstly a 150 µm mesh and then a 25 µm mesh. The 150 µm mesh collected mostly adults while the 25 µm mesh collected nauplii and copepodites. Only nauplii and copepodites collected on 25 µm mesh were used for feeding larvae. Hence, the adults collected on 150 µm mesh were returned to the culture tanks while *P. crassirostris* retained on 25 µm mesh were rinsed and then counted to determine their density before feeding to the larvae. The density estimation procedure was similar to that of rotifers as described above.

### 2.4 Microalgae culture

The Tahitian strain of *Isochrysis* species ('T-iso', class Prymnesiophyceae; CS-177) and *C. muelleri* ('Chaet', class Bacillariophyceae; CS-176), were inoculated from starter cultures supplied by the Commonwealth Scientific and Industrial Research Organization (CSIRO) Microalgal Supply Service, Hobart, Tasmania, Australia. Nutrients used for the culture of both species were f/2 concentration (Guillard and Ryther, 1962) with the addition of silicates for the

diatom *C. muelleri*.

Both microalgae were cultured indoor using 20 L polycarbonate carboys and were maintained in a temperature controlled room (25 ± 1 °C) using 1 µm-filtered and UV irradiated seawater at 30 ppt with vigorous aeration (0.2 µm filtered air). The photoperiod was set at light : dark cycle=12h : 12 h with a light intensity of approximately 5000 Lux. Concentrations of algal culture were assessed using a haemocytometer and a microscope (Leica CME).

### 2.5 *S. splendidus* incubation and experimental rearing system

The spawning of *S. splendidus* was found to generally occur approximately 30 min after sunset (Ricketts, 2012; from this laboratory). Prior to an experiment, eggs naturally spawned by the four pairs of broodstock on a same day were collected and immediately transferred to 3 L clear, cylindrical incubation buckets with temperature controlled at 27 ± 1 °C via a water bath. Incubation was carried out under constant light and without aeration. Under such condition, newly hatched larvae (also called prolarvae) generally took two days to develop into the first feeding larvae with pigmented eyes and functional mouth, hence the newly hatched larvae were left in the incubation buckets until 2 days post-hatching (DPH). On 2 DPH, healthy larvae were randomly selected and transferred from the hatching buckets into each of 3 L purposely-designed cylindrical rearing vessels (Moorhead, 2015). These vessels had inside walls painted black and have a double-floored bottom with an upper 173 µm mesh floor allowing rotifers and copepods to pass through but not early larvae (For *Artemia* feeding experiments, 1000 µm mesh was used to allow *Artemia* passing through but not late larvae). In-between the mesh floor and a solid PVC lower floor, there was an external

standpipe outlet that allows flushing out of live preys and detritus smaller than 173 µm (or 1000 µm) during daily water exchanges, which was done by switching on a controlled flow of seawater from an inlet connected to a recirculating system to the culture vessel. The aeration was supplied gently through an air-lift inlet directed toward the water surface of a culture vessel to gently circulate water within the vessel and to minimize negative effects of turbulence generated by aeration on fragile early larvae (Fig. 2.1)

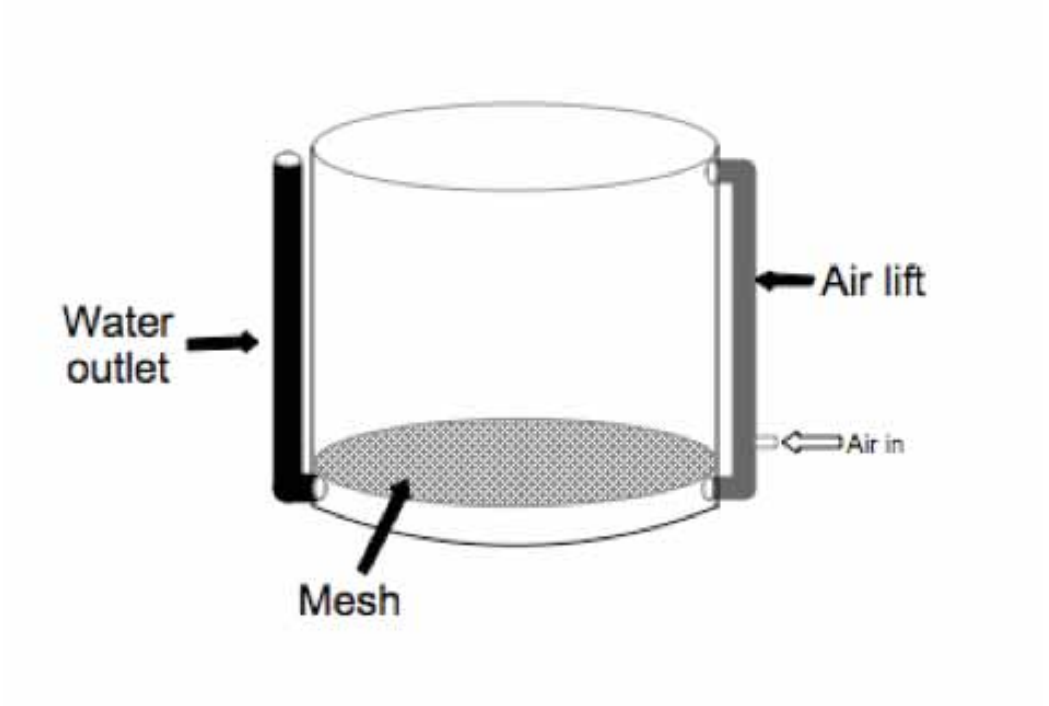


**Figure 2.1.** The schematic of a 3 L larval rearing vessel. The water outlet regulates the water level in the vessel while the nylon mesh separates the draw space and the culture chamber. Both airlift and standpipe inlets are located under the mesh in the draw space with the airlift outlet returning water drained from the drawn space to the culture chamber (Moorhead, 2015).

All culture vessels were placed within a temperature controlled water bath and individually connected to a recirculating system with a 500 L sump, a 10 µm filtration system, a biological trickle filter and a protein skimmer. During the

culture experiments, water exchange in the each replicate vessel was carried out twice daily at 09:00 am and 5:00 pm with a gentle flow (approximately 250 mL $min^{-1}$) of seawater from the recirculating system through a small inlet into each culture vessel from the inlet for 40-60 min. With the design of the culture vessels, such water exchange procedure flushed out almost all larval prey in each culture vessel, hence after each water exchange, larval prey were totally renewed by adding freshly harvested prey based on experimental design. The recirculating system itself was given a 30% water exchange twice daily with fresh natural seawater. Throughout the experiments, temperature was maintained 26-29 °C, salinity 34-38 ‰, pH 8.0-8.2, $NH_3$, $NO_2$ < 0.02 ppm, and $NO_3$ < 0.5 ppm and a constant light photoperiod was adopted.

# Chapter 3

## Insights into the importance of copepods as larval prey of *Synchiropus splendidus*: effects of copepod density, rotifer co-feeding and larval ingestion rate

### 3.1 Introduction

In recent decades, the interest in the trade of marine ornamental fish has increased significantly (Job, 2011; Moorhead and Zeng, 2010). However, it is estimated that between 90-98% of marine ornamental fish sold for aquarium trade are collected directly from the wild. The overexploitation of wild stocks and use of damaging harvesting techniques (eg. Cyanide and explosive) are believed to result in environmental damages for frail coral reefs and marine ecosystems (Vaz et al., 2012). Therefore, a number of different scientists have proposed studying the captive breeding techniques for some species most commonly used in the aquarium trade (Ignatius et al., 2001; Olivotto et al., 2006a; Olivotto et al., 2006b; Pena et al., 2004). Marine ornamental aquaculture (MOA) is still at a very early stage of development. Of various issues impeding the development of MOA, high mortality associated with early larval rearing, particularly during the first feeding of larvae, has been identified as one of the most crucial obstacles (Moorhead and Zeng, 2010; Olivotto et al., 2011; Ostrowski and Laidley, 2001).

Traditionally, rotifers and *Artemia* are the two live prey routinely used in fish hatcheries, however both of them have been found to be too big for larvae of species with small mouth gapes at the first feeding; and/or not induce sufficient larval predatory activity as well as with inadequate nutritional profiles (Olivotto et al., 2011). As the natural food of most marine fish larvae, copepods are often

found dominating the gut contents of fish larvae (Baensch and Tamaru, 2009a; Baensch and Tamaru, 2009b; Holt and Holt, 2000), they are hence widely considered as the best alternative to rotifers or *Artemia* as larval diets. The advantages of using copepods as the live prey for fish larvae include that their different developmental stages from nauplii to adults offer a wide range of sizes to suit various larvae, particularly their nauplii of smaller sizes than rotifers can be ingested by larvae with small mouth gaps (Rajkumar, 2006); their appetite stimulatory effects as they generally induce active foraging behavior in fish larvae (Olivotto et al., 2011; Zaleha et al., 2012); as well as their high essential fatty acid contents (Sargent et al., 1999a; Sargent et al., 1999b; Støttrup et al., 1999), good amino acid (Aragão et al., 2004; Rønnestad et al., 1999) and vitamin profiles (Hamre et al., 2008) and containing high levels of digestive enzymes that could be utilized by early larvae with low digest capacity (Conceição et al., 2010).

Among various copepod species that have been used for fish larval culture (Ajiboye et al., 2011; Barroso et al., 2013; Koldewey and Martin-Smith, 2010; Olivotto et al., 2010), the calanoida copepod, *Parvocalanus crassirostris*, have attracted much attention in recent years (Laidley et al., 2008; Olivotto et al., 2011). *P. crassirostris* is a small sized pelagic copepod species, its intensive culture techniques have been improved substantially in recent years (Alajmi and Zeng, 2013; 2014; Alajmi et al., 2014; 2015; Kline and Laidley, 2015), and its production is now considered sufficient for supplying early larvae culture of ornamental species as most ornamentals produced dramatically less larvae per spawning as compared to food fish. The green mandarin fish, *Synchiropus splendidus* (Herre, 1927) is a small colorful dragonet belonging to the family *Callionymidae* and is highly popular for marine ornamental trade worldwide

(Sadovy et al., 2001). Unfortunately, currently the green mandarin fish for the aquarium trade comes solely from the wild from the western Pacific. Moreover, it has been observed that most specimens on sale are males, suggesting there probably has been selectively harvested from the wild populations (Sadovy et al., 2001; Vincent and Sadovy, 1998). Clearly, there is a need to establish a reliable captive breeding technique for *S. splendidus* to supply world aquarium trade with captive bred ones to reduce the pressure on its natural populations.

The green mandarin fish is a pelagic-spawner and has been considered as a hard-to-culture species because its adults generally require live small crustaceans as feed, hence is relatively difficult to maintain under captive condition, and our limited knowledge in larval feeding habitats (Sadovy et al., 2001; Wittenrich et al., 2010). Furthermore, larvae of *Callionymids* family are among the smallest larvae of marine fish, ranging from 1.0 to 2.1 mm in total body length at hatching (Houde, 1984; Leis and Rennis, 1983). Previous research from this laboratory has shown that feeding rotifers alone, early larval survival was very low at only 3-5% on 11 days post-hatching (DPH). However, when *P. crassirostris* were co-fed with rotifers at 2 copepods $mL^{-1}$, larval survival dramatically increased to around 50% on 12 DPH (Ricketts, 2012; in this laboratory). However, it is still unknown if 2 copepods $mL^{-1}$ is the most appropriate copepod feeding density for *S. splendidus* early larvae and whether co-feeding rotifers is necessary. Moreover, what are underlying reasons for the superior quality of the copepod as live prey for early larvae as compared to rotifers? Through a series of purposely-designed experiments, the present study set out to search for answers to above questions.

## 3.2 Materials and Methods

### *3.2.1 Broodstock maintenance*

Broodstock source and husbandry were described in Chapter 2 (Section 2.1).

### *3.2.2 Live prey production*

Rotifers and copepods culture and harvest methods were described in Chapter 2 (Section 2.2 and 2.3).

### *3.2.3 Larval feeding experiment*

#### 3.2.3.1 General setup and procedures

The incubation and experimental system were described in Chapter 2 (Section 2.5).

For both following experiments, daily counting the number of surviving larvae in each replicate vessel was conducted in the morning immediately after water exchange when larvae were easier to see after all prey had been flushed out. At the end of experiments, all surviving larvae were anaesthetized with clove oil at 0.05 ml $L^{-1}$ (AQUI-S®, New Zealand LTD) and photographed under a dissecting microscope (Leica MZ 125) fitted with a digital camera (Olympus DB 25). Images were then imported to image analysis software (ImageJ, Freeware, Wayne Rasband, NIH, USA) for measuring larval notochord length (NL: straight line distance from tip of snout to posterior tip of notochord) and body depth (BD: the vertical distance between body margins (exclusive of fins) through the anterior margin of the pectoral fin base). Larvae were then rinsed with 0.5 M

ammonium formate to remove the salt (Bransden et al., 2005), and put in an oven set at 60 °C for 18 h for the measurement of the dry weight (DW) with a Cahn C-33 micro-balance (precision: 1 μg, Thermo Fisher Scientific Inc, Pittsburgh, Pa, USA).

To assess growth over time, specific growth rate (SGR) based on SL, BD of the 11 DPH larvae from each treatment were calculated using the following formula:

$$SGR\ (\%) = [\ln(F)-\ln(I)]*100/T$$

Where F and I represent the final and initial NL or BD, respectively, and T was the culture duration (days). DW was not included in SGR as the practical difficulty to weight 2 DPH pre-feeding larvae, and DW was not a crucial criteria for ornamental fish.

3.2.3.2 Copepod feeding density experiment

Since copepod production is still difficult and costly while the previous study from this laboratory did not investigate effects of copepod feeding density on *S. splendidus* early larvae (Ricketts, 2012), it is hence necessary to determine if lower copepod densities than 2 $mL^{-1}$ used in the previous study for co-feeding with rotifers could be adopted for larval rearing and achieve compatible result. This is particularly relevant considering that it has been reported that the existing of small number of copepods could stimulate strong larval feeding response on rotifers (Barroso et al., 2013; Farhadian et al., 2012). To this end, five feeding

regimes with *P. crassirostris* provided at 0, 0.1, 0.5, 1 and 2 copepods $mL^{-1}$ respectively with rotifers made up the remain of 10 prey $mL^{-1}$ in total for all treatments; i.e. 1) 2 copepods + 8 rotifers $mL^{-1}$; 2) 1 copepod + 9 rotifers $mL^{-1}$; 3) 0.5 copepod + 9.5 rotifers $mL^{-1}$; 4) 0.1 copepod + 9.9 rotifers $mL^{-1}$; 5) 0 copepod + 10 rotifers $mL^{-1}$, plus an unfed control were set up. Each treatment was allocated 3 replicates and each replicate stocked 20 pre-feeding larvae (2 DPH). The experiment terminated on 11 DPH as previous experiments showed that larval survival generally stabilized from this point on.

3.2.3.3 With or without rotifer co-feeding experiment

It is unclear if rotifers provided for co-feeding with copepods produced any beneficial effects in rearing early larvae of *S. splendidus*; similarly it was also unknown whether such effects might be related to rotifer density used. To investigate these, larval survival and growth were evaluated under 6 feeding treatments: 1) 1 copepod $mL^{-1}$; 2) 1 copepod + 9 rotifers $mL^{-1}$; 3) 0.5 copepod $mL^{-1}$; 4) 0.5 copepod + 9.5 rotifers $mL^{-1}$; 5) 0.1 copepod $mL^{-1}$ and 6) 0.1 copepod + 9.9 rotifers $mL^{-1}$, as well as an unfed control. Each treatment has 3 replicates and each replicate stocked 20 larvae. The experiment was similarly terminated on 11 DPH. Not including 2 copepod $mL^{-1}$ treatment in this experiment was based on the results from the copepod density experiment (3.2.3.2), which showed that copepod co-feeding density at 1 copepod $mL^{-1}$ produced compatible results to the 2 copepods $mL^{-1}$ treatment.

3.2.3.4 Larval ingestion rate and prey selection experiment

To gain insight into why the copepods performed superiorly than the rotifers as live prey for rearing early larvae of *S. splendidus*, ingestion rates of *S.*

*splendidus* larvae at different ages (i.e. 4, 6, 8 and 10 DPH) were compared when they were fed copepods at 1 copepod $mL^{-1}$ only, or rotifers at 10 rotifers $mL^{-1}$ only, as well as under four co-feeding conditions: 2 copepods $mL^{-1}$ + 8 rotifers $mL^{-1}$; 1.0 copepod $mL^{-1}$ + 9 rotifers $mL^{-1}$; 0.5 copepod $mL^{-1}$ + 9.5 rotifers $mL^{-1}$ and 0.1 copepod $mL^{-1}$ + 9.9 rotifers $mL^{-1}$.

In order to allow larvae to acclimatize to both live prey, prior to each feeding rate trial, larvae were fed 1 copepod + 9 rotifers $mL^{-1}$ in the purposely-designed 3 L vessels (Moorhead, 2015). On the days of a feeding rate trial, healthy larvae of a designated age were randomly selected and pre-starved for 6 h before being transferred to 2 L beakers containing prey from one of six different prey treatments specified above. Each treatment allocated five replicates and each replicate stocked forty 4 DPH larvae or twenty 6, 8 or 10 DPH larvae. To determine the mean larval ingestion rate under each feeding condition, eight 10 $mL^{-1}$ sub-samples were first taken from each beaker for counting prey densities, larvae were then added to the beaker and kept for 10 h before they were removed out and final prey density estimated by similarly taking eight 10 $mL^{-1}$ sub-samples. Number of both prey in each sub-sample was counted using a Bogorov's chamber under a microscope (Leica MZ 125). Three control beakers containing only prey items were setup for each treatment to correct for any changes in prey density over the experimental duration.

Ingestion rate ($I_R$) was calculated based on the following formula (Paffenhöfer, 1971):

$$I_R = [(C_0 - C_t)-((C_1 - C_2) * C_0 /C_1)] * [V/nt]$$

Where

$I_R$ = Ingestion rate, i.e. number of prey ingested $larva^{-1}$ $h^{-1}$

$C_0$ = initial prey density in each experimental beaker

$C_t$ = final prey density in each experimental beaker

$C_1$ = initial mean prey density of control beakers

$C_2$ = final mean prey density of control beakers

V = culture water volume of the experimental beaker

n = mean number of larvae at the beginning and the termination of experiment in each experimental beaker

t = experimental duration in hours

The electivity index (E) (Yurochko, 1976) was used to express prey selection by *S. splendidus* larvae when they were fed various combinations of copepods and rotifers:

$$E = (r - p) * (r + p)^{-1}$$

Where r is the percentage of prey type in total ingested diet by fish larvae and p is the percentage of same prey item in the experimental container. The electivity ranges from -1 to +1, indicating from complete avoidance to exclusive selection, respectively.

*3.2.4 Data analysis*

All measurements were expressed as mean ± standard deviation (SD). Data

were first assessed to confirm normality and homogeneity of variance (Levene's test for homogeneity of variances) and where necessary, arcsine-square root or logarithmic transformation was performed prior to analysis. A one-way ANOVA was used to determine significant differences among treatments. If any significant difference was detected, Tukey's multiple range test was then used as the mean separation procedure. A statistical probability of $p<0.05$ was accepted as significant. All statistical analysis were performed using SPSS statistic software version 22 (Statsoft$^{TM}$, Inc.).

## 3.3 Results

### *3.3.1 Copepod density experiment*

All larvae in the unfed control died by 6 DPH. For all other treatments, there were larvae survived to the end of the experiment but survival rates differed substantially ($p<0.05$)(Fig. 3.1). Larval survival to 11 DPH of the 2 copepods + 8 rotifers mL$^{-1}$ and 1 copepod + 9 rotifers mL$^{-1}$ treatments were similar, being 52% and 48 % respectively, which were significantly higher than all other treatments (20%, 10% and 12% for 0.5 copepod + 9.5 rotifers mL$^{-1}$, 0.1 copepod + 9.9 rotifers mL$^{-1}$ and 0 copepod + 10 rotifers mL$^{-1}$ treatment respectively) but no significant difference was detected between themselves ($p>0.05$).

Larval growth in terms of notochord length, body depth and dry weight at the end of the experiment were all not significantly different among treatments that copepods were provided, even copepods were offered at a very low level of 0.1 mL$^{-1}$. However, larval growth for the rotifer feeding only treatment (0 copepod + 10 rotifers mL$^{-1}$) was significantly lower than all treatments copepods were

provided ($p$<0.05)(Tab. 3.1). The specific growth (SGR), for NL was the highest (5.48 % day$^{-1}$) when larvae fed 2 copepods + 8 rotifers mL$^{-1}$, while the lowest SGR (NL: 1.96 % day$^{-1}$) was found for larvae fed 0 copepod + 10 rotifers mL$^{-1}$. The SGR (NL) of 0.1 copepod + 9.9 rotifers mL$^{-1}$ treatment was significantly lower than those of the other three treatments that copepods were provided but higher than that of rotifers feeding only treatment ($p$<0.05). The SGR for BD followed a similar trend as SGR based on NL, with the highest value (4.42 % day$^{-1}$) obtained for larvae fed 2 copepods + 8 rotifers mL$^{-1}$ and the lowest value (1.02 % day$^{-1}$) found for larvae fed 0 copepod + 10 rotifers mL$^{-1}$ (Tab. 3.1).

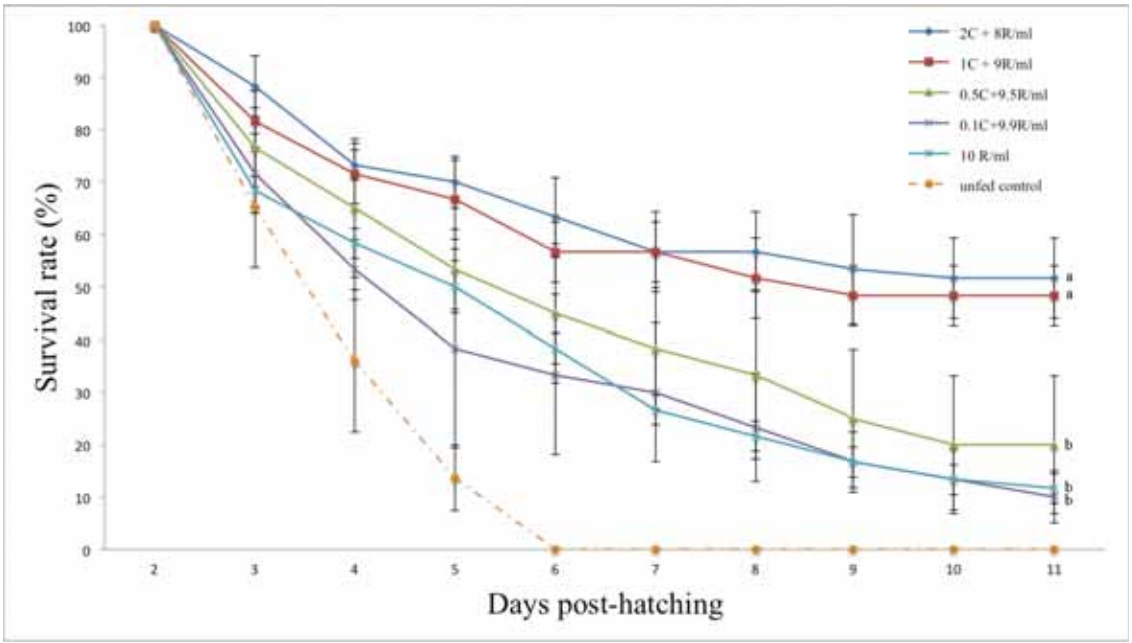


**Figure 3.1** Daily survival of *S. splendidus* larvae under different feeding conditions (copepod co-feeding densities). Different letters denote a significant difference ($p$<0.05). C and R as abbreviation for copepod and rotifer, respectively.

**Table 3.1** Notochord length, body depth, dry weight and specific growth rate of 11 DPH *S. splendidus* larvae under different feeding conditions (copepod co-feeding densities). Data are represented as mean ± SD. Values in a same column with different superscripts are significantly different ($p$<0.05).

| Treatments | 11 DPH larvae | | | SGR | |
|---|---|---|---|---|---|
| | NL (mm) | BD (mm) | DW (mg) | NL (% day$^{-1}$) | BD (% day$^{-1}$) |
| **2 C + 8 R mL$^{-1}$** | 2.69±0.11$^{a}$ | 0.70±0.06$^{a}$ | 0.24±0.07$^{a}$ | 5.48±0.46$^{a}$ | 4.42±0.97$^{a}$ |
| **1 C + 9 R mL$^{-1}$** | 2.66±0.37$^{a}$ | 0.68±0.09$^{a}$ | 0.20±0.07$^{a}$ | 5.28±1.60$^{ab}$ | 4.10±1.43$^{a}$ |
| **0.5 C + 9.5 R mL$^{-1}$** | 2.52±0.27$^{a}$ | 0.60±0.08$^{a}$ | 0.19±0.08$^{a}$ | 4.73±1.15$^{ab}$ | 2.67±1.46$^{a}$ |
| **0.1 C + 9.9 R mL$^{-1}$** | 2.35±0.20$^{a}$ | 0.60±0.04$^{a}$ | 0.17±0.07$^{a}$ | 3.95±0.95$^{b}$ | 2.69±0.81$^{a}$ |
| **0 C + 10 R mL$^{-1}$** | 1.97±0.19$^{b}$ | 0.52±0.03$^{b}$ | 0.14±0.10$^{b}$ | 1.96±1.10$^{c}$ | 1.02±0.57$^{b}$ |

C: copepod; R: rotifer; NL: notochord length; BD: body depth; DW: dry weight; SGR: specific growth rate

*3.3.2 With or without rotifer co-feeding experiment*

Similar to the copepod density experiment, the unfed control suffered a total mortality by 7 DPH while all fed treatments had some larvae survived to the end of the experiment. It is worth noting that no mortality was observed on the final two days (10 and 11 DPH) of the experiment in all fed treatments (Fig. 3.2).

When compared among treatments with different copepod feeding densities, larval survival of the 1 copepod mL$^{-1}$ treatments (>48%), no matter with or without rotifer co-feeding, were significantly higher than those of lower density treatments of 0.5 and 0.1 copepod mL$^{-1}$ (<24%), confirming the results from the previous copepod density experiment (3.3.1). However, when comparing the treatment pairs that larvae were fed a same copepod density but with or without

co-feeding rotifers, no significant difference on either larval survival or growth was detected at all three copepod density levels tested ($p$>0.05) (Fig. 3.2; Tab. 3.2).

Interestingly, despite significant differences in survival among larvae fed different copepod densities, the growth, including notochord length, body depth and dry weight, of the surviving larvae at the end of the experiment was not significantly different among all treatments ($p$>0.05)(Tab. 3.2). The specific growth (SGR), for NL was the highest (5.57 % $day^{-1}$) when larvae fed 1 copepod $mL^{-1}$ only, while the lowest SGR (NL: 4.11 % $day^{-1}$) was found for larvae fed 0.1 copepod + 9.9 rotifers $mL^{-1}$. The SGR for BD followed a similar trend as SGR based on NL, with the highest value (5.70 % $day^{-1}$) obtained for larvae fed 1 copepod $mL^{-1}$ and the lowest value (2.50 % $day^{-1}$) found for larvae fed 0.1 copepod $mL^{-1}$ (Tab. 3.2). While the differences in both SGR for NL and BD among all diet treatments were not statistically significant ($p$>0.05).

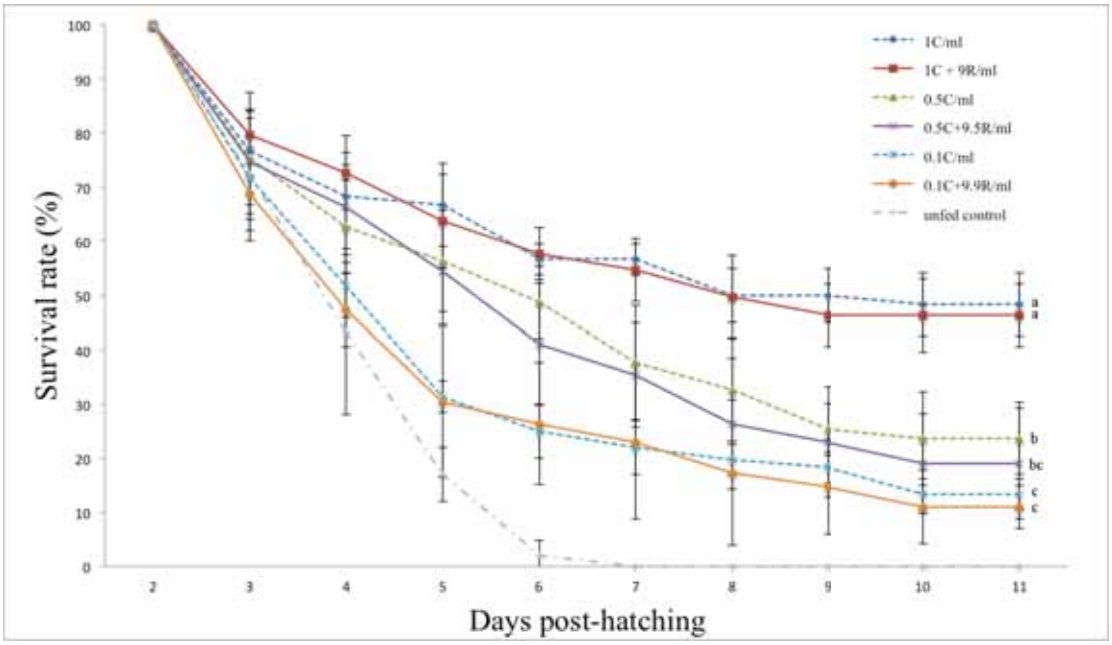


**Figure 3.2** Daily survival of *S. splendidus* larvae under different feeding conditions (with or without rotifer co-feeding). Different letters denote a significant difference ($p$<0.05). C and R as abbreviation for copepod and rotifer, respectively.

**Table 3.2** Notochord length, body depth, dry weight and specific growth rate of 11 DPH *S. splendidus* larvae under different feeding conditions (with or without rotifer co-feeding). Data are represented as mean ± SD. Values in a same column with different superscripts are significantly different ($p$<0.05).

| **Treatments** | **11 DPH larvae** | | | **SGR** | |
|---|---|---|---|---|---|
| | NL (mm) | BD (mm) | DW (mg) | NL (% day$^{-1}$) | BD (% day$^{-1}$) |
| **1 C mL$^{-1}$** | 2.71±0.21$^{a}$ | 0.72±0.09$^{a}$ | 0.25±0.06$^{a}$ | 5.57±0.90$^{a}$ | 5.70±1.31$^{a}$ |
| **1 C + 9 R mL$^{-1}$** | 2.63±0.28$^{a}$ | 0.70±0.08$^{a}$ | 0.23±0.06$^{a}$ | 5.15±1.20$^{a}$ | 5.26±1.39$^{a}$ |
| **0.5 C mL$^{-1}$** | 2.58±0.14$^{a}$ | 0.65±0.07$^{a}$ | 0.21±0.04$^{a}$ | 5.03±0.61$^{a}$ | 4.59±1.31$^{a}$ |
| **0.5 C + 9.5 R mL$^{-1}$** | 2.60±0.28$^{a}$ | 0.66±0.07$^{a}$ | 0.22±0.05$^{a}$ | 5.02±1.23$^{a}$ | 4.64±1.25$^{a}$ |
| **0.1 C mL$^{-1}$** | 2.42±0.32$^{a}$ | 0.54±0.06$^{a}$ | 0.19±0.09$^{a}$ | 4.26±1.41$^{a}$ | 2.50±1.19$^{a}$ |
| **0.1 C + 9.9 R mL$^{-1}$** | 2.39±0.30$^{a}$ | 0.55±0.07$^{a}$ | 0.19±0.08$^{a}$ | 4.11±1.39$^{a}$ | 2.74±1.31$^{a}$ |

C: copepod; R: rotifer; NL: notochord length; BD: body depth; DW: dry weight;

SGR: specific growth rate

*3.3.3 Ingestion rate and prey selection experiment*

Larval ingestion rates on the copepods and the rotifers under different feeding conditions are presented on Figure 3.3. The results showed that for all ages of larvae tested, they were capable of ingesting both prey. However, when both prey were presented under co-feeding conditions, the larvae showed a strong preference towards the copepods, and more so for early larvae. For example, the ratio of ingestion rates on the copepods and the rotifers was 3.8:1, 3.1:1, 1.7:1 and 1.5:1 for 4, 6, 8 and 10 DPH larvae respectively when they were fed 2 copepods+8 rotifers $mL^{-1}$ and 3.8:1, 2.6:1, 1.6:1 and 1.5:1 for 4, 6, 8 and 10 DPH larvae respectively when fed 1 copepods + 9 rotifers $mL^{-1}$. On the other hand, when larvae were offered only copepods at 1 $mL^{-1}$, larval ingestion rates on copepods were not significantly different from under co-feeding conditions in which copepods were offered at 1 or 2 copepods $mL^{-1}$ (i.e. 2 copepods + 8 rotifers $mL^{-1}$ and 1 copepod + 9 rotifers $mL^{-1}$ treatments) at all larval ages ($p>0.05$). In contrast, when only rotifers were offered at 10 $mL^{-1}$, larval ingestion rates on rotifers were significantly higher than all co-feeding conditions ($p<0.05$) at all larval ages except for the oldest 10 DPH larvae, no significant difference was found for the 0.5 copepods + 9.5 rotifers $mL^{-1}$ and 0.1 copepod + 9.9 rotifers $mL^{-1}$ treatments, suggesting improved ingestion on rotifers as larvae grew older (Fig. 3.3).

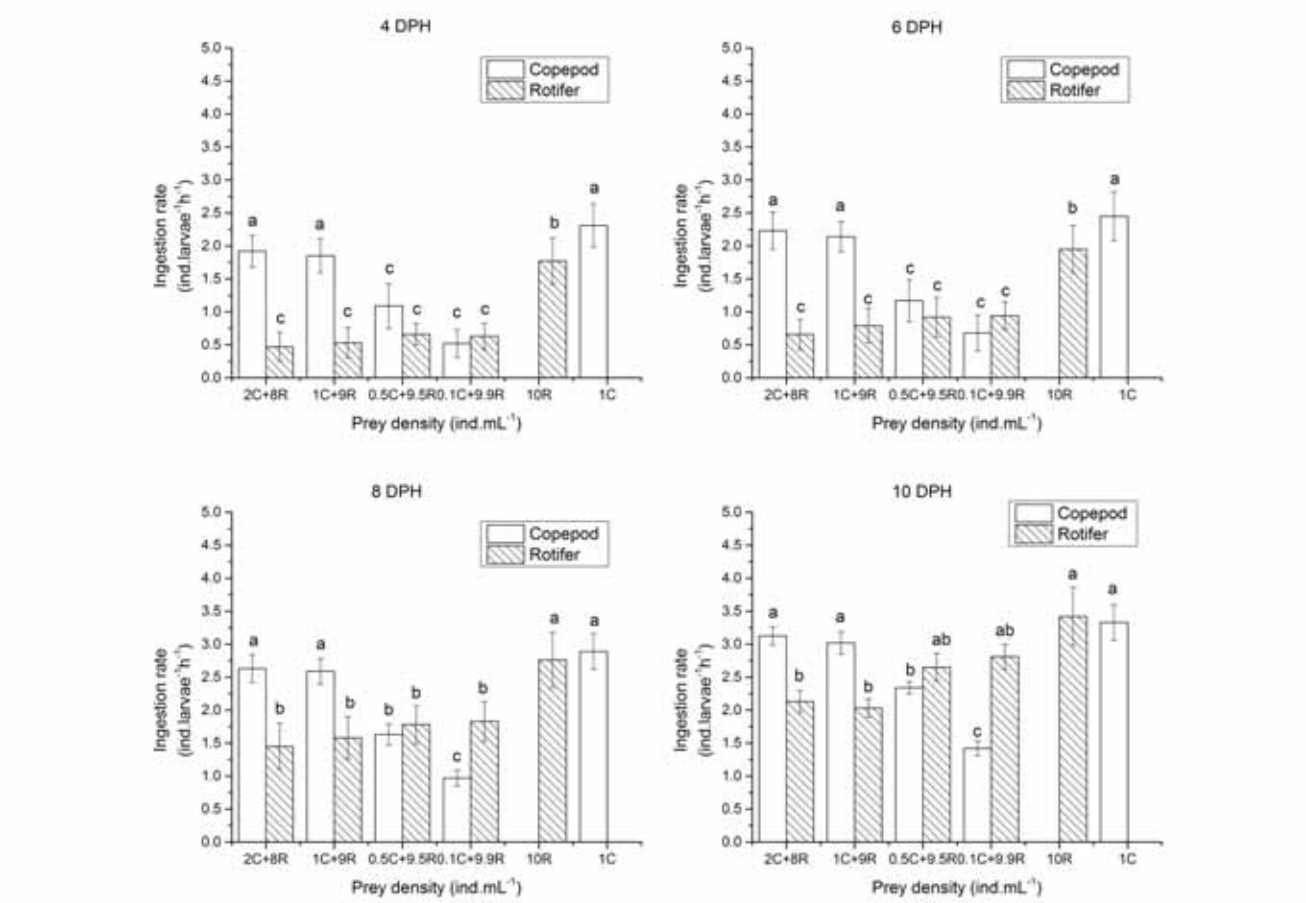


**Figure 3.3** Mean ingestion rates (prey larvae$^{-1}$ h$^{-1}$) of different aged green mandarin fish larvae on copepods and rotifers when the two prey were offered at different density combinations and singly. a) 4 DPH larvae; b) 6 DPH larvae; c) 8 DPH larvae and d) 10 DPH larvae. Values with different letters are significantly different ($p$<0.05). C, R and DPH stand for copepod, rotifer and days post-hatching, respectively.

The strong preference of *S. splendidus* larvae on the copepods over the rotifers is further evident by the electivity index *E*. It was shown that under any co-feeding conditions and for any ages of larvae tested, the electivity indices for copepods were all positive while for the rotifers were all negative (Tab. 3.3). There was also a clear trend that the electivity index for copepods increased significantly with decreasing copepod density at all larval ages, suggesting that

the larvae were capable of highly selective hunting for copepods when they were scarce. Additionally, there was a trend that electivity index for copepods decreased with increasing larval age under a same feeding condition although such a trend was more apparent at higher copepod densities, particularly when larvae were fed 2 copepods + 8 rotifers $mL^{-1}$. In contrast, *E* values for rotifers consistently improved with larval age under all co-feeding conditions, suggesting rotifers became more acceptable and were increasingly consumed by as larvae grew older (Tab. 3.3).

While it is clear that *S. splendidus* larvae are capable of hunting highly selectively for copepods under co-feeding conditions, larval ingestion rates on copepods did appear to be affected by copepod density, however such a density effect seemed to only exist when copepod density was under the threshold level of 1 copepod $mL^{-1}$. In fact, no significant difference in copepod ingestion rates were detected between the two treatments with copepod density ≥ 1 (i.e. 2 copepods + 8 rotifers $mL^{-1}$ and 1 copepod + 9 rotifers $mL^{-1}$ treatments) for all ages of larvae while the both treatments had significant higher copepod ingestion rates than the other two treatments with lower copepod density of 0.5 and 0.1 copepod $mL^{-1}$ (i.e. 0.5 copepod + 9.5 rotifers $mL^{-1}$ and 0.1 copepod + 9.9 rotifers $mL^{-1}$ treatments) ($p<0.01$). Meanwhile, larval ingestion rates on copepods of the 0.5 copepod + 9.5 rotifers $mL^{-1}$ treatment were significantly higher than that the 0.1 copepod + 9.9 rotifers $mL^{-1}$ treatment for the older 8 DPH and 10 DPH larvae ($p<0.05$), whereas for younger larvae, no significant different was detected (Fig. 3.3).

Finally, the ingestion rate of *S. splendidus* larvae generally increased with larval age. For example, when fed 2 copepods + 8 rotifers $mL^{-1}$, 1 copepod + 9

rotifers $mL^{-1}$ or 1 copepod $mL^{-1}$ only, the ingestion rate of 4 DPH larvae on copepods was 1.9, 1.9 and 2.3 copepods $larvae^{-1}$ $h^{-1}$, respectively; this increased to 3.1, 3.0 and 3.3 copepods $larvae^{-1}$ $h^{-1}$ for 10 DPH larvae. Comparatively, the trend of increasing ingestion rate on rotifers with larval age was more pronounced. For instance, the ingestion rate of 4 DPH larvae on rotifers was 0.5, 0.5 and 1.8 rotifers $larvae^{-1}$ $h^{-1}$ when fed 2 copepods + 8 rotifers $mL^{-1}$, 1 copepods + 9 rotifers $mL^{-1}$ or 10 rotifers $mL^{-1}$ only; it increased to 2.1, 2.0 and 3.4 rotifers $larvae^{-1}$ $h^{-1}$ respectively by 10 DPH larvae.

**Table 3.3** Electivity index on the copepods and the rotifers under various co-feeding conditions by different ages of *S. splendidus* larvae. C, R and DPH stand for copepod, rotifer and days post-hatching, respectively.

| **Co-feeding treatments** | **Prey species** | **4 DPH larvae** | **6 DPH larvae** | **8 DPH larvae** | **10 DPH larvae** |
|---|---|---|---|---|---|
| **2 C + 8 R $mL^{-1}$** | Copepod | + 0.6013 | + 0.5883 | + 0.5264 | + 0.4966 |
| | Rotifer | - 0.6053 | - 0.5558 | - 0.3848 | - 0.3274 |
| **1 C + 9 R $mL^{-1}$** | Copepod | + 0.7720 | + 0.7591 | + 0.7226 | + 0.7135 |
| | Rotifer | - 0.6033 | - 0.5390 | - 0.4075 | - 0.3825 |
| **0.5 C + 9.5 R $mL^{-1}$** | Copepod | + 0.8514 | + 0.8360 | + 0.8106 | + 0.8073 |
| | Rotifer | - 0.4316 | - 0.3667 | - 0.2908 | - 0.2829 |
| **0.1 C + 9.9 R $mL^{-1}$** | Copepod | + 0.9567 | + 0.9535 | + 0.9439 | + 0.9421 |
| | Rotifer | - 0.2875 | - 0.2609 | - 0.2047 | - 0.1969 |

### 3.4 Discussion

The first feeding in fish larvae can be considered as the period from which the ingestion is possible up to the moment when larval growth is detected. The main characteristic of this phase is that the source of nutrient and energy

necessary to continue the larval development changes from the yolk reserves to the ingested food (Yúfera and Darias, 2007). The first feeding period is widely considered as highly critical for successful larval culture as mass or total larval mortality often occurs after the exhaustion of the yolk-sac reserve if larvae were unable to hunt and ingest diets with appropriate nutritional quality (Mæhre et al., 2013). Many factors could affect the success of first feeding, which include larval foraging ability; food availability; and quality of available diets, such as appropriate size, attractiveness, palatability as well as nutritional values (Yúfera and Darias, 2007).

In a previous study from this laboratory, the first feeding larvae of *S. splendidus* were fed both enriched or unenriched rotifers alone at 10 $mL^{-1}$, larval survival to 11 DPH were very low (>3.3%) (Ricketts, 2012). The result showed that although *S. splendidus* early larvae could ingest rotifers and a few larvae could survive to 11 DPH solely on rotifers, low larval survival rendered it an unsuitable diet for commercial production. In the copepod feeding density experiment, co-feeding copepod *P. crassirostris* with rotifers at 2 copepods + 8 rotifers $mL^{-1}$ and 1 copepod + 9 rotifers $mL^{-1}$ produced a dramatically improved larval survival to 52% and 48 % on 11 DPH from 12% of the feeding rotifers alone (10 rotifers $mL^{-1}$) treatment. Moreover, the co-feeding treatments generated significantly larger larvae. Such significant improvements on both larval survival and growth via co-feeding of the copepods suggest that copepods are essential for larval culture of *S. splendidus*. However, despite recent significant improvements in culture techniques for copepods (Alajmi and Zeng, 2013; 2014; Alajmi et al., 2014; 2015; Kline and Laidley, 2015), production of large quantity of copepods is still challenging as well as costly, it is hence necessary to determine the minimum

level of the copepods needed for *S. splendidus* larval culture. The results of the copepod feeding density experiment showed that while all co-feeding treatments, even when the copepods were offered at very low level of only 0.1 copepod $mL^{-1}$, produced similar growth performance, larval survival of co-feeding 1 copepod $mL^{-1}$ with 9 rotifers $mL^{-1}$ was similar and not significantly different than that of co-feeding 2 copepods $mL^{-1}$ with 8 rotifers $mL^{-1}$. Both treatments had substantially higher larval survival than the other two treatments in which the copepods were offered at 0.5 and 0.1 $mL^{-1}$ for co-feeding with rotifers. Based on such results, co-feeding 1 copepod $mL^{-1}$ with 9 rotifers $mL^{-1}$ is recommended for early larval culture of *S. splendidus*. Interestingly, a very low density of 0.1 copepod $mL^{-1}$ provided for co-feeding with rotifers at 9.9 rotifers $mL^{-1}$ had significantly improved larval growth than feeding rotifers alone at 10 rotifers $mL^{-1}$, demonstrating that even a very small quantity of copepod supplementation could produce dramatic effects on larval growth of *S. splendidus*.

The significant benefits of copepods provided as either a primary or supplemental diet for marine fish larvae culture has been demonstrated by many past studies. For example, for the yellowtail clownfish *Amphiprion clarkia*, larvae fed nauplii and copepodites of the copepod *Centropage typicus* showed significant better survival and growth compared to those fed rotifer/*Artemia* (10 rotifers $mL^{-1}$ followed by 6 *Artemia* $mL^{-1}$ ) by 11 DPH (Olivotto et al., 2008a). Similarly, feeding larvae the benthic copepod *Tisbe* spp. was shown to significantly improve larval survival, growth as well as reduce the time to metamorphosis for the striped blenny *Meiacanthus grammistes* (Olivotto et al., 2010b). Similar results were also reported for other marine ornamental fish, including various seahorses (Job et al., 2006; Koldewey and Martin-Smith, 2010;

Olivotto et al., 2008b), the peacock hind *Cephalopholis argus* (Ajiboye et al., 2011), the small-mouthed pipefish *Stigmatopora argus* (Payne et al., 1998) and various angelfish *Centropyge* sp. (Laidley et al., 2008; Olivotto et al., 2006a). In the food fish, Toledo et al. (1999) likewise reported that larvae of the grouper, *Epinephelus coioides*, showed significantly increased survival and growth when their rotifer *B. rotundiformis* diet was supplemented with copepod (mixed with *Acartia tsuensis, Pseudodiaptomus* spp., and *Oithona* sp.) nauplii at only 0.1 $mL^{-1}$. Similarly, larvae of the northern red snapper, *Lutjanus campechanus*, fed with nauplii of *Parvocalanus* sp. exhibited significantly greater survival and were larger in size by 7 DPH (Shields et al., 2005).

As the important of the copepods as the diet for early larvae of *S. splendidus* was unequivocally demonstrated, the subsequent experiment investigated whether co-feeding rotifers with copepods had beneficial effects. The results showed that at a same copepod feeding density, additionally feeding rotifers neither significantly improve larval survival nor larval growth as compared to the corresponding treatment in which copepods were fed alone, even at the lowest copepod feeding density of 0.1 copepod $mL^{-1}$. Such results suggested that rotifer co-feeding is not necessary for *S. splendidus* early larval culture. The experiment further showed that similar to the earlier copepod feeding density experiment with rotifer co-feeding, while no significant difference in larval growth was found among the treatments that copepods were fed alone at densities of 1, 0.5 and 0.1 copepod $mL^{-1}$, larval survival of the 1 copepod $mL^{-1}$ treatment (>48%) was significantly higher than the other two lower density treatments. Therefore, summarizing the results from the two experiments, feeding larvae with 1 copepod $mL^{-1}$ can be recommend as the standard feeding regime for early larval culture of

*S. splendidus*. In fact, similar results were reported for the fat snook *Centropomus parallelus* larval culture by Barroso et al. (2013), who compared treatments in which larvae were co-fed rotifers *B. rotundiformis* with copepod *Acartia tonsa* vs. those fed the copepods alone and no significant difference was found in larval survival and growth on 14 DPH, as well as the occurrence of functional swimming bladder and notochord flexion. However, in the flatfish *Hippoglossus hippoglossus*, co-feeding nauplii of the copepod *Tisbe holoturiae* with the rotifer *B. rotundiformis*, larval survival and growth rate were found improved compared to fed either the rotifers or the copepods only (Evjemo et al., 2003; Støttrup and Norsker, 1997). Such difference likely reflects species-specific difference in larval feeding and dietary requirements; or alternatively, may be related to the fact that adult *T. holoturiae* are benthic thus were less available to fish larvae than planktonic species, such as *P. crassirostris* used in this study. It is worth noting that in both experiments, larval survival stabilized around 9-10 DPH. It indicates the crucial larviculture period for *S. splendidus* is relatively short, suggesting high promise for commercial breeding of this popular ornamental species.

As the superior prey quality of the copepod *P. crassirostris* as compared to the rotifers became clear via the two larval culture experiments, it is important to gain insights into the underlying mechanisms that why copepods performed so much better than rotifers. The results of the larval feeding rate experiment and electivity index calculated based on the results clearly elucidated that under different co-feeding ratio conditions, *S. splendidus* larvae of all ages tested (4, 6, 8 and 10 DPH) positively selected copepods over rotifers. Such result could be attributed to the strong appetite stimulatory effects, both behaviorally and chemically, of copepods to fish larvae (Støttrup and Norsker, 1997; Takeuchi et

al., 2001). For example, it has been reported that compared to the slow circular motion of rotifers, the characteristic jerking (i.e. zigzag) swimming motion of copepod nauplii stimulated predatory behavior in first feeding larvae of angelfishes (*Pomacanthus* spp.) (Leu et al., 2009; Moe, 1997). Furthermore, taurine is known as an appetite stimulant and essential dietary nutrient for marine larvae (Helland et al., 2003; Yokoyama et al., 2001); it was found to be the most abundant free amino acid in copepods but devoid in rotifers (Aragão et al., 2004; Helland et al., 2003).

Other factors could also affect larval prey selection and ingestion rate, such as prey size and density as well as larval foraging ability (Olivotto et al., 2010a). It was suggested at the first feeding, prey size smaller than larval mouth gap to allow ingestion is of paramount important (Yúfera and Darias, 2007). The size of both *P. crassirostris* and the ss-type rotifers used in this study clearly met the criteria as both prey were ingested by *S. splendidus* first feeding larvae. Prey selection may also related to prey density as higher densities create higher chance of encounter (Farhadian et al., 2012). However, our results showed that the copepods were consistently positive selected in all co-feeding treatments for all ages of larvae, even under the co-feeding condition in which rotifer density was almost 100 times higher than that of the copepods (i.e. under the co-feeding condition of 0.1 copepod + 9.9 rotifers $mL^{-1}$). This is even more amazing considering that the swimming speed of the copepods were substantially faster than that of the rotifers and early larvae had relative weak foraging ability by themselves. In fact, similar results were also found in the group *E. coioides* larvae, although rotifers was provided much more abundant than the copepod nauplii, the grouper larvae reportedly consumed significantly more copepod nauplii than

rotifers (Toledo et al., 1999).

While adding copepods has been shown in other research to have feeding stimulating effects, leading to increased larval ingestion rates on rotifers or cladocerans (Barroso et al., 2013; Farhadian et al., 2012), this appears not the case for *S. splendidus* larvae. The present results showed that larval ingestion rate on rotifers was significantly higher when they were fed rotifers alone at 10 $mL^{-1}$ than all co-feeding treatments, including the 0.1 copepod + 9.9 rotifers $mL^{-1}$ treatment of very similar rotifer density, for 4, 6 and 8 DPH larvae. For older 10 DPH larvae, the rotifer ingestion rate of the feeding rotifers only treatment was also higher than all the co-feeding treatments although significant differences were only found for the 2 copepod + 8 rotifers $mL^{-1}$ and 1 copepod + 9 rotifers $mL^{-1}$ treatments.

Finally, the copepod ingestion rates of feeding 2 copepods + 8 rotifers $mL^{-1}$, 1 copepods+9 rotifers $mL^{-1}$ and 1 copepod $mL^{-1}$ only were not significantly different for all ages of larvae but they were significantly higher than the other two co-feeding treatments at lower copepod densities (0.5 copepod + 9.5 rotifers $mL^{-1}$, 0.1 copepod + 9.9 rotifers $mL^{-1}$), this supports the conclusion from the larval culture experiments that *P. crassirostris* fed at 1 $mL^{-1}$ is sufficient for early larval culture of *S. splendidus* and should be adopted as standard feeding regime. Furthermore, since there was an increasing trend towards rotifer consumption as larval grew older, suggesting a promising prospective for transferring copepod feeding to rotifers in elder *S. splendidus* larvae.

# Chapter 4

## Effects of timing for transition of prey from copepods to rotifers on larval survival, growth, development, feeding behavior and efficiency of the green mandarin fish, *Synchiropus splendidus*

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### 4.1 Introduction

It is well documented that for early larvae of coral reef fish, particularly marine ornamental fish, copepods are generally considered as more suitable diets than rotifers. Previous studies have reported that feeding larvae with copepods significantly improved larval survival and growth in a range of taxonomically very diverse species, including the yellowtail clownfish *Amphiprion clarkia* (Olivotto et al., 2008b), the striped blenny *Meiacanthus grammistes* (Olivotto et al., 2010), various seahorses *Hippocampus* spp. (Job et al., 2006; Koldewey and Martin-Smith, 2010; Olivotto et al., 2008a), the peacock hind *Cephalopholis argus* (Ajiboye et al., 2011), the small-mouthed pipefish *Stigmatopora argus* (Payne et al., 1998), the flame angelfish *Centropyge loriculus* (Laidley et al., 2008) and the lemonpeel angelfish *Centropyge flavissimus* (Olivotto et al., 2006). The superior performance of copepods as larval prey as compared to traditional rotifers and *Artemia* has been attributed to a range of factors, including a wide range of size from nauplii to adults to suite larvae, unique movement that inciting strong larval feeding responses, and high content of highly unsaturated fatty acids and enzymes that meet larval nutritional requirements (Rasdi and Qin, 2014).

However, although copepod culture techniques have improved substantially in recent years (Alajmi and Zeng, 2014, 2015; Alajmi et al., 2014, 2015; Kline and Laidley, 2015), their culture are still difficult and culture densities achieved

are only two to three orders of magnitude lower than that of rotifers. Additionally, the copepod production is also very costly, demanding large and sophisticated facilities as well as highly skilled personnel, hence the supply of intensively cultured copepods for larval culture consistently presents as a major problem (Ianora et al, 1996; Støttrup and Norsker, 1997; Olivotto et al, 2008). Consequently, copepods are often used only as a supplement to the traditional rotifers + *Artemia* feeding regimes for fish larvae, particularly early larvae (Olivotto et al., 2008a; Olivotto et al., 2008b).

While previous studies from this laboratory have identified the optimal feeding regime for early larvae of the green mandarin fish *Synchiropus splendidus* as feeding them copepods as the sole prey at 1 copepod $mL^{-1}$ (Chapter 3). However with larval development, their consumption on copepods increasing rapidly, copepod supply increasingly became a problem. It has been shown that in various fish, copepods only needed for first a few days during larval culture (Gopakumar et al., 2009; Olivotto et al., 2011) since with fast larval development, rotifers or *Artemia* soon became acceptable to the larvae and hence could be used to replace copepods. Our larval ingestion rate study also showed that there was a trend of increasing ingestion rate on rotifers as *S. splendidus* larvae grew older (Chapter 3). Therefore, it is necessary to investigate whether and when rotifers might be introduced to substitute copepods for feeding *S. splendidus* larvae.

Rotifers and copepods differ substantially in their morphology and swimming ability and pattern. Calanoid copepods swim fast and are more evasive than rotifers, who move swirly and much slower in the water column (Ajiboye et al., 2011). Comparative studies on larval feeding behavior on different prey can

provide important insights into their capability and efficiency in capturing different prey, prey-predator interactions, and their selectivity on various prey species, which is helpful in devising appropriate feeding regime (Graeb et al., 2004; von Herbing and Gallager, 2000). In a previous study, larval ingestion rates and electivity indices on rotifers and copepods were estimated and the results showed that despite that even newly hatched larvae were capable of catching and ingesting rotifers, rotifers were negatively selected over copepods by *S. splendidus* early larvae (Chapter 3). However, it was not clear which factor or factors prior to prey ingestion had limited the utilization of rotifers by the larvae, hence further comparative study on larval feeding behavior on these two preys should be helpful in better understanding underlying behavioral mechanisms.

The green mandarin fish *S. splendidus* is a small, brilliantly colored fish, who is among the most desirable species to marine aquarium hobbyists worldwide (Sadovy et al., 2001). As a pelagic-spawner, *S. splendidus* produces very small eggs and its larvae are considered relatively hard to rear (Sadovy et al., 2001). Built on previous studies that established the optimal feeding regime of 1 copepod $mL^{-1}$ for *S. splendidus* early larvae, the present study set out to determine whether and when copepods may be replaced by rotifers as larval prey. The feeding behavior of larvae at different ages on rotifers and copepods were also compared to provide insights into possible ontogenetic changes in larval feeding response and preference on the two preys.

## 4.2 Materials and Methods

### *4.2.1 Broodstock maintenance*

Broodstock source and husbandry were described in Chapter 2 (Section 2.1).

### *4.2.2 Live feed culture*

Rotifers and copepods culture and harvest methods were described in Chapter 2 (Section 2.2 and 2.3).

### *4.2.3 Prey transition from copepods to rotifers experiment*

This experiment was designed to determine the suitable time for introducing rotifers to substitute copepods for feeding *S. splendidus* larvae. This was based on the consideration that copepod production is still much more difficult and costly than rotifers; and that it has been reported that copepods are often only indispensable during the first few days after mouth opening in larval rearing of various fish (Gopakumar et al., 2009; Olivotto et al., 2011).

The incubation and experimental system were described in Chapter 2 (Section 2.5). 2 DPH larvae in the 12 replicate rearing vessels were initially fed identically on copepods at 1 ind. $mL^{-1}$. However in three treatments, larval diet was subsequently switched from copepods to 10 rotifers $mL^{-1}$ on 4, 8 and 12 DPH, respectively. In a fourth treatment, larvae were fed copepods throughout at 1 ind. $mL^{-1}$ and served as a control. All treatments were triplicated and each replicate stocked 30 larvae. The experiment was terminated on 15 DPH as previous trials showed survival of *S. splendidus* larvae largely stabilized 15 DPH onward.

Daily counting the number of surviving larvae in each replicate vessel was conducted in the morning immediately after water exchange when larvae were easier to see after all prey had been flushed out. At the end of experiment, all surviving larvae were anaesthetized with clove oil at 0.05 ml $L^{-1}$ (AQUI-S®, New Zealand LTD) and photographed under a dissecting microscope (Leica MZ 125) fitted with a digital camera (Olympus DB 25). Images were then imported to an image analysis software (ImageJ, Freeware, Wayne Rasband, NIH, USA) for the measurement of larval standard length (SL: the length from the tip of the snout to the posterior extremity of the hypural plate) and body depth (BD: the vertical distance between body margins through the anterior of the pectoral fin base, exclusive of fins). Larval development was also assessed by examining if larvae had reached the stage of initial notochord flexion (i..e the initial dorsal way bending of the notochord tip). The larvae were then rinsed with 0.5 M ammonium formate to remove the residual salt (Bransden et al., 2005) and put in a 60 °C oven for 18 h until reached constant weight. The measurement of individual dry weight (DW) of the larvae was carried out with a Cahn C-33 microbalance (precision: 1 μg; Thermo Fisher Scientific Inc, Pittsburgh, Pa, USA).

To assess growth over time, specific growth rate (SGR) based on SL, BD of the 15 DPH larvae from each treatment were calculated using the following formula:

$$SGR\ (\%) = [\ln(F)-\ln(I)]*100/T$$

Where F and I represent the final and initial SL or BD, respectively, and T was the culture duration (days). DW was not included in SGR as the practical difficulty to weight 2 DPH pre-feeding larvae, and DW was not a crucial criteria for ornamental fish.

*4.2.4 Larval feeding behavior experiment*

Ontogenetic changes in larval feeding behavior and prey selection were investigated by comparing feeding responses on copepods and rotifers by 6, 8, 10 and 12 DPH larvae, respectively. Prior to being picked randomly for the feeding behavior study, all larvae were cultured under identical condition and fed them 1 rotifer + 1 copepod $mL^{-1}$ to allow acclimatization to both live prey since it has been documented that prey identification and capture success could be affected by whether larvae had exposed to them previously (Brown and Laland, 2001; Cox and Pankhurst, 2000). On each of the designated DPH during larval development, active individual larva were randomly selected and pre-starved for 6 h before being introduced into a 10 mL petri dish for 20 min to allow acclimatization to the experimental condition. Pilot trials have shown that this was enough for larvae to recover from handling stress and to assume their normal behavior. Following the acclimatization period, preys of 1 rotifer + 1 copepod $mL^{-1}$ were introduced. Subsequently, the numbers of targeting (larva turned and oriented toward the prey), attack (larva moved toward the prey), capture (prey was inside the mouth of the larva), rejection (larva rejected prey following capture) and ingestion (larva ingested prey) were monitored and recorded for 10 min with a dissecting microscope (Nikon SMZ645). A total of 15 larvae were assessed for each larval

age tested and the results were averaged to obtain means. The mean values were then used to calculate attack rate (number of prey attacks as a percentage of targetings), capture rate (prey captures as a percentage of attacks), rejection rate (prey rejections as a percentage of captures), and finally the overall feeding efficiency (number of prey ingested as a percentage of targetings).

A further experiment was conducted to examine the average time interval from capture and consumption a prey to when the next feeding attempt was resumed by larvae of different ages on either rotifers or copepods, respectively. This time period encompassed two components of larval feeding: 1) prey handling time, i.e. the time between a prey being successfully captured to it was fully ingested; and 2) the time of digestive pause, which was defined as the time after a prey being ingested till the time a larva started next active searching for prey and is termed 'feeding interval'. The experimental procedure was similar to what was mentioned above except that prey were introduced as either 2 rotifers $mL^{-1}$ only or 2 copepods $mL^{-1}$ alone (both rotifers and copepods were provide at 2 ind. $mL^{-1}$ to allow equal total prey number per mL to the previous experiment). To avoid potential gut fullness influenced the results, only the first capture event was recorded to obtain the data. Similarly to the previous feeding behavioral experiment, 6, 8, 10 and 12 DPH larvae were tested, hence a total of eight treatments of different prey + larval age combinations. There were 20 larvae monitored as replicates for each treatment.

*4.2.5 Data analysis*

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

data were assessed to confirm normality and homogeneity of variance (Levene's test for homogeneity of variances), and where necessary, arcsine-square root or logarithmic transformation was performed prior to the ANOVA analysis. A one-way ANOVA was employed to determine significant differences among treatments for each parameter obtained. When significant difference was detected, Tukey's multiple range test was used as the means separation procedure. A statistical probability of $p<0.05$ was accepted as significant for all tests. All statistics were performed using Statistica software, version 22 (Statsoft$^{TM}$, Inc.)

## 4.3 Results

### *4.3.1 Prey transition experiment*

Larval survival at the end of experiment on 15 DPH was significantly affected by the time at which larval prey were switched from copepods to rotifers ($p<0.01$)(Fig. 4.1). The treatment with the prey switching occurred on 4 DPH had the poorest survival of 7.8 ± 1.9%, which was significantly lower than all other treatments ($p<0.01$). The treatment with the prey switching occurred on 8 DPH fared better, but still had a survival (25.6 ± 3.9%) significantly lower than the treatments with the diet change occurred on 12 DPH and the continuous copepod feeding control ($p<0.01$). Larval survival of the feeding copepod throughout control and prey switched on 12 DPH were the best and very similar, reached 42.2 ± 3.9% and 40.0 ± 3.3%, respectively ($p>0.05$).

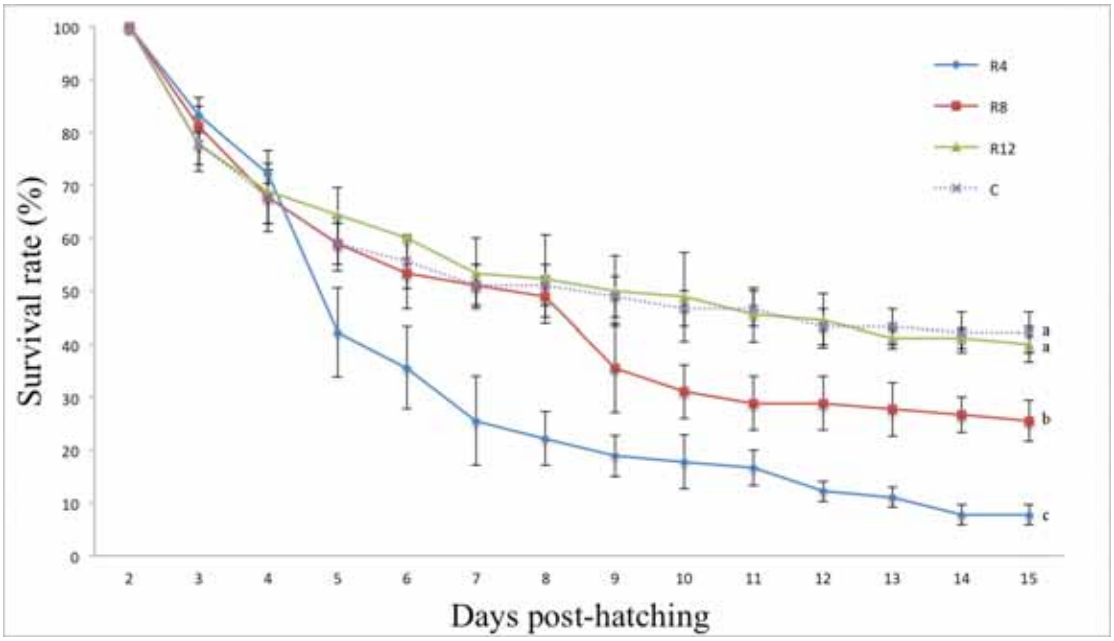



**Figure 4.1** Daily survival of *S. splendidus* larvae of the prey transition experiment, showing the effects of the timing of introducing rotifers to replace copepods. R4 (—◆—); R8 (—■—) and R12 (—▲—): larval diet changed from copepods to rotifers on 4, 8 and 12 DPH, respectively; C (···※···): larvae fed copepods throughout. a. b. and c: different letters denote significant differences in larval survival on 15 DPH ($p$<0.01).

All growth parameters of surviving *S. splendidus* larvae on 15 DPH showed a consistent trend of improving with longer period of copepods feeding and the best growth performance was found in the continuous copepod feeding control (Tab. 4.1). Similar to survival, the treatment with the prey switching occurred the earliest on 4 DPH suffered the poorest growth with significantly inferior standard length (SL) and dry weight (DW) than all other treatments ($p$<0.05). The treatment also had the lowest body depth (BD) among all treatments although significant difference was only detected when compared to the copepod feeding control. The SL, BD and DW of larvae with prey switching occurred on 8 DPH were significantly lower than those of the copepod feeding control ($p$<0.05) but not significant different from the treatment with prey switched on 12 DPH

($p$>0.05). Finally, all growth parameters of the prey switching on 12 DPH treatment were not significantly different from the copepod feeding control ($p$>0.05)(Tab. 4.1). The specific growth (SGR), for SL was the highest in copepod feeding control (5.34 % $day^{-1}$) and prey switching on 12 DPH treatment (4.31 % $day^{-1}$), but no significant difference was found between them ($p$>0.05). The lowest SGR (SL: 1.57 % $day^{-1}$) was found in larvae with prey switching occurred on 4 DPH, which was significantly lower than the other three treatments ($p$<0.05). The SGR for BD was also the highest (4.83 % $day^{-1}$) in the continuous copepod feeding treatment, which was significantly higher than that in treatments with the prey switching occurred on 4 DPH (1.78 % $day^{-1}$) and 8 DPH (2.59 % $day^{-1}$) ($p$<0.05) but similar with that of prey switching on 12 DPH treatment (3.49 % $day^{-1}$) ($p$>0.05)(Tab. 4.1).

Larval development at the end of the experiment, indicated by the percentage of larvae reached initial notochord flexion stage, showed a same trend as growth with longer period of copepod feeding generally enhanced larval development (Tab. 4.1). The copepod feeding control had the most larvae reached initial notochord flexion (88.9 ± 12.7%), which was followed by 54.6 ± 16.7% and 16.9 ± 4.6% from the prey switching on 12 and 8 DPH treatments, respectively. However, none of the surviving larvae from the treatment with prey switched on 4 DPH showed initial notochord flexion on 15 DPH (Tab. 4.1).

**Table 4.1** Growth and development of *S. splendidus* larvae on 15 DPH from the prey transition experiment. Data are represented as mean ± SD. Values in a same column with different superscripts are significantly different ($p$<0.05).

| Treatments | 15 DPH larvae | | | | SGR | |
|---|---|---|---|---|---|---|
| | NF | SL | BD | DW | SL | BD |
| | (%) | (mm) | (mm) | (mg) | (% day$^{-1}$) | (% day$^{-1}$) |
| **R4** | 0 | 2.01±0.10$^{a}$ | 0.60±0.04$^{a}$ | 0.19±0.06$^{a}$ | 1.57±0.41$^{a}$ | 1.78±0.57$^{a}$ |
| **R8** | 16.9±4.6$^{a}$ | 2.33±0.17$^{b}$ | 0.66±0.07$^{a}$ | 0.29±0.08$^{b}$ | 2.68±0.57$^{b}$ | 2.59±0.88$^{a}$ |
| **R12** | 54.6±16.7$^{b}$ | 2.88±0.26$^{bc}$ | 0.75±0.14$^{ab}$ | 0.30±0.08$^{bc}$ | 4.31±0.68$^{c}$ | 3.49±1.42$^{ab}$ |
| **C** | 88.9±12.7$^{c}$ | 3.30±0.35$^{c}$ | 0.89±0.10$^{b}$ | 0.36±0.04$^{c}$ | 5.34±0.85$^{cd}$ | 4.83±0.89$^{b}$ |

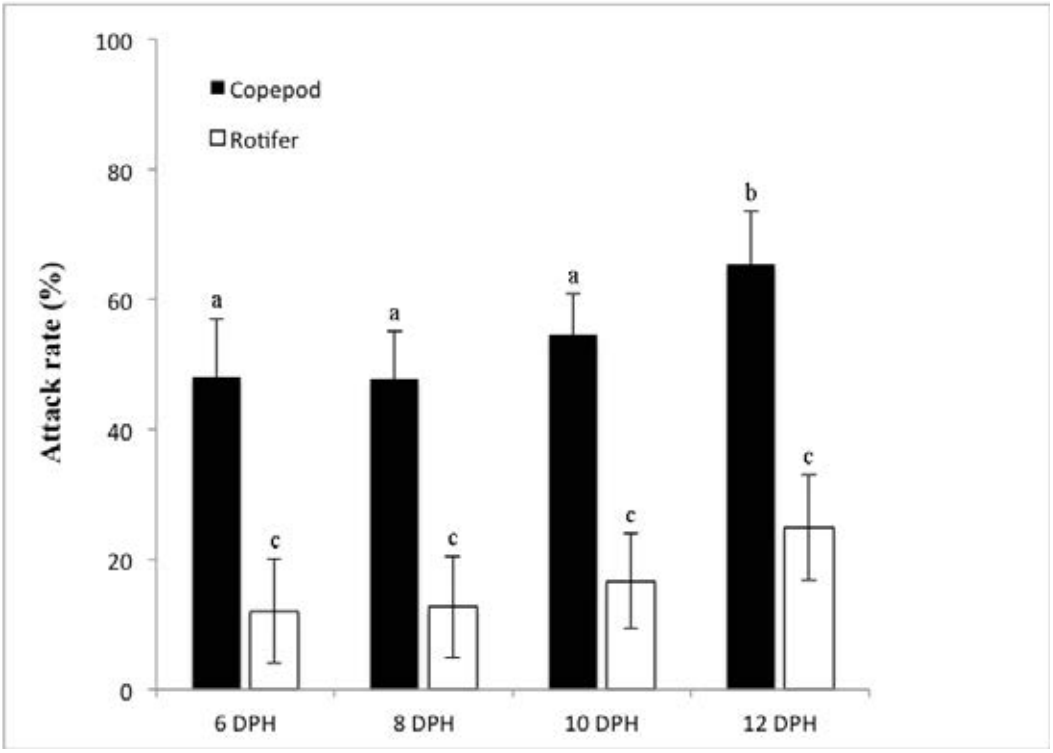
R4; R8 and R12: larval diet changed from copepods to rotifers on 4, 8 and 12 DPH, respectively; C: larvae fed copepods throughout control; NF: notochord flexion; SL: standard length; BD: body depth; DW: dry weight; SGR: specific growth rate

*4.3.2 Larval feeding behavior experiment*

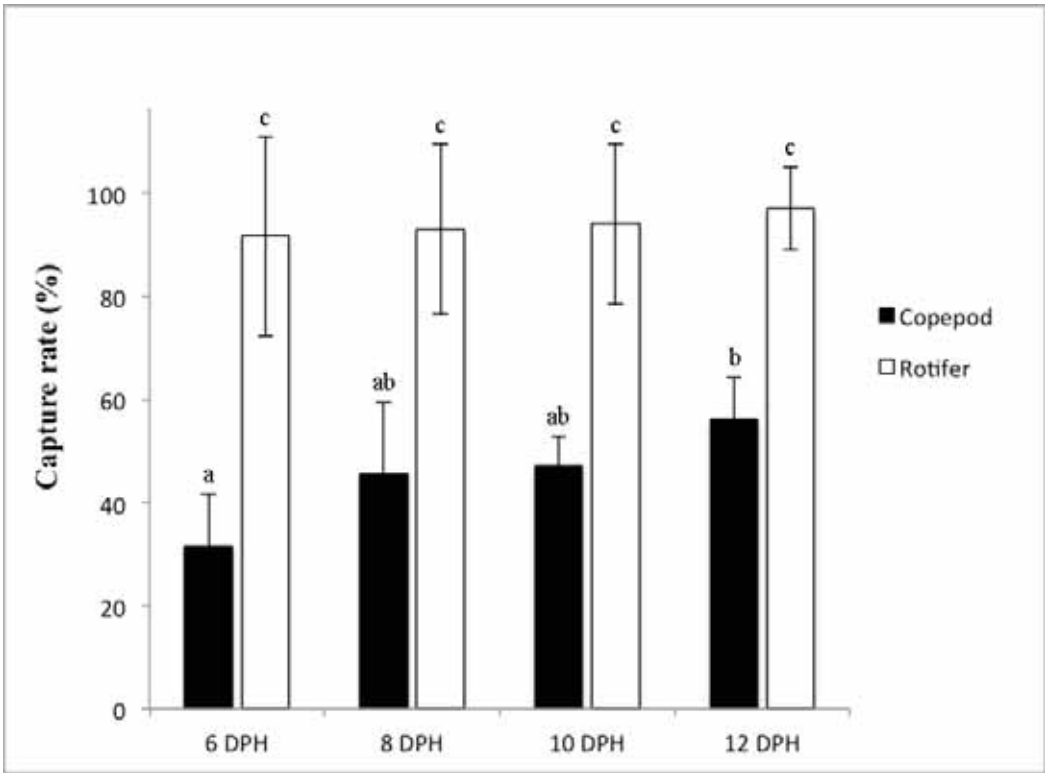
The results of the feeding behavior experiment showed that at all larval development stages tested, larval capture rate (captures as % of attacks) on the rotifers were actually very high (ranging from 91.7% to 97.0%), and significantly better than on the copepods (from 31.3% to 56.2%)($p$<0.01)(Fig. 4.2B). However, the attacking rate (attacks as % of targetings) on rotifers were dramatically lower than on copepods (attacking rate on rotifers vs. on copepods: 6 DPH larvae: 12.1% vs. 48.1%; 8 DPH larvae: 12.7% vs. 47.9%; 10 DPH larvae: 16.7% vs. 54.5%; and 12 DPH larvae: 24.9% vs. 65.5%)($p$<0.01)(Fig. 4.2A), showing a strong preference towards copepods by larvae of all developmental stages. Such a clear and interesting contrast in larval feeding behavior suggested that even though rotifers were much easier for the larvae to catch, they were much more likely to launch an attack on copepods than rotifers. Furthermore, copepods were never

observed being rejected by the larvae after being captured, whereas the rejection rate (rejections as % of captures) on rotifers by 6, 8, 10 and 12 DPH larvae were 59.2%, 33.3%, 22.6% and 15.8%, respectively (Fig. 4.2C). The clear decreasing trend of rejection rate on rotifers with larval development demonstrated that rotifers were becoming increasingly acceptable to the larvae of *S. splendidus*. Finally, as substantial lower attacking rates plus significant higher rejection rates after capture on rotifers have more than cancelled out higher capture rate on them, the eventual overall feeding efficiency (ingestions as % of targetings) was significantly higher on copepods for all ages of larvae tested (feeding efficiency on rotifers vs. on copepods: 6 DPH larvae: 4.5% vs. 15.3%; 8 DPH larvae: 7.0% vs. 21.5%; 10 DPH larvae: 11.9% vs. 25.8%; and 12 DPH larvae: 19.6% vs. 36.6%) ($p<0.01$)(Fig. 4.2D).

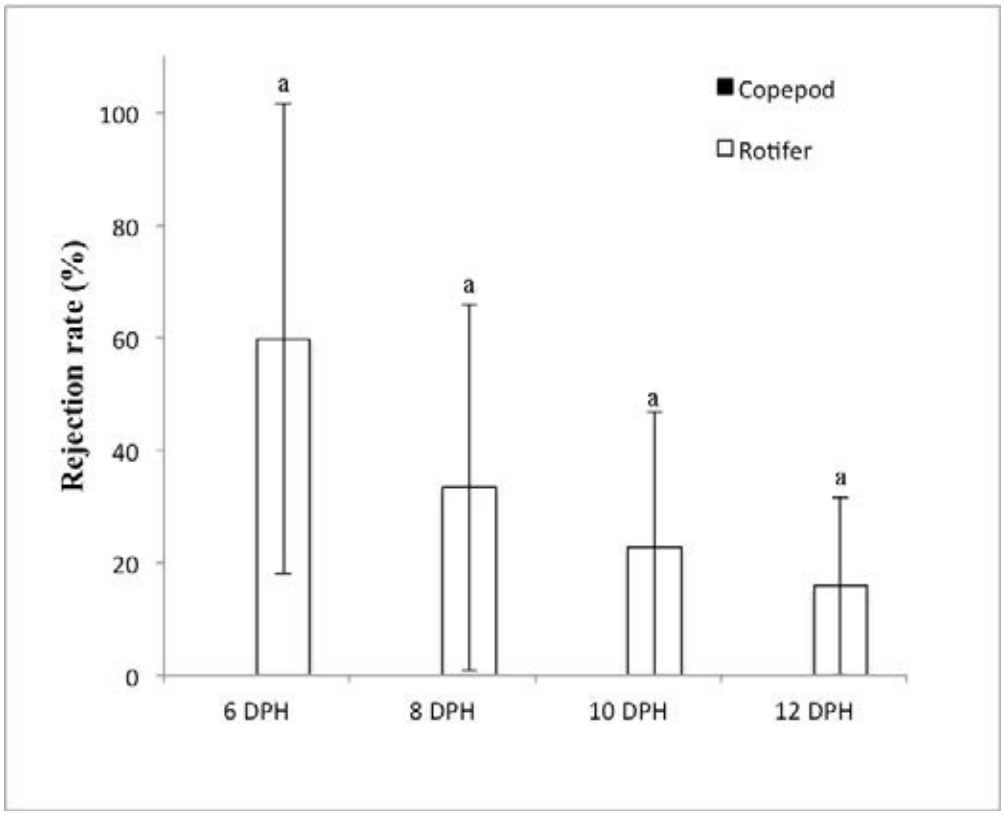
The results also showed that as larvae grew older, the attacking rate, capture rate and feeding efficiency on copepods increased significantly for 12 DPH larvae as compared to the 6 DPH larvae ($p<0.05$)(Fig. 4.2). For instance, the feeding efficiency on copepods increased from 15.3% by the 6 DPH larvae to 36.6% by the 12 DPH larvae (Fig. 4.2D). Higher feeding efficiency on rotifers was also found in the 12 DPH larvae (19.6%) compared to the 6 DPH larvae (4.5%), reconfirming rotifers were becoming increasingly acceptable to the larvae of *S. splendidus*.


Attack rate (%)
100
80
60
40
20
0
Copepod
Rotifer
a
c
a
c
a
c
b
c
6 DPH
8 DPH
10 DPH
12 DPH

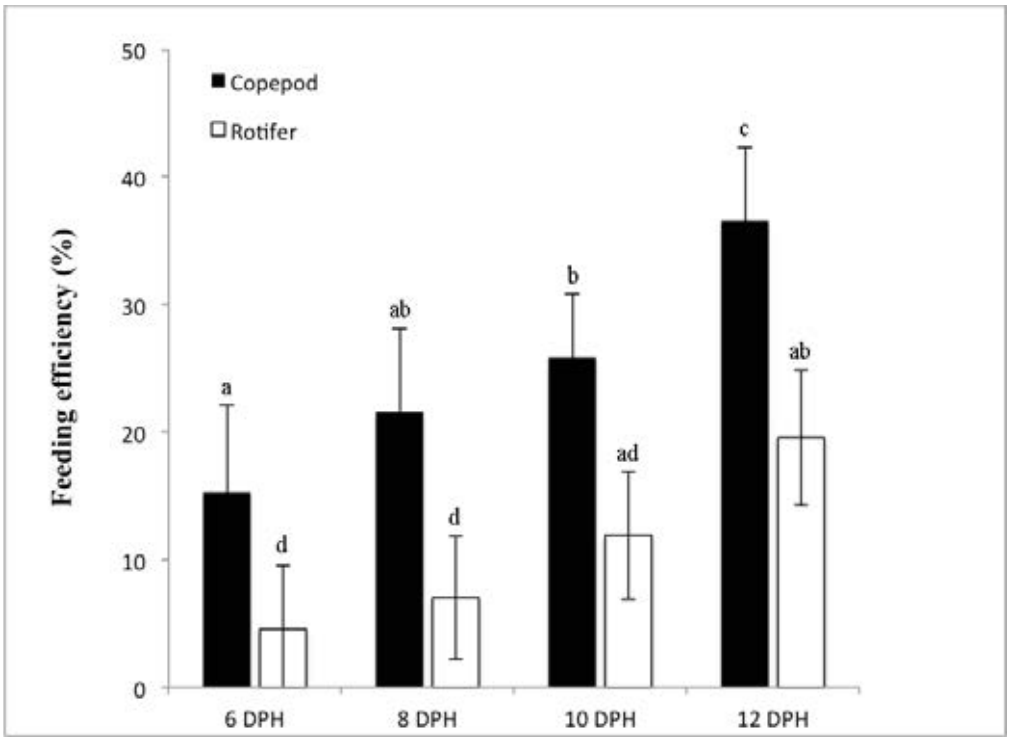
(A)


Capture rate (%)
100
80
60
40
20
0
a
c
ab
c
ab
c
b
c
Copepod
Rotifer
6 DPH
8 DPH
10 DPH
12 DPH

(B)


Copepod
Rotifer
Rejection rate (%)
100
80
60
40
20
0
a
a
a
a
6 DPH
8 DPH
10 DPH
12 DPH

(C)


Copepod
Rotifer
Feeding efficiency (%)
50
40
30
20
10
0
a
d
ab
d
b
ad
c
ab
6 DPH
8 DPH
10 DPH
12 DPH

(D)

**Figure 4.2** The attack rate (attacks/targetings*100%)(A); capture rate (prey captures/attacks*100%) (B); rejection rate (prey rejections/prey captures*100%) (C); and overall feeding efficiency (prey ingestions/targetings*100%)(D), on rotifers and copepods, respectively by different developmental stage larvae of *S. splendidus* when offered a mixed prey of 1 copepod + 1 rotifer $mL^{-1}$. Bars with different letters on the tops denote significant differences.

When copepods or rotifers were offered alone at 2 ind. $mL^{-1}$, the feeding intervals (defined as the time interval form capture and consumption a prey to when the next feeding attempt was launched) on copepods were significantly shorter than on rotifers by larvae of all ages tested ($p$<0.05)(Fig. 4.3). The feeding interval on copepods and rotifers was 28.47 vs. 45.2 seconds by 4 DPH larvae; 25.67 vs. 44.67 seconds by 8 DPH larvae; 25.53 vs 42.47 seconds by 10 DPH larvae, and 20.40 vs. 39.00 seconds by 12 DPH larvae, respectively. The results showed that compared to rotifers, copepods were much more appetizing to the larvae of all ages and incited significant more frequent feeding responses. Although not statistically significant, there was a slight trend of decreasing in feeding intervals on both prey as larvae grew older (Fig. 4.3).

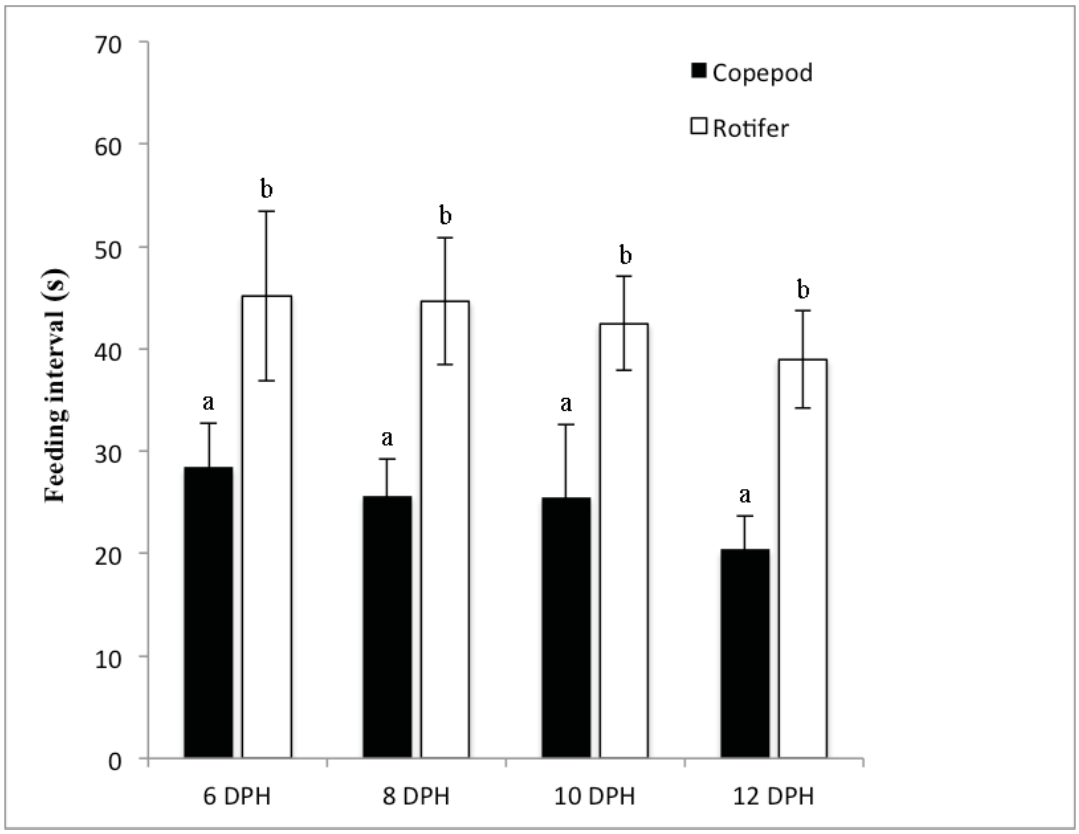



**Figure 4.3** The feeding interval (in seconds) on copepods and rotifers by *S. splendidus* larvae of different ages when they were fed either copepods or rotifers at 2 ind. $mL^{-1}$. Bars with different letters on the tops denote significant differences.

### 4.4 Discussion

It is well known that fish larvae, particularly early larvae, are very fragile, their survival largely relies on availability of prey in both appropriate quality and quantity (Yúfera and Darias, 2007). If their requirement on either prey quality or quantity is not met, mass mortality could occur quickly. In the prey transition experiment, the survival of *S. splendidus* larvae dropped sharply after copepods was replaced by the rotifers on 4 or 8 DPH; the surviving larvae also suffered significant poor growth and development as compared to the continuous copepod feeding control. Such significant deterioration in larval survival, growth and development suggested that it was too early to switch larval prey from copepods

to the rotifers prior to 8 DPH for *S. splendidus* larvae. On the other hand, when the prey switch occurred on 12 DPH, no significant difference was detected in both larval survival and growth when compared to the copepod feeding control although larval development appeared to be retarded, which was indicated by substantially fewer larvae reached initial notochord flexion at the end of experiment. The result suggests that larval development is a more sensitive parameter responding to the prey switching, which is supported by the fact that none of the surviving larvae of the prey switching on 4 DPH treatment showed initial notochord flexion at the end of experiment.

Interestingly, it was reported that when larvae of the sapphire devil damselfish, *Chrysiptera cyanea*, were fed a diet of two copepods mixture (*Euterpina acutifrons* and *Pseudodiaptomus serricaudatus*) initially but switched to the ss-type rotifers from 7 DPH onward until 15 DPH gave a comparable result to those fed the copepods continuously during 1-15 DPH (Gopakumar et al., 2009). Such a result showed that unlike *S. splendidus*, copepods could be fully substituted by rotifers for *C. cyanea* larvae from 6 DPH onward, demonstrating that larval feeding requirements are highly species-specific. It is therefore necessary to establish larval feeding regime for each targeted species for culture through appropriately desinged experimentations.

In the present study, it was noticed that larvae fed on copepods started notochord flexion on 11 DPH and 88.9% of larvae showed initial notochord flexion by 15 DPH. Wittenrich et al. (2010) reported a different result in which *S. splendidus* larvae started notochord flexion on 17 DPH, and completed on 23 DPH. The difference may be attributed to different feeding regimes used since in

the study by Wittenrich et al. (2010), larvae was fed a mixture of rotifers and wild plankton dominated by copepods. However, using copepod-dominated wild plankton only, Sadovy et al. (2001) reported a similar result as the present study with the occurrence of notochord flexion between 12-14 DPH in *S. splendidus* larvae. Such a difference highlights the issue with using wild-caught copepods to feed the larvae; that is, no control on their species compositions and hence inconsistent nutritional values, to the larvae. Another possible factor that might lead to the difference could be that photoperiod adopted by Wittenrich et al. (2010) was 14 h light : 10 h dark while in the present study and that of Sadovy et al. (2001), a constant light was adopted. Constant light has been reported to significantly improved larvae performance in various marine fish than other photoperiods used (Arvedlund et al., 2000; Barlow et al., 1995; Olivotto et al., 2003；Avella et al., 2007; Moorhead and Zeng, 2011; Olivotto et al., 2006; Shei et al., 2010).

Better understanding larval feeding behavior is critical to the development of appropriate feeding regime as well as help revealing underlying reasons on larval prey selection and feeding efficiency. Results from larval rearing experiment of the present study as well as those previous ones (Chapter 3) have clearly shown that copepod is a superior prey for *S. splendidus* early larvae than rotifer. While the prey size relative to the mouth gape of fish larvae is crucial in determining whether the prey could be ingested by the larvae (Yúfera and Darias, 2007), the larval ingestion rate experiment (Chapter 3) showed that both copepods and rotifers were in fact ingested by *S. splendidus* early larvae, suggesting that either prey was not too big for the mouth gape of the larvae. Hence prey size appeared not likely a limiting factor on prey selection by the larvae.

Asides from size, other prey characteristics, including pattern of swimming motion and velocity, predator avoidance behavior and relative availability have all been found to affect prey selection by fish larvae (Checkly Jr, 1982; Meng and Orsi, 1991). Rotifers have much slow swimming velocity as compared to copepods and generally don't show strong evasive response to predators, which means early larvae with limited swimming ability are likely to be more efficient in capturing them. In fact, the present study found that the capture rate (successful captures as % of attacks) on rotifers by *S. splendidus* early larvae were very high, ranging from 91.7% to 97%, which were substantially higher than on copepods. Similar result was reported for larval airbreathing catfish *Heterobranchus longifilis*, it was found that capture rate on rotifers ranged from 96% by 3 DPH larvae to 100% by 10 DPH larvae (Ajah, 2010).

Despite capture rate on rotifers was very high, larval attack rate (attacks as % of targetings) on rotifers were dramatically lower on rotifers than on copepods for larvae of all ages. This suggested that despite larvae could catch rotifers with high efficiency, they appeared to be much less likely to launch an attack on rotifers than on copepods, confirming their positive selection on copepods over rotifers. Saravanan et al. (2013) also found that for the newly hatched larvae of two-striped cardinalfish *Apogon quadrifasciatus,* the L-type rotifer *Brachionus plicatilis* was not a preferred prey even though larval mouth gape size was large enough to consume them. Similar positive selection on copepods was also reported in larvae of the greenback flounder *Rhombosolea tapirina* and the long-snouted flounder *Ammotretis rostratus* (Jenkins, 1987).

Larval preference on characteristic swimming motion of copepods has been suggested as the reason for positive selection on copepods by larvae of the lemonpeel angelfish *Centropyge flavissimus* and the orchid dottyback *Pseudochromis fridmani* (Moe, 1997; Olivotto et al., 2006). In a study on larval feeding of the perch *Perca fluviatilis*, Furnass (1979) concluded that the amplitude of prey movement was an important influencing factor in prey selection since the smaller prey preferably selected by the larvae was substantially more active than the larger prey simultaneously available. Similarly, Utne-Palm (1999) reported that prey with higher activity increased the chances of prey detection and reaction distance of larval *Gobiusculus flavescens*. For *S. splendidus* larvae, the characteristic irregular, zigzag fast swimming motion of the copepods as oppose to consistent whirling movement of the rotifers may serve as a critical factor in triggering larval feeding response. The feeding behavioral study showed that *S. splendidus* larvae of all ages significantly more frequent attacked on copepods than rotifers when the two prey were offered at a same density, indicating the motion pattern of the copepods may have stimulatory effects on larval appetite. This could be explained by the fact that copepods are the natural prey for most fish larvae, hence larvae have innate preference to their swimming motion.

The feeding behavior study further showed that aside from eliciting strong larval feeding responses, copepods were never rejected by the larvae once captured. In contrast, rejections after capture on rotifers were observed in larvae of all ages, suggesting that copepods were also substantially more palatable to the *S. splendidus* larvae. Therefore, due to innate preference towards the swimming motion and high palatability of the copepods, the significant higher attack rates plus no rejection once capture had more than cancelled out lower capture rate,

resulted in the overall significant higher feeding efficiency (ingestions as % of targetings) on copepods than on rotifers, which help explaining the superiority of copepods as larval prey than rotifers for early larvae of *S. splendidus.*

As larval developed, the increase in body length and gape size generally improve larval ability on prey capture (Saravanan et al., 2013). Meanwhile, larval vision also improves with age, the increased visual acuity also contributes to enhanced feeding success (Peña‐Aguado et al., 2007). As the result, the ability of fish larvae to target, attack, capture and ingest prey generally increases with their age. This study showed that the attack rate and capture rate on copepods by 12 DPH larvae increased significantly from 6 DPH larvae, which did not occur for rotifers. Such a result supported the conclusion of the prey switching experiment that copepods should not be replaced earlier than 12 DPH for *S. splendidus* larvae.

Optimal foraging theory predicts that predators should select more profitable prey in terms of energy gain per unit foraging effort (Stephens and Krebs, 1986). The feeding interval on copepods by larvae of all ages were found significantly lower than rotifers, whereas overall feeding efficiency on the copepods was significantly higher than on rotifers by larvae of all ages. This suggested that the foraging costs on the rotifers were sustainably higher, and the copepods provide higher energetic benefits to the *S. splendidus* larvae than rotifers. Hence, shorter feeding interval and higher feeding efficiency would increase net energy gain and consequently enhance growth and survival (Wahl et al., 1995). Moreover, it was also found that 12 DPH larvae had significantly higher overall feeding efficiency on rotifers compared to the younger larvae, hence the high energetic cost of long feeding interval on the rotifers probably were partly offset by the high feeding

efficiency, which promised that prey shifting occurred on 12 DPH may be feasible. However, when prey shift occurred on 12 DPH, larval development was still retarded, suggesting that the total replacement of the copepods with the rotifers should not occur earlier than 12 DPH.

# Chapter 5

## Ontogenetic changes in the fatty acid composition of the green mandarin fish, *Synchiropus splendidus* from the eggs, newly hatched and growing larvae

### 5.1 Introduction

The first feeding of fish larvae is defined as the period when endogenous nutrition is exhausted and larvae must start feeding on exogenous sources to support their survival and development (Watanabe and Kiron, 1994; Olivotto et al, 2003). Because of the high mortality experienced during this transitional period, it has been considered the most critical period for successful larval culture (Ignatius et al, 2001; Olivotto et al, 2006; Pena et al, 2004). The failures of many larvae to survive when using live prey for first feeding could be attributed to biological and environmental factors, including shape and size of prey (Gordon and Hecht, 2002; Ignatius et al, 2001), visibility of prey (Pena et al, 2004), swimming behavior and speed of prey (Sarma et al, 2003; Ostrowski and Laidley, 2001; Wittenrich et al, 2007), nutritional quality of prey (Vega-Orellana et al, 2006), larval digestive abilities (Vega-Orellana et al, 2006; Watanabe and Kiron, 1994), resistance to starvation of larvae, and sub-optimal environmental conditions for culture (Pena et al, 2004; Watanabe and Kiron, 1994).

As the superior prey quality of the copepod, *Parvocalanus crassirostris* as compared to the rotifers, *Branchionus rotundiformis* became clear via a series of larval rearing experiments in Chapters 3 and 4, it is important to gain insights into the underlying mechanisms that why copepods performed so much better than rotifers. Considering the marine ornamental fish larvae, the most important factor

influencing the success of first feeding is the physical size of prey compared to the mouth gape of larvae. However, the size of both *P. crassirostris* and *B. rotundiformis* used in this study clearly met the criteria as both prey were ingested by *S. splendidus* first feeding larvae. Although clear innate preference and significant higher ingestion rates on copepods by *S. splendidus* early larvae under co-feeding conditions helped explain the superior performance of copepods over rotifers in larval culture (Chapters 3 and 4), this appears to be only a part of the story since larvae did ingest substantial number of rotifers when they were fed rotifers only, and the surviving larvae of the prey switching to rotifers showed significantly lower percentage with initial notochord flexion compared to the continuous copepod feeding control (Chapters 3 and 4). These findings seem to be possibly attributed to the different nutrition profiles of the prey. Of major nutrients, lipids play important roles in providing metabolic energy (Sargent et al., 2002), maintenance of structure and function of cellular biomemmbranes and precursors of eicosanoids (Coutteau et al., 1997). Therefore, the quality and quantity of lipids supplied to the larvae via their prey could significantly affect survival, development and growth of first feeding larvae. A number of studies have clearly demonstrated that the essential fatty acids would help marine ornamental larvae achieve better survival and growth (Olivotto et al., 2003, 2005, 2006a, 2006b, 2008a; Avella et al., 2007), as well as decrease anomalies in the development of the central nervous system (Avella et al., 2007). DHA must be present in the diet to maximize larval survival of the coral reef damselfish, *Acanthochromis polyacanthus* (Southgate and Kavanagh, 1999). However, the dietary lipid requirement of *S. splendidus* early larvae is largely unknown.

Generally lipid composition of marine fish eggs provides a valuable

approximation of the optimal lipid composition of a larval diet, it has been suggested that the ideal diet for fish larvae should have a lipid composition close to that of fish egg (Sargent et al., 1999b). Therefore, an examination of lipid composition of *S. splendidus* eggs as well as rotifers and copepods could gain insights into underlying reasons of copepods' superior performance for *S. splendidus* first feeding larvae from the perspective of nutrition value. An additional analysis of the ontogenetic changes in fatty acids composition of *S. splendidus* could provide further information of lipid requirements of *S. splendidus* early larvae and an understanding of the relative importance of specific fatty acids during larval development (Fraser et al., 1988). Hence, the present study sets out to determine: 1) Fatty acids compositions of newly-spawned eggs of *S. splendidus* as well as rotifers and domesticated copepods from this laboratory; 2) Ontogenetic changes in lipid composition during larval development of *S. splendidus*. The findings gained in this study would provide baseline information on the basic lipid metabolism of early stage larvae and larval lipid nutrition as well as a complete answer to the superior performance of copepods as the first feeding diet.

## 5.2 Materials and Methods

### *5.2.1 Broodstock maintenance*

Broodstock source and husbandry were described in Chapter 2 (Section 2.1).

### *5.2.2 Live prey production*

Rotifers and copepods culture and harvest methods were described in

Chapter 2 (Section 2.2 and 2.3).

*5.2.3 Lipid and fatty acid analysis*

In order to compare nutritional values of the copepods and rotifers used for feeding *S. splendidus* larvae as well as their effects on the larvae, the total lipid and fatty acid composition of the newly-spawned eggs, 2 DPH pre-feeding larvae, 4 DPH larvae fed rotifers only; 4, 8 and 12 DPH larvae fed copepods only as well as rotifers and copepods cultured under standard condition were analyzed.

The newly-spawned eggs were collected and pooled together from four pairs of *S. splendidus* broodstock. Since the eggs were tiny and each pair of broodstock generally only produced between 80 to 2,100 eggs daily, it took a full two months of continuous collection to amass enough egg samples (0.6 g dry weight) for the lipid and fatty acid analysis. Similarly, the pre-feeding larvae were sampled after two days of development from the newly hatched larvae in the incubation buckets and samples collected over 70 days were pooled together to enable the analysis. All eggs and pre-feeding larvae samples were stored at -70 °C until analyzed.

Based on the results of copepod density experiment in Chapter 3 (Section 3.2.3.2), *S. splendidus* larvae of different ages (4, 8 and 12 DPH) fed copepods at 1 $mL^{-1}$ only, or 4 DPH larvae fed rotifers at 10 $mL^{-1}$ only were also sampled for lipid and fatty acid analysis. Unfortunately, due to high larval mortality beyond 4 DPH when fed on rotifers alone, it was not possible to collect enough samples of 8 and 12 DPH larvae when feeding on rotifers only for comparison. To obtain the larval samples, 60 pre-feeding larvae were stocked into four 10 L cylindrical

tanks ran as static system with a 100% water exchange daily. The larvae were firstly cultured to 4 DPH and sampled, when sufficient larval sample of 4 DPH was accumulated, larval culture then extended to 8 DPH for sampling and finally to 12 DPH. The procedure was repeated until accumulated enough larval samples of each age for the analysis and all larval samples were stored at -70 °C. Rotifers and copepods cultured under standard condition (Chapter 2, section 2.3) were also sampled three times (each time as a replicate) over the period of larval culture.

Prior to fatty acid analysis, each sample was freeze-dried and homogenized separately. The total lipid (TL) was then extracted with chloroform-methanol (2:1,v/v) (Folch et al., 1957). For fatty acid analysis, fatty acid methyl esters (FAME) were prepared by transesterification with boiling 14% borontrifluoride/methanol (w/w) following the method of Morrison and Smith (1964). FAMEs were analytically verified by flame ionization detection (FID) after injecting a sample into an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) fitted with an Omegawax 320 fused silica capillary column (30m×0.32mm; Supelco, Billefonte, PA, USA). The detailed method of fatty acid analysis was provided in Shao et al. (2013). Fatty acid composition was defined as percentage of a particular fatty acid to the total fatty acids (%). Three replicates were analyzed for each sample.

### *5.2.4 Data analysis*

All measurements were expressed as mean ± standard deviation (SD). Data were first assessed to confirm normality and homogeneity of variance (Levene's test for homogeneity of variances) and where necessary, arcsine-square root or

logarithmic transformation was performed prior to analysis. A one-way ANOVA was used to determine significant differences among treatments. If any significant difference was detected, Tukey's multiple range test was then used as the mean separation procedure. A statistical probability of $p<0.05$ was accepted as significant. All statistical analysis were performed using Statistica software version 22 (Statsoft™, Inc.).

### 5.3 Results

Table 5.1 shows total lipids (% dry weight) and fatty acid compositions (% total fatty acids) of the copepods and rotifers used as the larval prey, the *S. splendidus* newly-spawned eggs, 2 DPH pre-feeding larvae and 4 DPH larvae fed rotifers and copepods respectively, as well as 8 DPH and 12 DPH larvae fed copepods. The results show that of the two prey used, while their total lipids and total saturated fatty acids (SFAs) were very similar ($p>0.05$), the rotifers contained significantly higher total monounsaturated fatty acids (MUFAs) but significantly lower polyunsaturated (PUFAs) and long-chain polyunsaturated fatty acids (LC-PUFAs) than those of the copepods ($p<0.001$). The most striking difference in fatty acid profiles between the two prey appeared to be that the LC-PUFA profile of copepods was dominated by docosahexaenoic acid (DHA, 22:6n-3; 22.32%), which was almost 16 times greater than that of the rotifers (1.41%). On the other hand, eicosapentaenoic acid (EPA, 20:5n-3) contents were similar between the two prey (copepods: 5.39%; rotifers: 6.16%) while the rotifers contained significantly higher arachidonic acid (ARA, 20:4n-6) that the copepods (copepods: 0.55%; rotifers: 1.44%). As the result, the copepods had substantially higher DHA/EPA ratio than that of the rotifers (copepods: 4.14; rotifers: 0.23), and was very similar to that of newly-spawned eggs (4.73).

Of the most important essential fatty acids (EFAs), DHA and ARA of the 2 DPH pre-feeding larvae were significantly lower ($p$<0.001) and almost only half of that the newly-spawned eggs (decreased from 15.35% to 7.95% and from 7.13% to 3.94%, respectively) whereas EPA remained relatively stable and was not significantly different. This indicated that both DHA and ARA were heavily utilized during embryonic development and the yolk-sac period while EPA was not. On the other hand, after larvae started feeding for only two days, both DHA and ARA increased significantly in 4 DPH larvae fed rotifers or copepods. However, the increase in DHA (from 7.95% to 18.10% and 12.37% when fed copepods and rotifers respectively) were much more substantial than ARA (from 3.94% to 4.91% and 4.19% when fed copepods and rotifers respectively). Furthermore, DHA in the larvae fed copepods was significantly higher than those fed rotifers (18.10% vs. 12.37%), which appeared to mirror DHA levels of these two prey (Tab. 5.1).

For the larvae fed copepods, the total lipids as percentage of dry weight continued to increase significantly as they grew older, more than doubled from 13.30 ± 1.89% of 2 DPH larvae to 33.26 ± 2.55% in 12 DPH larvae. Of various fatty acid classes, SFAs remained relatively stable with the major constituents being 16:0 and 18:0 fatty acids. Among MUFAs, 18:1n-9 was the dominant fatty acid and on 12 DPH, MUFAs reached the highest level (27.79%; $p$<0.001). In contrast, LC-PUFAs was found to be at the lowest level (15.45%) for 12 DPH larvae. Among the most important LC-PUFAs, DHA was the most dominant one across all ages of the feeding larvae, ranging from 13.27% to 18.10%. As larval grew older, there was a significant increasing trend for DHA/EPA ratio but a decreasing trend for ARA/EPA ratio from 4 DPH to 12 DPH (Tab. 5.1).

**Table 5.1** Total lipids (% dry weight) and fatty acid compositions (% total fatty acids) of the ss-type rotifers *Brachionus rotundiformis* and the copepods *Parvocalanus crassirostris*, the green mandarin fish *Synchiropus splendidus* newly-spawned eggs, 2 DPH pre-feeding larvae, 4 DPH larvae fed the rotifers and the copepods, respectively, and 8 DPH, 12 DPH larvae fed the copepods. Values are represented as mean ± SD. Values within a same row with different superscripts are significantly different ($p<0.05$)

| | Rotifers | Copepods | Newly-spawned | 2 DPH prefeeding | 4 DPH larvae-fed | 4 DPH larvae-fed | 8 DPH larvae-fed | 12 DPH larvae-fed |
|---|---|---|---|---|---|---|---|---|
| | *B.rotundiformis* | *P.crassirostris* | Eggs | larvae | rotifers | copepods | copepods | copepods |
| Total Lipid (%) | 10.75±1.04$^{a}$ | 11.23±1.29$^{a}$ | 19.61±2.34$^{b}$ | 13.30±1.89$^{a}$ | 24.50±1.84$^{c}$ | 23.60±1.21$^{c}$ | 28.54±1.76$^{d}$ | 33.26±2.55$^{e}$ |
| Fatty acids (%) | | | | | | | | |
| 14:0 | 2.74±0.47$^{a}$ | 8.79±0.11$^{b}$ | 0.96±0.25$^{c}$ | 2.84±0.16$^{a}$ | 1.34±0.29$^{cd}$ | 1.42±0.21$^{d}$ | 3.03±0.43$^{a}$ | 3.76±0.23$^{e}$ |
| 16:0 | 21.43±0.64$^{a}$ | 16.53±0.11$^{b}$ | 20.24±1.16$^{a}$ | 23.03±0.39$^{a}$ | 27.11±2.01$^{c}$ | 26.83±1.97$^{c}$ | 26.99±1.43$^{c}$ | 28.36±0.78$^{c}$ |
| 18:0 | 6.29±0.22$^{b}$ | 5.20±0.09$^{b}$ | 14.19±0.41$^{ac}$ | 11.02±0.56$^{a}$ | 18.22±1.91$^{d}$ | 18.03±1.86$^{d}$ | 15.63±0.63$^{c}$ | 11.15±0.88$^{a}$ |
| ∑SFA | 31.23±0.43$^{b}$ | 31.17±0.11$^{b}$ | 35.70±0.91$^{a}$ | 37.30±0.89$^{a}$ | 47.31±2.40$^{c}$ | 46.80±2.06$^{c}$ | 46.33±1.85$^{c}$ | 44.05±2.54$^{c}$ |
| 16:1n-7 | 13.25±0.18$^{b}$ | 4.50±0.11$^{a}$ | 1.65±0.21$^{c}$ | 3.98±0.30$^{a}$ | 2.36±0.63$^{cd}$ | 1.75±0.34$^{c}$ | 2.88±0.64$^{d}$ | 4.72±1.11$^{a}$ |
| 18:1n-7 | 4.17±0.37$^{c}$ | 3.55±0.07$^{b}$ | 2.46±0.75$^{a}$ | 2.94±0.09$^{a}$ | 3.49±0.06$^{b}$ | 3.09±0.10$^{a}$ | 4.20±0.23$^{c}$ | 4.58±0.21$^{c}$ |
| 18:1n-9 | 6.73±0.74$^{a}$ | 7.14±0.11$^{a}$ | 10.22±0.66$^{b}$ | 13.50±0.45$^{b}$ | 11.10±0.92$^{b}$ | 10.30±0.87$^{b}$ | 9.84±0.44$^{b}$ | 16.99±1.22$^{c}$ |
| ∑MUFA | 24.47±0.98$^{a}$ | 17.04±0.99$^{b}$ | 15.25±1.02$^{b}$ | 22.97±1.61$^{a}$ | 17.82±1.34$^{b}$ | 15.72±1.03$^{b}$ | 17.52±0.77$^{b}$ | 27.79±1.87$^{c}$ |
| 18:2n-6 | 7.87±0.24$^{c}$ | 4.75±0.07$^{b}$ | 2.29±0.18$^{a}$ | 2.51±1.00$^{a}$ | 2.30±0.20$^{a}$ | 0.31±0.04$^{d}$ | 2.18±0.09$^{a}$ | 2.67±0.21$^{a}$ |
| 18:3n-6 | 1.25±0.05$^{b}$ | 0.21±0.01$^{c}$ | 0.50±0.10$^{a}$ | 0.40±0.04$^{a}$ | 0.52±0.04$^{a}$ | 0.66±0.06$^{a}$ | 1.85±0.11$^{d}$ | 3.29±0.13$^{e}$ |

| | Rotifers | Copepods | Newly-spawned | 2 DPH prefeeding | 4 DPH larvae-fed | 4 DPH larvae-fed | 8 DPH larvae-fed | 12 DPH larvae-fed |
|---|---|---|---|---|---|---|---|---|
| | *B.rotundiformis* | *P.crassirostris* | Eggs | larvae | rotifers | copepods | copepods | copepods |
| 20:3n-6 | 2.65±0.16$^{b}$ | 0.73±0.02$^{a}$ | 2.99±0.10$^{b}$ | 1.82±0.01$^{c}$ | 0.15±0.01$^{d}$ | n.d. | 0.23±0.02$^{e}$ | 0.41±0.15$^{e}$ |
| 20:4n-6(ARA) | 1.44±0.10$^{a}$ | 0.55±0.07$^{b}$ | 7.13±0.31$^{c}$ | 3.94±0.22$^{d}$ | 4.19±0.17$^{d}$ | 4.91±0.29$^{e}$ | 1.53±0.19$^{a}$ | 0.73±0.22$^{b}$ |
| 20:5n-3(EPA) | 6.16±0.18$^{a}$ | 5.39±0.26$^{a}$ | 3.25±0.22$^{b}$ | 3.93±0.27$^{b}$ | 2.91±0.56$^{bc}$ | 2.53±0.38$^{c}$ | 1.77±0.21$^{d}$ | 1.08±0.13$^{e}$ |
| 22:6n-3(DHA) | 1.41±0.23$^{a}$ | 22.32±0.32$^{d}$ | 15.35±0.34$^{c}$ | 7.95±0.78$^{b}$ | 12.37±0.54$^{e}$ | 18.10±0.52$^{f}$ | 17.34±1.48$^{c}$ | 13.27±0.92$^{e}$ |
| ∑PUFA | 29.42±0.39$^{a}$ | 41.71±0.85$^{c}$ | 36.51±2.06$^{b}$ | 29.47±1.09$^{a}$ | 23.00±1.20$^{de}$ | 26.60±0.97$^{e}$ | 25.29±1.54$^{e}$ | 21.50±0.85$^{d}$ |
| ∑LC-PUFA | 17.05±0.40$^{a}$ | 32.40±0.74$^{b}$ | 30.73±0.55$^{b}$ | 23.53±1.76$^{c}$ | 20.01±0.78$^{d}$ | 25.63±1.01$^{c}$ | 21.26±1.21$^{d}$ | 15.45±1.04$^{a}$ |
| ∑n-3PUFA | 15.33±0.61$^{c}$ | 34.74±0.60$^{a}$ | 21.37±0.86$^{b}$ | 18.64±1.54$^{b}$ | 15.65±0.54$^{c}$ | 20.76±0.85$^{b}$ | 19.51±1.23$^{b}$ | 14.35±0.65$^{c}$ |
| ∑n-6PUFA | 14.09±0.26$^{c}$ | 6.92±0.31$^{a}$ | 15.14±1.22$^{c}$ | 10.84±0.59$^{b}$ | 7.35±0.32$^{a}$ | 5.84±0.12$^{d}$ | 5.78±0.22$^{d}$ | 7.16±0.24$^{a}$ |
| n-3/n-6 | 1.09±0.04$^{a}$ | 5.03±0.03$^{c}$ | 1.41±0.08b | 1.72±0.15$^{b}$ | 2.13±0.09$^{d}$ | 3.56±0.21$^{e}$ | 3.89±0.32$^{e}$ | 2.00±0.17$^{bd}$ |
| DHA/EPA | 0.23±0.03$^{c}$ | 4.14±0.14$^{a}$ | 4.73±0.22$^{a}$ | 2.02±0.50$^{b}$ | 4.33±0.68$^{a}$ | 7.23±0.90$^{d}$ | 9.80±0.33$^{e}$ | 12.33±0.57$^{f}$ |
| ARA/EPA | 0.23±0.01$^{a}$ | 0.10±0.01$^{b}$ | 2.20±0.06$^{c}$ | 1.00±0.07$^{d}$ | 1.46±0.23$^{e}$ | 1.95±0.19$^{e}$ | 0.85±0.10$^{d}$ | 0.68±0.03$^{f}$ |

n.d: value was not detectable or <0.1%; DPH: days post-hatching; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; LC-PUFA: long-chain polyunsaturated fatty acids.

## 5.4 Discussion

Clear preference and significant higher ingestion rates on copepods by *S. splendidus* early larvae under co-feeding conditions help explain the superior performance of copepods over rotifers in larval culture, however this appeared to be only a part of the story since larvae did ingest substantial number of rotifers when they were fed rotifers only. The nutritional profiles of copepods have been reported to better match larval requirements (Mæhre et al., 2013) and hence in this study, total lipids and fatty acids of both the rotifers and the copepods as well as newly-spawned eggs and different aged larvae were analyzed and compared. Lipids generally form an important part of marine fish eggs and are a major source of energy during embryonic and early larval development prior to first feeding (Bell and Sargent, 2003). The present study found that the mean total lipid content of the newly-spawned eggs of *S. splendidus* was 19.61%, it decreased rapidly to 13.30% in the 2 DPH pre-feeding larvae. However following feeding, larval total lipids increased substantially to 33.26% in 12 DPH larvae. The copepods used in this study contained only slightly higher total lipids than that of the rotifers and the difference was not significant, hence the difference in total lipids of the two prey was unlikely a major contributor to their performance difference for *S. splendidus* larval culture.

Although total lipid content may be similar, differences in fatty acid profiles of both rotifers and copepods, particular essential fatty acids (EFAs), could very much determine their dietary values (Bell and Sargent, 2003). Generally lipid composition of marine fish eggs provides a valuable approximation of the optimal lipid composition of a larval diet, it has been suggested that the ideal diet for fish larvae should have a lipid composition close to that of fish egg (Sargent et al.,

1999b). The newly-spawned eggs of *S. splendidus* contained 15.25% of MUFA, 36.51% of PUFA and 30.73% of LC-PUFA, which was similar to 17.04% of MUFA, 41.71% of PUFA and 32.40% LC-PUFA of the copepods but very different from 24.47% of MUFA, 29.42% of PUFA and 17.05% LC-PUFA of the rotifers.

More importantly, first feeding fish larvae need live prey to provide sufficient EFAs to meet their development requirements (Lall and Lewis-McCrea, 2007; Park et al., 2006) and larval requirements for LC-PUFAs belonging to the n-3 LC-PUFA group is higher than those of juveniles and pre-adults in marine fish (Sargent et al., 2002). The intake of from diets is the only way for marine fish to obtain EFAs, such as EPA, DHA and ARA, since marine larvae are unable to synthesize these fatty acids from their precursors (Cahu and Infante, 2001; Kanazawa, 2003; Sargent et al., 1999a). The most striking difference in EFA contents between the copepods and rotifers was that the copepods contained high level of DHA (22.32%) while the rotifers were deficient in DHA (1.41%), which was only approximately 1/11 of that newly-spawned eggs (15.35%). Hamre and Harboe (2008) showed that the nervous and sensory systems in vertebrates, including fish, contain high levels of DHA and its supply during early life stages was considered critically important for the development of cognitive and sensory function. Hence, it is possible that the larvae fed copepods had better developed vision and neural system, and were more successful predators.

Finally, the important DHA/EPA ratio of the copepods was 4.1, which is very similar to 4.7 of the newly-spawned eggs, but the ratio for the rotifers was dramatically different at 0.2. In fact, a decreasing trend was found on the

DHA/EPA ratio during the embryonic development of *S. splendidus*, resulting in a significantly lower DHA/EPA ratio in 2 DPH pre-feeding larvae (from 4.7 to 2.0), which confirmed the heavy utilization of DHA during early development of *S. splendidus* prior to the first feeding and hence likely subsequent high demands for DHA by the larvae from their diets.

Aside from the superior fatty acid profile of the domesticated copepods, *P. crassirostris* as compared to the rotifers, copepods reportedly also possess superior compositions of other essential nutrients, such as high quality proteins, amino acids (e.g. Taurine) and minerals (e.g. Selenium) (Hamre et al., 2008; Mæhre et al., 2013). Moreover, early larvae of many marine fish often do not have a well-developed digestive system and may need exogenous supply of digestive enzymes from live prey to help digestion (Yúfera and Darias, 2007), copepods are known to contain high level of digestive enzymes as well as higher proportion of phospholipids, which are more effectively digested by fish larvae than triacylglycerols (Conceição et al., 2010), hence are likely to promote more effective utilization of ingested food by the larvae.

The differences of fatty acid composition between 4 DPH larvae fed rotifers and copepods generally reflected their diets. During larval development of *S. splendidus*, total lipid and fatty acids composition underwent significant changes. The 12 DPH *S. splendidus* larvae fed copepods were found to have the highest total lipid content among all larval ages, and the possible explanation for this: it is likely related to the high energy and nutritional reserves required to facilitate crucial process of notochord flexion and metamorphosis. From 4 to 12 DPH, there was a decreasing trend of SFA, particularly 18:0 in the larvae fed copepods,

indicating that these were preferentially mobilized as energy sources for growth (Sargent et al., 2002). Furthermore, 12 DPH *S. splendidus* larvae contained significantly lower percentage of ARA, EPA and DHA than younger larvae, which suggested that dietary LC-PUFAs might become increasingly important during early larval physiologically demanding periods. However, the increasing DHA/EPA ratio while decreasing ARA/EPA ratio were detected from 4 DPH onwards, which could be attributed to the diet: copepod (DHA/EPA=4.14; ARA/EPA=0.10).

# Chapter 6

## Identifying the suitable time for introducing *Artemia* nauplii and subsequently enriched metanauplii in either live or dead form for larval rearing of green mandarin fish, *Synchiropus splendidus*

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### 6.1 Introduction

Unlike freshwater ornamental species, of which over 90% are captive produced, the vast majority of marine aquaria species are currently sourced from wild-caught specimens (Job, 2011; FAO, 2012). Marine ornamental collectors for developing countries, which are the world's largest exporters, often employ destructive fishing techniques, such as cyanide and explosives, to stun the fish for easy collection (Laidley and Ostrowski, 2000). The use of techniques is known not only harms targeted, but also non-targeted reef fish and has damaging effects on coral reefs (Moorhead and Zeng, 2010; Vaz et al., 2012). The current dependence of marine aquarium industry on wild-caught specimens, which have poor survival rates, to supply the rapid growing demands worldwide on marine ornamentals is clearly unsustainable. Therefore, captive breeding of marine ornamentals has been proposed as the only viable as well as substantial alternative (Pomeroy et al., 2006； Moorhead and Zeng, 2010； Olivotto et al., 2011).

The green mandarin fish, *Synchiropus splendidus*, is among the most heavily collected marine ornamentals due to its popularity among aquarium hobbyist. Belonging to the family *Callionymidae*, *S. splendidus* is a pelagic-spawner that produces small eggs and hence considered relatively hard to breed (Sadovy et al.,

2001). Previous attempts to develop larval culture techniques for *S. splendidus* in this laboratory has established feeding regime for early larvae up to 11 DPH by feeding copepods solely at 1 $mL^{-1}$ (Chapter 3).

However, the use of copepods as larval prey is constrained by a lack of reliable and cost effective culture methods (Ianora et al., 1996; Støttrup and Norsker, 1997; Olivotto et al., 2008; Olivotto et al., 2011), and with larval development, their consumption on copepods increasing rapidly and hence copepod supply became increasingly a problem. Another further study was consequently conducted to assess whether and when copepods could be replaced by rotifers during *S. splendidus* larval culture, the results showed that if copepods were replaced by rotifers on 12 DPH, larvae generally had similar survival and growth as those from the continuous copepod feeding control although larval development was negatively impacted (Chapter 4). This result suggests that if copepod production is a problem or too costly, a rotifers feeding transitional period could be implemented before larvae accept newly hatched *Artemia* nauplii, which is based on the assumption that for those rotifer fed larvae, once they start to accept *Artemia* nauplii, their development would catch up.

The typical feeding regime for marine fish larvae is to start with rotifers, followed by newly hatched *Artemia* nauplii, which are subsequently replaced by larger enriched *Artemia* metanauplii (Zambonino Infante and Cahu, 1994; Dhert et al., 2001; Sorgeloos et al., 2001) before being weaned into formulated feed. Such sequential changes in larval feeds must be timed correctly to match the morphological, physiological and foraging competency of the larvae to obtain best results (Léger and Sorgeloos, 1984; Léger et al., 1986; Nhu et al., 2009). For

example, the newly hatched instar I *Artemia* nauplii is smaller in size with weaker swimming ability, making them a more suitable prey for younger larvae that can be weaned off rotifers. On the other hand, enriched instar II *Artemia* metanauplii are larger (about 50% larger), more mobile and elusive to marine larvae, but more nutritional after enrichment (Sorgeloos et al., 2001), hence better suit more developed larvae following a few days feeding on *Artemia* nauplii. Clearly, establishing an appropriate and well-defined larval feeding regime throughout larval development through a series properly designed experiments are necessary to ensure consistent high larval survival (Samocha et al., 1989; Holt et al., 2007). Unfortunately, such an approach is largely lacking in the past attempts to develop captive breeding techniques for most marine ornamental species.

In this study, two experiments were designed and conducted to investigate the best timing of utilizing different forms of *Artemia* for feeding green mandarin fish larvae. The first experiment sought to determine the best timing for introducing *Artemia* nauplii as *S. splendidus* larval diet as well as evaluated the effects of with and without an intermittent rotifer feeding period between copepod and *Artemia* nauplii feeding on such transition. The second experiment subsequently investigated the appropriate time for the introduction of enriched *Artemia* metanauplii as well as compared the effects of enriched *Artemia* metanauplii being introduced as live vs. dead form. These experiments enabled the establishment of a complete larval feeding regime for *S. splendidus*.

## 6.2 Materials and Methods

### *6.2.1 Broodstock husbandry*

Broodstock source and husbandry were described in Chapter 2 (Section 2.1).

### *6.2.2 Live prey production*

Rotifers and copepods culture and harvest methods were described in Chapter 2 (Section 2.2 and 2.3).

*Artemia* cysts (Great Salt Lake strain, INVE Aquaculture) were hatched daily to obtain newly hatched nauplii. After 24 h of hatching, newly hatched *Artemia* nauplii were harvested for feeding larval directly or late enrichment. The enrichment of *Artemia* was carried out in 10 L bucket with a commercial enrichment emulsion following manufacture's instruction (Selco S. presso, INVE Aquaculture; ∑LC-PUFA: 150 mg/g DW; DHA/EPA=9). After enrichment, the enriched *Artemia* metanauplii were harvested and thoroughly rinsed with freshwater to remove residual emulsion before being used for feeding to the larvae. Before the experiment 2 started, the live enriched *Artemia* metanauplii were harvested and separated into 20 small containers then immediately frozen in a -70 °C fridge to death. When needed for feeding larvae, the dead enriched *Artemia* metanauplii that was prior prepared in each container were removed from freezer and placed in a beaker with clean seawater to thaw prior to the feeding. The average size of the enriched *Artemia* metanauplii used was 709 ± 8 µm.

*6.2.3 Larval rearing experiments*

6.2.3.1 Initial communal rearing of larvae and general experimental procedures

For both experiments, larvae hatched on a same day were collected and initially reared communally under an identical feeding condition (Fig. 6.1). The tank used for communal rearing was 100 L fiberglass conical tank as described by Moorhead (2015) and lit overhead by fluorescent ceiling lights. During the period of communal culture, the water was exchanged 50% daily at around 09:00 am with gentle inflow of clean filtered water from a large recirculating system. Temperature in the culture tank was maintained between 27 to 29.5 °C, salinity 35 to 37 ‰, pH 8.0 to 8.2, $NH_3$, $NO_2$ < 0.2 ppm, and $NO_3$ < 5 ppm.

To start an experiment with different treatments as shown in Figure 6.1, healthy larvae on a designated day of development based on the experimental design were randomly transferred from the communal rearing tank to 3 L replicate culture vessels. The culture vessels and experimental system were described in Chapter 2 (Section 2.5). Each replicate vessel was maintained as a semi-static system. During the experiments, water exchange was carried out twice daily (09:00 am and 05:00 pm) with gentle flow (250 mL $min^{-1}$) of filtered seawater from the recirculating system for 40-60 min to flush out uneaten prey and detritus before new prey were added. In experiment 2, for treatments that thawed dead enriched *Artemia* metanauplii were used to feed larvae, 400 µm mesh was used to form the upper floor of the vessels to prevent the dead *Artemia* metanauplii sunk onto the PVC bottom floor, and the uneaten *Artemia* and detritus were carefully siphoned out twice daily.

The feeding behavior was observed immediately after adding prey to the

culture vessels. Each morning, the number of surviving larvae in each replicate was counted and noted to obtain daily survival data. On the final day of the experiments, all surviving larvae were anesthetized with clove oil at 0.05 ml $L^{-1}$ (AQUI-S®, New Zealand LTD) and photographed under a dissecting microscope (Leica MZ 125) fitted with a digital camera (Olympus DB 25) for measuring standard length (BL: the length from the tip of the snout to the posterior extremity of the hypural plate) and body width (BW: the transverse distance between body margins at the pectoral fin base). Larvae were then rinsed with 0.5 M ammonium formate to remove the residual salt (Bransden et al., 2005) before being placed in a 60 °C oven for 18 h. The measurement of the DW of the larvae was carried out with a Cahn C-33 micro-balance (precision: 1 μg, Thermo Fisher Scientific Inc, Pittsburgh, Pa, USA).

6.2.3.2 Experiment 1: Determining the timing for introducing *Artemia* nauplii and the effects of with and without a transitional rotifer feeding period

This experiment was conducted to determine the best time to introduce newly hatched *Artemia* nauplii as well as the effects of with and without a rotifer feeding transitional period between copepod and *Artemia* nauplii feeding. A transitional rotifer feeding period should reduce the pressure on copepod production and supply, which is still costly with low productivity. To start the experiment, newly hatched *S. splendidus* larvae were firstly reared communally on 1 copepod $mL^{-1}$ (see Section 6.2.3.1 for details). On the morning of 12 DPH, healthy larvae were randomly collected and transferred into the 21 × 3 L culture vessels (Section 6.2.3.1) assigned to seven treatments: three treatments were fed continuously on copepods at 1 $mL^{-1}$ but switched to *Artemia* nauplii at 5 $mL^{-1}$ on 15, 18 and 21 DPH, respectively; larval prey in other three treatments were firstly

transited from copepod to rotifer feeding (10 $mL^{-1}$) on the day of transfer (12 DPH) and then switched again to *Artemia* nauplii (5 $mL^{-1}$) on 15, 18 and 21 DPH, respectively. There was a control with continuous copepod feeding at 1 $mL^{-1}$ throughout the experiment duration (Fig. 6.1A). All treatments were triplicated and each replicate stocked 20 larvae. The experiment terminated on 25 DPH.

6.2.3.3 Experiment 2: Determining the timing for introducing enriched *Artemia* metanauplii and the effects of providing them in live vs. dead form

The aim of this experiment was to determine the best time for introducing enriched instar II *Artemia* metanauplii to post-settlement *S. splendidus*, and to examine the effects of providing enriched *Artemia* in either live or dead form. The second part of the experiment was based on following considerations: 1) dead enriched *Artemia* settled on the bottom hence may be more suitable for increasingly benthic *S. splendidus*; 2) dead enriched *Artemia* prevent the self-metabolism by *Artemia* on enriched lipid nutrition; and 3) dead *Artemia* can be produced in large quantity and stored for late use, reducing costs associated with daily *Artemia* hatching. For the experiment, the larvae were firstly reared communally and fed the copepods at 1 $mL^{-1}$ between 2-17 DPH, and then newly hatched *Artemia* nauplii at 5 $mL^{-1}$ between 18-21 DPH, since such a feeding regime produced the best survival and growth based on previous experiments.

On 22 DPH, healthy *S. splendidus* from the communal culture were randomly allocated to the replicate 3 L vessels and subjected to seven different feeding treatments. This experiment adopted a crossed two-factor design with different times on introducing enriched *Artemia* metanauplii (at 5 $mL^{-1}$ on 22, 25 and 28 DPH, respectively) and two forms of enriched *Artemia* metanauplii (live

and dead) and as the fixed factors (Fig. 6.1B). A control in which the *S. splendidus* were fed *Artemia* nauplii at 5 $mL^{-1}$ till the end of the experiment was also set up. All treatments were triplicated and each replicate stocked 15 larvae. The experiment terminated on 42 DPH.

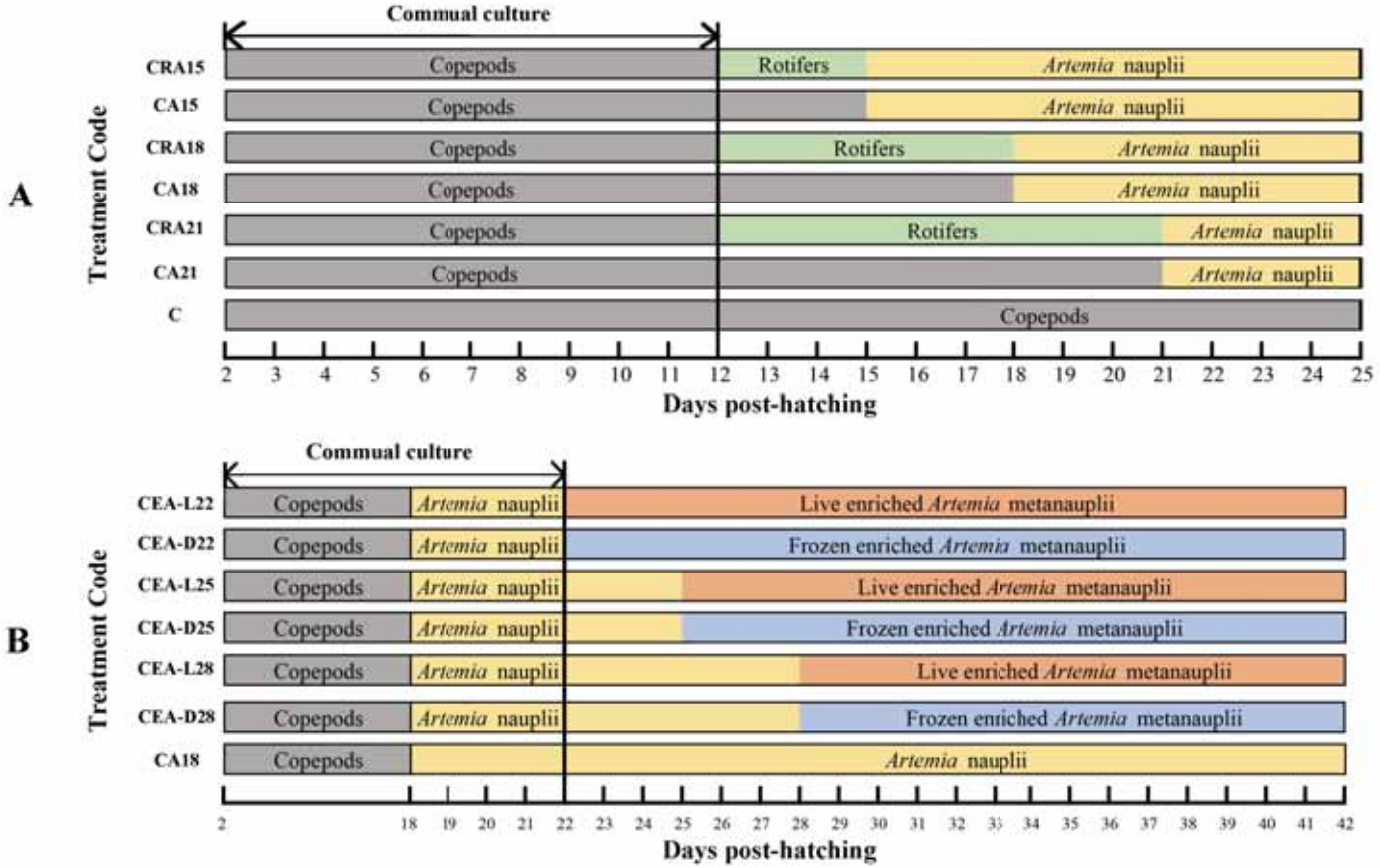


**Figure 6.1** Experiment designs of the experiment 1 and 2: showing different feeding regimes. Black vertical lines indicate the time

larvae were transferred from the communal culture tank to the replicate experimental vessels to subject to different treatments. (**A)** Experiment 1: all larvae were firstly cultured communally and fed copepods at 1 $mL^{-1}$ until 11 DPH. CRA15, CRA18 and CRA21: larvae subsequently fed rotifers from 12 DPH until the time of introducing *Artemia* nauplii on 15, 18 and 21 DPH, respectively; CA15, CA19 and CA21: larval diet switched from copepods to *Artemia* nauplii directly (without a rotifers feeding period) on 15, 18 and 21 DPH, respectively; C: larvae fed copepods at 1 $mL^{-1}$ throughout. **(B)** Experiment 2: all larvae were firstly cultured communally and fed copepods at 1 $mL^{-1}$ until 17 DPH. CEA-L22, CEA-L25 and CEA-L28: larvae fed on newly hatched *Artemia* nauplii from 18 DPH onward until the introduction of live enriched *Artemia* metanauplii on 22, 25, 28 DPH, respectively; CEA-D22, CEA-D25 and CEA-D28: larvae fed on newly hatched *Artemia* nauplii from 18 DPH onward until the introduction of dead enriched *Artemia* metanauplii on 22, 25 and 28 DPH, respectively; CA18: larvae fed on newly hatched *Artemia* nauplii from 18 DPH until the end of experiment on 42 DPH.

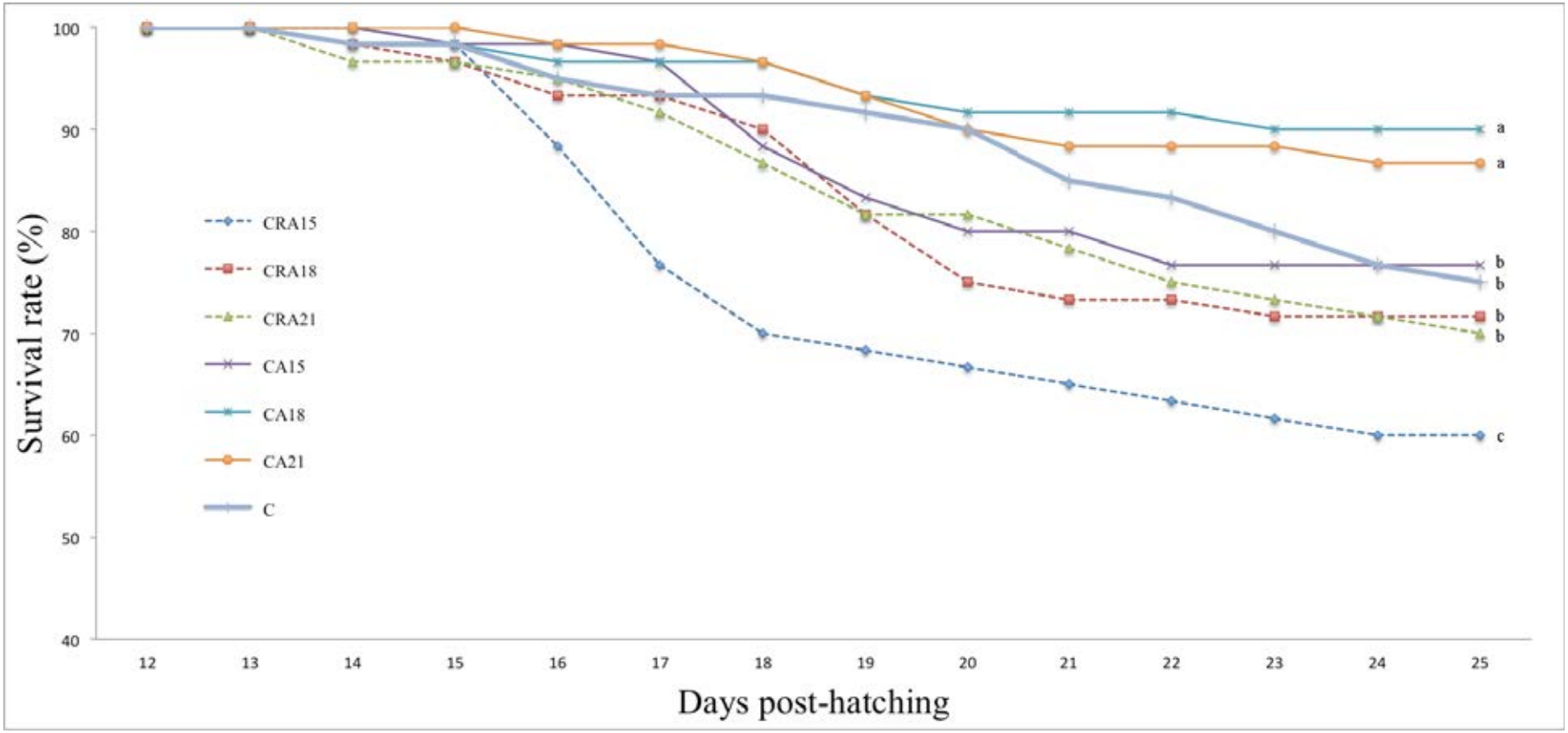


**Figure 6.2** Daily survival of *S. splendidus* larvae from experiment 1. Different letters denote significant differences ($p<0.05$). Standard deviations (SD) have been omitted for clarity. Please refer to Fig. 6.1A for explanation on treatment codes.

**Table 6.1** Growth performance of surviving *S. splendidus* on 25 DPH from experiment 1. Values in the same row with different superscripts are significantly different ($p<0.05$).

| | Treatments | | | | | | | two-way ANOVA ($p$-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CRA15 | CRA18 | CRA21 | CA15 | CA18 | CA21 | C | Prey | Time | Prey*Time |
| Standard Length (mm) | $4.35 \pm 0.20^{a}$ | $4.33 \pm 0.22^{a}$ | $4.02 \pm 0.28^{ab}$ | $4.32 \pm 0.19^{a}$ | $4.37 \pm 0.24^{a}$ | $4.13 \pm 0.27^{ab}$ | $3.97 \pm 0.08^{b}$ | 0.742 | 0.119 | 0.874 |
| Body Width (mm) | $1.57 \pm 0.19^{a}$ | $1.45 \pm 0.20^{a}$ | $1.22 \pm 0.19^{ab}$ | $1.55 \pm 0.16^{a}$ | $1.76 \pm 0.19^{a}$ | $1.37 \pm 0.21^{ab}$ | $1.06 \pm 0.11^{b}$ | 0.173 | 0.875 | 0.113 |
| Dry Weight (mg) | $1.01 \pm 0.16^{a}$ | $1.02 \pm 0.21^{a}$ | $0.88 \pm 0.11^{ab}$ | $0.97 \pm 0.17^{a}$ | $1.09 \pm 0.19^{a}$ | $0.83 \pm 0.10^{ab}$ | $0.69 \pm 0.09^{b}$ | 0.932 | 0.135 | 0.782 |

For treatment code descriptions please refer to Fig. 6.1A. C is not included for the two-way ANOVA.

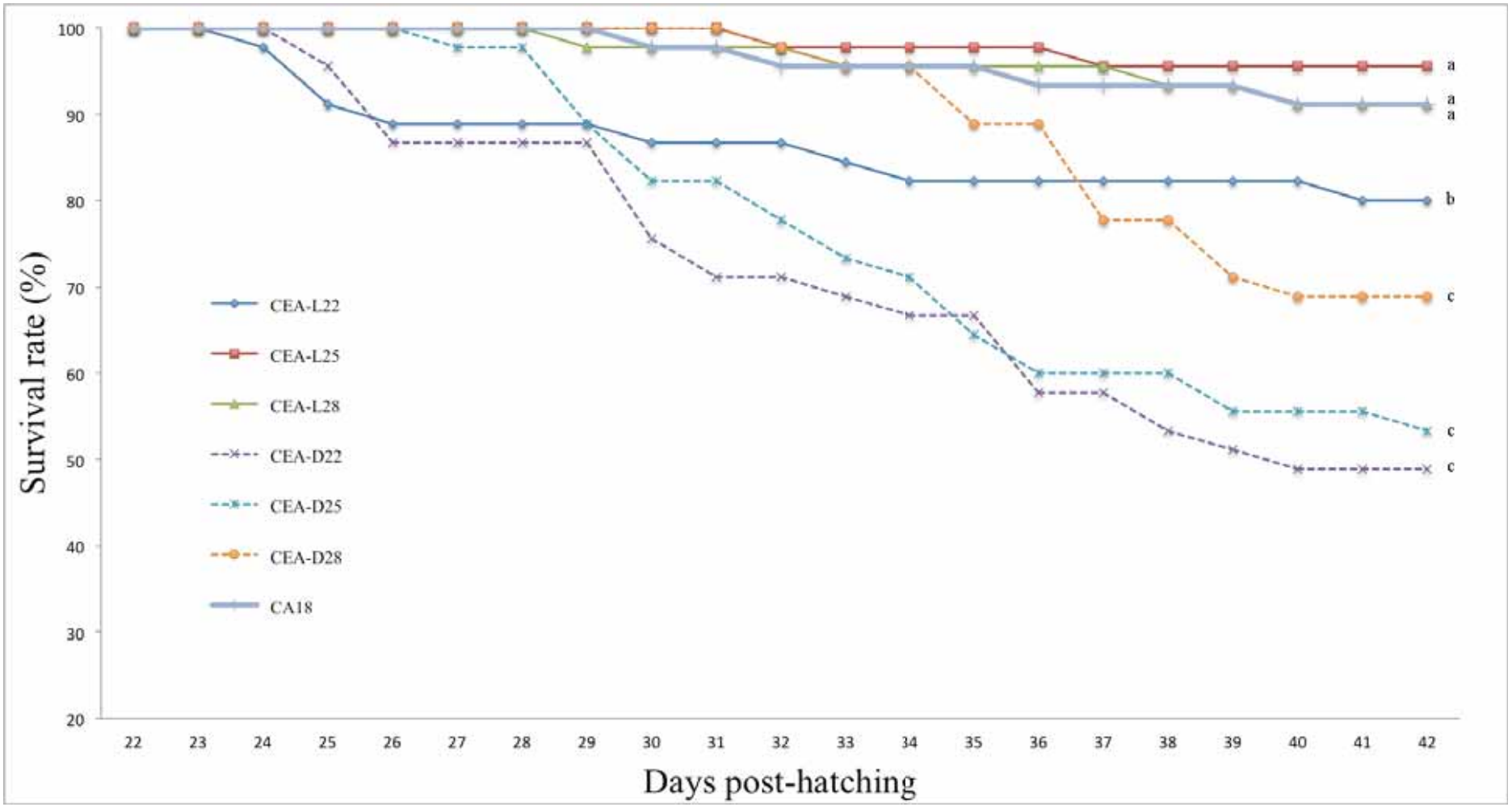


**Figure 6.3** Daily survival of post-settlement *S. splendidus* from experiment 2. Different letters denote significant differences ($p$<0.05). Standard deviations (SD) have been omitted for clarity. Please refer to Fig. 6.1B for explanation on treatment codes.

**Table 6.2** Growth parameters of surviving post-settlement *S. splendidus* on 42 DPH from experiment 2. Values in the same row with different superscripts are significantly different ($p$<0.05).

| | Treatments | | | | | | | two-way ANOVA ($p$-value) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CEA-L22 | CEA-L25 | CEA-L28 | CEA-D22 | CEA-D25 | CEA-D28 | CA18 | Prey | Time | Prey* Time |
| Standard Length (mm) | $8.56 \pm 0.48^{a}$ | $8.74 \pm 0.65^{a}$ | $8.38 \pm 0.54^{a}$ | $5.88 \pm 0.52^{b}$ | $6.10 \pm 0.54^{b}$ | $5.99 \pm 0.44^{b}$ | $6.07 \pm 0.37^{b}$ | 0.000 | 0.714 | 0.882 |
| Body Width (mm) | $3.44 \pm 0.21^{a}$ | $3.42 \pm 0.24^{a}$ | $3.35 \pm 0.24^{a}$ | $2.24 \pm 0.17^{b}$ | $2.27 \pm 0.21^{b}$ | $2.21 \pm 0.20^{b}$ | $2.35 \pm 0.20^{b}$ | 0.000 | 0.845 | 0.975 |
| Dry Weight (mg) | $4.11 \pm 0.25^{a}$ | $4.31 \pm 0.28^{a}$ | $4.12 \pm 0.22^{a}$ | $2.02 \pm 0.31^{b}$ | $2.17 \pm 0.35^{b}$ | $2.03 \pm 0.29^{b}$ | $2.25 \pm 0.19^{b}$ | 0.000 | 0.739 | 0.984 |

For treatment code descriptions please refer to Fig. 6.1B. CA18 is not included for the two-way ANOVA.

*6.2.4 Data analysis*

All data are presented as mean ± standard deviation (SD). Data were confirmed normality and homogeneity of variance (Levene's test for homogeneity of variances) before subjected to one-way ANOVA analysis. If significant differences were found, a Tukey's multiple comparisons test was performed to identify significantly different treatments. A statistical probability of $p<0.05$ was accepted as significant. Interactive effects between "dietary prey" and "time of prey switch" were determined using two-way ANOVA. All statistical analyses were performed using Statistics, Version 22.

## 6.3 Results

*6.3.1 Experiment 1*

Larval survival of *S. splendidus* at the end of the experiment (25 DPH) was significantly affected by both the timing of *Artemia* nauplii introduction and whether a transitional rotifer feeding interval was incorporated (Fig. 6.2)($p < 0.05$). The highest larval survival between 12 and 25 DPH (metamorphosis period) were from the two treatments in which larval prey were switched directly from the copepods to *Artemia* nauplii on 18 DPH (CA18) and 21 DPH (CA21) without a rotifer feeding period, reaching 90.0 ± 0.0% and 86.7 ± 2.9% respectively (Fig. 6.2). The continuous copepod feeding control (C) had comparable larval survival to the two treatments until 20 DPH but decreased sharply afterward, resulting in a significant lower survival of 75.0 ± 5.0% during the metamorphosis period ($p<0.01$). Prey switching from copepods to *Artemia* nauplii earlier on 15 DPH (CA15) without a rotifer feeding period resulted in a survival (76.7 ± 2.9%)

similar to the control, but significantly lower than the two best performed treatments (i.e. CA18 and CA21) ($p<0.01$).

For treatments in which a rotifer feeding period was incorporated from 12 DPH onward, an early larval diet switch from rotifers to *Artemia* nauplii on 15 DPH (CRA15) sustained mass mortality (ca. 30 %) between 15 to 18 DPH, resulting in a significantly lower final survival (60.0 ± 0.0%) than all other treatments ($p<0.05$) (Fig. 6.2). On the other hand, when switching rotifers to *Artemia* nauplii occurred later on 18 (CRA18) and 21 DPH (CRA21), larval survival fared much better (71.7 ± 2.9% and 70.0 ± 5.0%, respectively). However when compared to corresponding treatments in which no rotifer feeding period was incorporated (i.e. CA18 and CA21), the final survival were still significantly lower (Fig. 6.2)($p<0.05$).

Except the continuous copepod feeding control, the standard length (SL), body width (BW) and dry weight (DW) of the surviving larvae at the end of the experiment did not differ significantly among all treatments, whether a rotifer feeding period was incorporated (Tab. 6.1). The continuous copepod feeding control had significantly lower SL, BW and DW than those of the larvae from the treatments in which *Artemia* nauplii were introduced on 15 and 18 DPH whether or not a rotifer feeding period was incorporated ($p<0.05$). However, when compared to the two treatments in which prey switching to *Artemia* nauplii occurred later on 21 DPH (CRA21 and CA21), no significant difference in growth was detected (Tab. 6.1)($p>0.05$).

*6.3.2 Experiment 2*

Both the timing of switching *Artemia* nauplii to enriched metanauplii and providing enriched *Artemia* metanauplii in either live or dead form significantly affected *S. splendidus* survival ($p$<0.05)(Fig. 6.3). The highest survival at the end of the experiment was from the treatment in which enriched *Artemia* metanauplii were introduced live on 25 DPH (CEA-L25: 95.6 ± 2.9%, please note this survival is for 20 days between 22-42 DPH only and the same applied for subsequent survival for experiment 2) and 28 DPH (CEA-L28: 91.1 ± 2.9%), respectively. Similar survival was obtained from the control in which *Artemia* nauplii were fed throughout (CA: 91.1 ± 2.9%)(Fig. 6.3). Earlier switch to live enriched *Artemia* metanauplii on 22 DPH bore a significant poorer survival (CEA-L22: 80.0 ± 5.0%) than the above three best performed treatments ($p$<0.05). However, still significant higher than all treatments in which dead enriched *Artemia* metanauplii were introduced for feeding on 22 DPH (CEA-D22: 48.9 ± 2.9%), 25 DPH (CEA-D25: 53.3 ± 13.2%) and 28 DPH (CEA-D28: 68.9 ± 10.4%), respectively ($p$<0.05)(Fig. 6.3).

Significant differences in growth performance at the end of the experiment were detected among different treatments ($p$<0.05), which appears to relate to the form of enriched *Artemia* used but not the time of their introduction (Tab. 6.2). For instance, regardless the time for their introduction, surviving *S. splendidus* from the treatments fed live enriched *Artemia* (CEA-L22, CEA-L25 and CLA-L28) had significant superior BL (~1.5 folds), BW (~1.4 folds), and DW (~2.0 folds) than those from the treatments fed dead enriched *Artemia* metanauplii (CEA-D22, CEA-D25 and CLA-D28) ($p$<0.05). On the contrast, no significant difference in growth performance was detected among treatments fed same live or

dead enriched *Artemia* but with different introduction times ($p>0.05$). Finally, the control in which *Artemia* nauplii were fed throughout had significantly inferior SL (6.07 ± 0.37 mm), BW (2.35 ± 0.20 mm) and DW (2.25 ± 0.19 mg) than the treatments fed live enriched *Artemia* ($p<0.01$), but not those treatments fed dead enriched *Artemia* metanauplii (Tab. 6.2).

### 6.4. Discussion

The present study demonstrated the importance of timing the right widow period during early life history development of *S. splendidus* for introducing both newly hatched *Artemia* nauplii to feed its larvae and enriched *Artemia* metanauplii to feed its post-larvae, and clear superiority of live enriched *Artemia* metanauplii as compared to dead ones for feeding post-settlement *S. splendidus*. Our results showed that newly hatched *Artemia* nauplii should be introduced for feeding larvae between 18 to 21 DPH, while 22 to 25 DPH was recommended for the introduction of live enriched *Artemia* metanauplii for feeding *S. splendidus*. The present experiments, together with those previous ones investigating feeding protocols for early larvae, has successfully established a complete feeding regime for *S. splendidus* from newly hatched larvae through up to 42 DPH.

Although techniques for intensive copepod culture have been improved substantially in experimental scale in recent years (Alajmi and Zeng, 2013; 2014; Alajmi et al., 2014; 2015; Kline and Laidley, 2015), the usage of intensively cultured copepods in commercial scale larval culture is still severely constrained by low productivity and high production costs of copepods (Olivotto et al., 2011). As with larval development, their consumption on copepods increasing rapidly, copepod production represents an even more severe problem as it generally has

hard times in catching up with fast growing demands. In order to alleviate such a main constrain, this study further investigated the feasibility of a transitional rotifer feeding period be implemented to replace copepods for older *S. splendidus* larvae before they could effectively feeding on larger newly hatched *Artemia* nauplii. Our results demonstrated that such a rotifer feeding period is feasible between 12 to 18 DPH with acceptable larval survival (survival >70% during the metamorphosis period) and growth, although if copepods supply is not a problem, feeding copepods up to 18 DPH should generate significant better results.

As larvae developed further, rotifers normally become unsuitable prey for fish larvae; however with significantly improved foraging ability, the elimination of rotifers/copepods in favour of newly hatched *Artemia* nauplii, which offer off-the-shelf convenience, becomes feasible (Samocha et al., 1989; Sorgeloos et al., 2001; Ruscoe et al., 2004; Nhu et al., 2009; Van et al., 2014). Our results showed that for *S. splendidus, Artemia* nauplii could be introduced to replace copepods as early as 18 DPH for feeding the larvae to minimize the use of copepods. In the alternative case that a rotifer feeding period was incorporated from 12 DPH onward, larval diet could also be switched from rotifers to *Artemia* nauplii on 18 DPH, which resulted in an acceptable survival despite significantly lower than in case that copepods were fed to 18 DPH.

The results of this study showed that when a rotifer feeding period incorporated from 12 DPH, larval diet switched to *Artemia* nauplii on 15, 18 and 21 DPH resulted in consistent significantly lower larval survival than the corresponding treatments without a rotifer feeding period. This is likely linked to slower development of rotifer fed larvae with lower capability to ingest *Artemia*

nauplii as larger prey as our previous studies has showed that compared to continuous copepod feeding larvae, rotifers feeding occurred on 12 DPH although not significantly affected larval survival and growth, larval development appeared to be retarded (Chapter 4). These underdeveloped larvae were likely to have a lower physiological and cognitive capacity to adapt quickly to the diet switch to *Artemia* nauplii, hence resulted in lower survival. The present results also showed that the larval survival of continuous copepod feeding control decreased sharply after 20 DPH, and the growth of surviving larvae fed copepods throughout was significantly lower than those from the treatments in which *Artemia* nauplii were introduced on 15 and 18 DPH. These unexpected poor results are most likely due to food quantity issue (1 copepod $mL^{-1}$ vs. 5 *Artemia* $mL^{-1}$). As the food consumption of growing larvae increased dramatically, the limited copepods in the culture vessels were not enough to satisfy the feeding demands of older larvae, therefore they were probably starved most of time, hence sustained the poorer survival and growth.

The introduction of larger enriched instar II *Artemia* metanauplii as prey has two important implications. Firstly, they are larger hence can serve as a size step-up in the larval feeding regime; correctly timing their introduction can therefore improve the energetic gain for each successful predatory attempt by larvae (Polo et al., 1992; Cox and Pankhurst, 2000; Scharf and Schlicht, 2000). Secondly, as *Artemia* is known to deficient in essential fatty acids, if not enriched often lead to poor development and high mortality in late larvae or juveniles (Vagelli, 2004). The instar II *Artemia* metanauplii are the first stage in the life cycle of *Artemia* to start filter feeding, allowing nutritional enrichment through simply applying enrichment emulsion (Van Ballaer et al., 1985; Verreth et al.,

1994; Sorgeloos et al., 2001; Woods, 2003; Figueirdo et al., 2009; Haché and Plante, 2011). Hence knowing when post-settlement *S. splendidus* are able to accept larger *Artemia* metanauplii is important. This study demonstrated that the best survival and growth were resulted when live enriched *Artemia* metanauplii were introduced on 25 DPH.

The previous observation suggested that the post-settlement *S. splendidus* ingested dead enriched *Artemia*, as at this stage they became increasingly benthic, thus they may prefer dead enriched *Artemia* that are settled on the bottom. If *S. splendidus* could utilize dead enriched *Artemia* metanauplii effectively, it would be also nutritionally beneficial as dead *Artemia* would not metabolize enriched lipid nutrition by themselves (Navarro et al., 1999) and should also reduce costs associated with daily *Artemia* hatching and enrichment. However, our results showed that dead enriched *Artemia* were clearly inferior to live ones for post-settlement *S. splendidus*, resulting in significant poorer survival and growth. Similar inferior performance of dead *Artemia* as compared to live ones have been noted for the African catfish larvae, *Heterobranchus longifilis*, the striped bass larvae, *Morone saxatilis* and the Senegal sole, *Solea senegalensis* (Webster and lovell, 1990; Kerdchuen and Legendre, 1994; Villalta et al., 2007). They found that survival was significantly higher for *M. saxatilis* larvae consuming live *Artemia* (28.5%) than those fed frozen *Artemia* (9.3%), and the percentage of larvae consuming food was also higher for striped bass fed live *Artemia* (85.8%) than those fed frozen ones (38.2%), indicating the poorer growth and survival of *M. saxatilis* larvae fed frozen *Artemia* may be contributed to the lower rate of food consumption. However, in the whitefish, *Coregonus fera*, it has been reported that dead *Artemia* were accepted equally well by *C. fera* larvae as live

ones, led to similar survival and growth (Flüchter, 1980).

Grabner et al. (1981) has suggested that inferior performance of dead *Artemia* as compared to the live ones in several fish species was mainly due to the losses of essential nutrients during thawing process. While the losses of nutrients during thawing may be a contributor to inferior performance of dead *Artemia* in the present study; we observed that although *S. splendidus* indeed ingested on the dead *Artemia*, their consumption on dead *Artemia* was not good. It was observed that when the *S. splendidus* were presented with the dead enriched *Artemia*, they generally carefully scrutinized them and then rejected most of them before occasionally stroke and ingested one. In contrast, when live enriched *Artemia* were offered, the *S. splendidus* usually attacked every one that they visually "locked-on". Such a difference in feeding behavior suggested that dead *Artemia* did not trigger as good feeding responses as live ones. This could be as a result of inactive or physical damages of the dead *Artemia* during freezing and thawing, which rendered them "unattractive" or unrecognizable as prey. Therefore, it appeared that "unattractiveness" of the dead *Artemia* to the *S. splendidus* leading to their low consumption was the main causative reason for the poor performance in survival and growth. It is hence concluded that live enriched *Artemia* should be used preferentially over the dead ones for post-settlement *S. splendidus* culturing.

In summary, based on the past and present studies, a complete larval feeding regime for *S. splendidus* is recommend as follows: initially feeding copepods at 1 ind. $mL^{-1}$ to 17 DPH; the newly hatched *Artemia* nauplii could then be introduced at 5 ind. $mL^{-1}$ from 18 DPH onward while the introduction of live enriched *Artemia* metanauplii at 5 ind. $mL^{-1}$ for post-settlement *S. splendidus* should start

from 25 DPH onward. In the case that copepod supply are limited or considered too costly, an alternative feeding regime with a transitional rotifer-feeding period to replace copepods between 12 to 17 DPH can be adopted, which should yield a comparatively low but still acceptable overall survival.

# Chapter 7

## The effects of graded concentrations of dietary DHA (22:6n-3) on growth, survival and tissue fatty acid profile of the post-settlement green mandarin fish, *Synchiropus splendidus*

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### 7.1. Introduction

The green mandarin fish, *Synchiropus splendidus* (Herre, 1927), is a small dragonet found in the tropical Pacific and Indian Oceans, and is highly popular for marine ornamental trade worldwide due to its unique and beautiful color pattern (Sadovy et al., 2001). Recent studies in this laboratory on its captive breeding have shown that after initially feeding on copepods, larval survival rate were generally very high and stable from 18 days post hatching (DPH) onward by feeding them on *Artemia*, however their growth was very slow after metamorphosis (Chapter 6), which represents as a major constraint for the captive breeding efforts on this popular ornamental species.

*Artemia* is widely used in fish larval culture because its readily available and off-the-shelf convenience (Sorgeloos et al., 2001; Van et al., 2014). However, *Artemia* is considered as an incomplete feed for many marine larvae due to its lack of essential n-3 long-chain polyunsaturated fatty acids (LC-PUFAs). For example, as the natural prey for marine fish larvae, copepods generally contain 25-45% docosohexanoic acid (DHA, as % of total fatty acids), 10-20% eicosapentaenoic acid (EPA) and 0-2.6% arachidonic acid (ARA) (Van der Meeren et al., 2008), however unenriched *Artemia* contain only approximately 5%

EPA, very low level of ARA, and hardly DHA (Hamre and Harboe, 2008). DHA, together with EPA and ARA, are known as essential fatty acids (EFAs) for marine fish, since most of marine fish can not or with very limited ability to biosynthesize these fatty acids themselves (Tocher, 2003). It is well documented that a deficiency in LC-PUFAs could severely impact marine fish larvae on various ways, including significantly reduced larval growth and heath; increased physical anomalies in the central nervous system and visual development (Avella et al., 2007; Velu and Munuswamy, 2003); challenged metamorphosis (Watanabe and Kiron, 1994); significantly reduced stress resistance (Vagelli, 2004; Olivotto et al., 2003; Lee and Ostrowski, 2001) and abnormal swimming and feeding behavior (Avella et al., 2007; Olivotto et al., 2006; Lee and Ostrowski, 2001). In most cases, providing improved dietary levels of LC-PUFAs through enrichment of *Artemia* with specialist emulsion oil, is crucial to successful cultivation of marine fish larvae (Sorgeloos et al., 2001). However, in some instances, overly high dietary LC-PUFAs level have also been reported to have negative effects, such as significantly reduced growth in larvae of sole, *Solea* spp. (Rodriguez et al., 1994; Morais et al., 2004; Villalta et al., 2005; Lund et al., 2007).

While some past studies have suggested that levels of individual LC-PUFA are the key factor for achieving optimal development in fish larvae (Sargent et al., 1997; Næss and Lie, 1998), others have pointed to the DHA:EPA ratio as the most crucial. For example, Watanabe et al. (1989) observed that poor larval growth and survival in striped jack, *Pseudocaranx dentex*, fed an EFA-deficient diets were effectively improved by supplementing EPA and DHA to their diet. However, supplementing EPA alone did not prevent high mortality or improve growth; a replacement of a half of the EPA with DHA effectively improved larval

performance. Various past studies have also suggested that the DHA:EPA ratio for marine fish larvae should normally be higher than 1, indicating DHA played a more important role than EPA in promoting larval growth (Rodriguez et al., 1997; Gapaspin and Duray, 2001; Wu et al., 2002; Ma et al., 2014).

To date, the EFA requirements by *S. splendidus* larvae have not been studied. Previous studies from this laboratory have consistently showed very slow growth of post-settlement *S. splendidus* and it has been suspected that this may be in related to nutritional deficiencies, in particular DHA deficiency. Using *Artemia* enriched with proposed formulated experimental emulsions to produce graduated DHA profiles, the present study was hence carried out to examine the effect of dietary DHA level on performance of the post-settlement green mandarin fish.

## 7.2. Materials and Methods

### *7.2.1 Broodstock maintenance*

Broodstock source and husbandry were described in Chapter 2 (Section 2.1).

### *7.2.2 Live prey production and Artemia enrichment*

Rotifers and copepods culture and harvest methods were described in Chapter 2 (Section 2.2 and 2.3).

*Artemia* cysts (Great Salt Lake strain, INVE Aquaculture, Belgium) were hatched daily at 26-28 °C and salinity 28-30 ‰ to produce newly hatched nauplii for the purposed experiment. After 24 h of hatching, newly hatched nauplii

(430-520 μm) were harvested for subsequent enrichment. The *Artemia* enrichment was conducted in 500 mL beakers with a density of 200-250 *Artemia* $mL^{-1}$ with 0.6 g L of each of purposely formulated emulsion oils (Sui et al., 2007). During enrichment, the temperature and salinity was maintained at 26-28 °C and 30 ‰, respectively. Following enrichment, enriched *Artemia* metanauplii were harvested next morning and thoroughly rinsed with freshwater to remove traces of the emulsion before they were estimated for density and a portion of them fed to the post-settlement *S. splendidus*.

Five *Artemia* enrichment emulsions containing different DHA levels were formulated by incorporating 0% (E-1), 19% (E-2), 38% (E-3), 57% (E-4) and 77% (E-5) of a DHA-rich oil (Neuromins, Martek Biosciences, USA) into the formulation, respectively (for the details of the formulations, please refer to Table 7.1). In addition to DHA oil, the formulations of the emulsions included identical amounts of an ARA-rich oil (Bulk Nutrients TAS, Australia) and different amounts of, a DHA-ARA-free olive oil and a DHA-ARA-free blended vegetable oil (canola and soy bean oils) to provide ARA and other essential fatty acids (Tab. 7.1). Finally, soy lecithin was added at the same 7% as emulsifier while 2% of α-tocopherol acetate was added as antioxidant for all emulsions (Estévez et al., 2001; Villalta et al., 2005). Each emulsion oil was then added an equal amount of distilled water and emulsified by using an Ultra-turrax T25 homogenizer (IKA works inc, Wilmington, North Carolina, USA) operating at 10,000 rpm for 60 seconds. They were subsequently stored in five labeled bottles and kept at 4 °C until used.

**Table 7.1.** The formulation (mg/g) of the five experimental emulsion oils used for *Artemia* enrichment.

| Emulsion Oil | 0% | 19% | 38% | 57% | 77% |
|---|---|---|---|---|---|
| Neuromins oil[a] | 0 | 191 | 382 | 574 | 766 |
| Olive oil[b] | 860 | 647 | 432 | 216 | 0 |
| ARA oil[c] | 50 | 50 | 50 | 50 | 50 |
| Blended vegetable oil[d] | 0 | 22 | 46 | 70 | 94 |
| α-tocopherol[e] | 20 | 20 | 20 | 20 | 20 |
| Soy lecithin[f] | 70 | 70 | 70 | 70 | 70 |

[a] Martek Biosciences, USA.
[b] Moro Congafoods, Australia
[c] Bulk Nutrients, Australia
[d] Blended canola and soy bean oils
[e] Blackmors Ltd, Warriwood, Australia
[f] Sigma-Aldrich Pty Ltd, Castle hill, Australia

*7.2.3 Experimental design and setup*

The incubation and experimental system were described in Chapter 2 (Section 2.5). The newly hatched larvae were left in the incubation buckets until 2 DPH when healthy larvae were transferred to a 50 L black rectangular polypropylene tank for culture. Based on previous experiments, the larvae were initially fed copepods *Parvocalanus crassirostris* at a density of 1 $mL^{-1}$ until 17 days post hatching (DPH), they were then fed 5 *Artemia* nauplii $mL^{-1}$ from 18 to 24 DPH. The experiment started with 25 DPH larvae sourced from those communally cultured in the 50 L tank. On 25 DPH, 300 *S. splendidus* were randomly selected from the 50 L tank and evenly transferred into fifteen 3 L purposely designed cylindrical rearing vessels (i.e. 20 each) (Moorhead, 2015). They were then divided into five groups of three vessels (replicates) each and fed 5 ind. $mL^{-1}$ *Artemia* metanauplii enriched with one of the five enrichment emulsions with graded concentrations of DHA. An additional 20 *S. splendidus* were sampled from the 50 L communally cultured tank on 25 DPH for the

measures of initial standard length, body width and dry weight and also for subsequent fatty acid analysis. The experiment terminated on 45 DPH. Throughout the experiment, the major physical parameters were maintained as follows: temperature 26-29 °C, salinity 34-38 ‰, pH 8.0-8.2, $NH_3$, $NO_2$ < 0.02 ppm, and $NO_3$ < 0.5 ppm.

*7.2.4 Sampling and data collection*

Daily counting the number of surviving *S. splendidus* in each replicate vessel was conducted in the morning immediately after water exchange when water is clean as *Artemia* had been flushed out. At the end of experiment on 45 DPH, all the surviving *S. splendidus* were anaesthetized with clove oil (0.05 ml $L^{-1}$)(AQUI-S®, New Zealand Ltd) and photographed under a dissecting microscope (Leica MZ 125) fitted with a digital camera (Olympus DB 25). Images were then imported to an image analysis software (ImageJ, Freeware, Wayne Rasband, NIH, USA) for the measurement of larval standard length (SL: the length from the tip of the snout to the posterior extremity of the hypural plate) and body width (BW: the transverse distance between body margins at the pectoral fin base). Then the fish samples were rinsed with 0.5 M ammonium formate to remove any residual salt (Bransden et al., 2005), and immediately freeze-dried for 24 h until reached constant weight. The measurement of individual dry weight (DW) was carried out with a Cahn C-33 micro-balance (precision-1μg, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). After dry weight measurement, all samples were stored at -70 °C until biochemical analysis. The enriched *Artemia* metanauplii from all five treatments were also sampled three times on 25, 35 and 45 DPH, respectively (ca.1-2 g/sample) and stored at

-70 °C for lipid and fatty acid analysis.

In additional to SL, BW and DW, growth performance was measured as specific growth rates (SGR) based on those data, which were calculated for each replicate based on the following formula:

$$SGR\ (\%) = [\ln(F)-\ln(I)]*100/T$$

Where F and I represent the final (45 DPH) and initial (25 DPH) SL, BW or DW, respectively, and T represents the culture duration (days).

*7.2.5 Total lipid and fatty acid analysis*

Prior to fatty acid analysis, each sample of *S. splendidus* or enriched *Artemia* metanauplii was freeze-dried and homogenized separately. The total lipid (TL) was extracted with chloroform-methanol (2:1, v/v) (Folch et al., 1957). For fatty acid analysis, fatty acid methyl esters (FAME) were prepared by transesterification with boiling 14% borontrifluoride/methanol (w/w) following the method of Morrison and Smith (1964). FAMEs were analytically verified by flame ionization detection (FID) after injecting a sample into an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) fitted with an Omegawax 320 fused silica capillary column (30m×0.32mm; Supelco, Billefonte, PA, USA). The detailed method of fatty acid analysis was provided in Shao et al. (2013). Fatty acid composition were defined as a fatty acid as percentage of the total fatty acids (% total fatty acids) while fatty acid content was defined as mg fatty acid per g sample dry weight (mg/g DW).

*7.2.6 Data analysis*

Homogeneity of variance of all data was first tested with Levene's test. Where necessary, arcsine-square root or logarithmic transformation was performed prior to analysis. One-way ANOVA was used to determine significant differences for various parameters among treatments. If any significant difference was detected, Tukey's multiple range test was used as the means separation procedure. Pairwise t-test was used to determine statistically significant of relationship between dietary DHA level and different growth parameters measured. The relationships between dietary DHA level and different growth parameters were further analyzed with second-order polynomial regression. When a normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Kruskal-Wallis H non-parametric test followed by the Games-Howell non-parametric multiple comparison test. $p<0.05$ was regarded as the statistically significant level. All statistics were performed using SPSS package 23 (Statsoft$^{TM}$, Inc.).

## 7.3. Results

*7.3.1 Total lipid and fatty acid profiles of Artemia metanauplii enriched with emulsions of graded DHA concentrations*

The total lipid and fatty acid composition of *Artemia* metanauplii enriched with five emulsion oils containing different levels of DHA are presented in Table 7.2. Total lipid contents of the enriched *Artemia* were very similar among five treatments. However, with increasing DHA level of the emulsion oils from E-1 to E-5 (Tab. 7.1), a clear increasing trend in C16:0 (Palmitic acid)(increased from

11.11% of E-1 to 15.71% total fatty acids of E-5) and total saturated fatty acid (SFA)(increased from 17.77% of E-1 to 22.68% total fatty acids of E-5) was detected in the *Artemia* enriched with these emulsion oils (Tab. 7.2). In contrast, a decreasing trend in C18:1n-9 (OA)(decreased from 38.16% of E-1 to 24.84% total fatty acids of E-5) and total monounsaturated fatty acids (MUFA)(decreased from 50.92% of E-1 to 37.82% total fatty acids of E-5) was found in the enriched *Artemia*.

**Table 7.2** Total lipid contents (% dry weight) and fatty acid composition (% total fatty acid) of *Artemia* metanauplii enriched with five emulsions (E1 to E5: 0%, 19%, 38%, 57% and 77%) with graduated DHA concentrations.

| | *Artemia* enriched with E-1 | *Artemia* enriched with E-2 | *Artemia* enriched with E-3 | *Artemia* enriched with E-4 | *Artemia* enriched with E-5 |
|---|---|---|---|---|---|
| Total lipid (%) | 23.20 | 23.56 | 24.35 | 24.65 | 23.30 |
| Fatty acids | | | | | |
| 16:0 | 11.11 | 12.88 | 13.11 | 14.19 | 15.71 |
| 18:0 | 5.33 | 5.65 | 5.85 | 5.66 | 5.03 |
| ∑SFA | 17.77 | 20.06 | 20.56 | 21.67 | 22.68 |
| 16:1n-7 | 1.30 | 1.11 | 1.19 | 1.03 | 1.03 |
| 16:1n-5 | 2.24 | 2.99 | 2.65 | 2.55 | 2.42 |
| 18:1n-9(OA) | 38.16 | 34.41 | 29.52 | 27.50 | 24.84 |
| 18:1n-7 | 6.93 | 7.69 | 8.23 | 7.77 | 7.11 |
| 20:1n-9 | 1.03 | 1.11 | 1.10 | 1.11 | 1.07 |
| ∑MUFA | 50.92 | 48.69 | 43.77 | 40.98 | 37.82 |
| 18:2n-6(LOA) | 6.85 | 6.92 | 6.75 | 6.60 | 6.92 |
| 18:3n-3(ALA) | 14.75 | 15.38 | 16.38 | 15.98 | 16.43 |
| 18:3n-6 | 2.44 | 2.48 | 2.29 | 2.76 | 2.50 |
| 20:4n-6(ARA) | 1.82 | 1.92 | 2.22 | 2.22 | 2.05 |
| 20:5n-3(EPA) | 2.23 | 2.48 | 2.89 | 3.12 | 3.31 |
| 22:6n-3(DHA) | 0.01 | 0.97 | 2.53 | 3.98 | 5.69 |
| ∑PUFA | 29.14 | 31.25 | 34.31 | 36.55 | 38.78 |
| ∑LC-PUFA | 4.49 | 5.83 | 8.19 | 10.13 | 11.90 |
| ∑n-3PUFA | 17.13 | 18.99 | 22.00 | 23.48 | 25.89 |
| ∑n-6PUFA | 11.59 | 11.82 | 11.79 | 12.49 | 12.07 |
| n-3/n-6 | 1.48 | 1.61 | 1.87 | 1.93 | 2.14 |
| DHA/EPA | 0.01 | 0.39 | 0.88 | 1.27 | 1.72 |
| ARA/EPA | 0.82 | 0.78 | 0.77 | 0.71 | 0.62 |

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; LC-PUFA: long-chain polyunsaturated fatty acid; Totals (∑) included some minor components not shown.

Among the polyunsaturated fatty acids (PUFAs), enriched *Artemia* from different treatments generally contained close ranges of C18:2n-6 (LOA; ranging between 6.60-6.92% total fatty acids), C18:3n-3 (ALA; ranging between 14.75

-16.43% total fatty acids), C18:3n-6 (ranging between 2.29-2.76% total fatty acids) and C20:4n-6 (ARA; ranging between 1.82-2.22% total fatty acids). However as expected, the enrichment of *Artemia* with five emulsion oils containing graduated DHA contents resulted in sharp increases in DHA in a magnitude of 569 times (increased from 0.01% of E-1 to 5.69% total fatty acids of the E-5 treatment) while EPA (C20:5n-3) also increased modestly (steadily increased from 2.23% of E-1 to 3.31% total fatty acids of the E-5 treatment), which resulted in a sharp increased DHA/EPA ratio from 0.01 of E-1 to 1.72 of E-5 while a modestly increased n-3 PUFA/n-6 PUFA ratio from 1.48 to 2.14. A significant positive correlation was detected between DHA contents of enriched *Artemia* and incorporation level of DHA-rich oil in the five emulsions (Fig. 7.1, n=5, r=0.997, $p<0.01$).

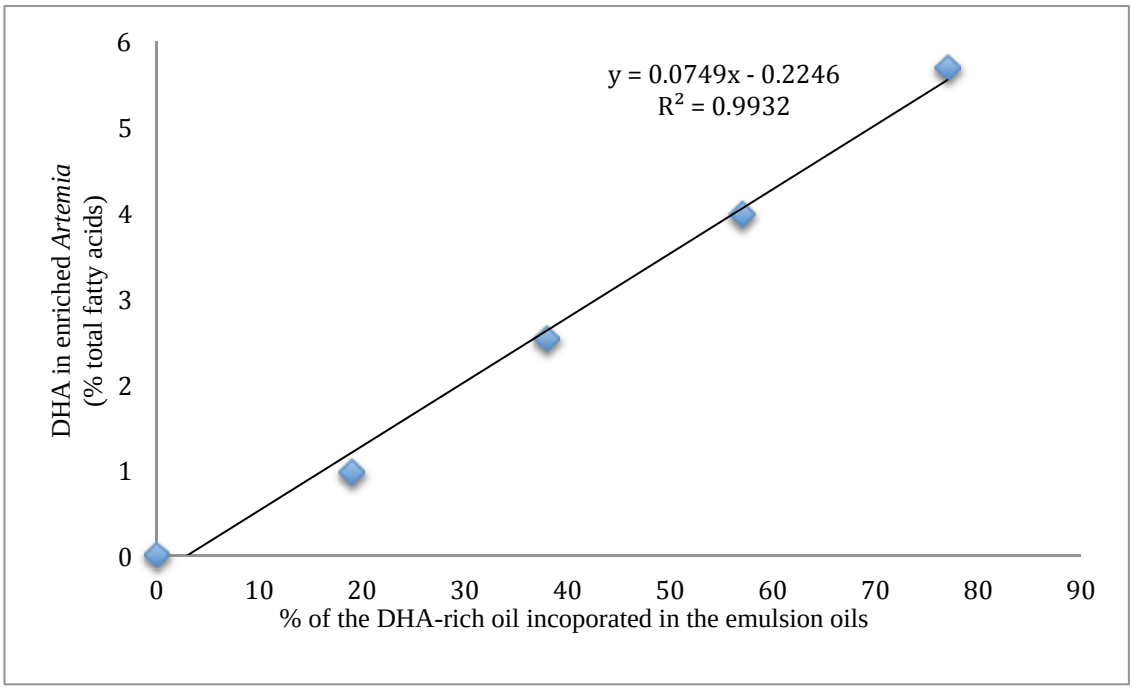



**Figure 7.1** The relationship between DHA contents of enriched *Artemia* and the incorporated level (%) of the DHA-rich oil in the five emulsion oils.

Table 7.3 presents the absolute contents (mg/g DW) of principal fatty acids of *Artemia* enriched with the five emulsions. Similarly, while the total fatty acid (TFA) contents remained very similar among all the treatments (ranging between 212.73-224.57 mg/g DW), the DHA contents increased more than 426 times from 0.03 mg/g DW in the *Artemia* enriched with E-1 to 12.79 mg/g DW in those enriched with E-5 (Tab. 7.3).

**Table 7.3** Principal fatty acid contents (mg/g DW) of *Artemia* metanauplii enriched with five emulsions (E1 to E5: 0%, 19%, 38%, 57% and 77%) with graduated DHA concentrations.

| | *Artemia* enriched with E-1 | *Artemia* enriched with E-2 | *Artemia* enriched with E-3 | *Artemia* enriched with E-4 | *Artemia* enriched with E-5 |
|---|---|---|---|---|---|
| Fatty acids | | | | | |
| 16:0 | 23.86 | 27.58 | 29.66 | 31.66 | 35.32 |
| 18:0 | 11.45 | 12.10 | 13.32 | 12.63 | 11.30 |
| ∑SFA | 38.15 | 42.96 | 46.50 | 48.33 | 50.97 |
| 16:1n-5 | 4.80 | 6.41 | 5.99 | 5.70 | 5.45 |
| 18:1n-9 (OA) | 81.94 | 73.70 | 66.77 | 61.35 | 55.84 |
| 18:1n-7 | 14.88 | 16.47 | 18.62 | 17.32 | 15.98 |
| ∑MUFA | 109.33 | 104.28 | 99.01 | 91.41 | 85.01 |
| 18:2n-6(LOA) | 14.70 | 14.82 | 15.26 | 14.71 | 15.55 |
| 18:3n-3 (ALA) | 31.66 | 32.93 | 37.01 | 35.65 | 36.93 |
| 18:3n-6 | 5.24 | 5.30 | 5.17 | 6.15 | 5.61 |
| 20:4n-6(ARA) | 3.90 | 4.12 | 5.01 | 4.95 | 4.60 |
| 20:5n-3(EPA) | 4.79 | 5.31 | 6.53 | 6.96 | 7.44 |
| 22:6n-3(DHA) | 0.03 | 2.08 | 5.71 | 8.88 | 12.79 |
| ∑PUFA | 62.57 | 66.93 | 77.61 | 81.53 | 87.17 |
| ∑LC-PUFA | 9.63 | 12.48 | 18.53 | 22.59 | 26.76 |
| ∑n-3LC-PUFA | 5.11 | 7.74 | 12.75 | 16.73 | 21.26 |
| ∑n-3PUFA | 36.77 | 40.67 | 49.76 | 52.38 | 58.19 |
| ∑n-6PUFA | 24.88 | 25.31 | 26.67 | 27.20 | 27.14 |
| Total fatty acids | 212.73 | 213.36 | 224.57 | 221.30 | 224.42 |

DW: dry weight; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; LC-PUFA: long-chain polyunsaturated fatty

acid; Totals (∑) include some minor components not shown.

*7.3.2 Survival and growth performance of S. splendidus*

Throughout the whole duration of experiment, no mortality was observed in any of the treatments. Hence, the survival of the post-settlement *S. splendidus* was not affected by the dietary DHA levels.

At the end of the experiment, the standard length (SL), body width (BW) and dry weight (DW) of the 45 DPH *S. splendidus* from all dietary treatments increased significantly from those at the beginning of the experiment on 25 DPH (SL: 4.27 mm; BW: 1.68 mm; DW: 1.11 mg)($p$<0.05; Tab. 7.4). Among the DHA treatments, *S. splendidus* fed *Artemia* enriched with medium level of DHA (E-3) had the highest SL, BW and DW (8.72 mm, 3.37 mm and 4.48 mg, respectively), which were followed by E-4 treatment (8.09 mm, 3.07 mm and 3.61 mg, respectively). The SL and BW of these two treatments were not significantly different ($p$>0.05) but DW of E-3 treatment was significantly heavier than that of E-4 ($p$<0.05). The SL, BW and DW of these two treatments were significantly higher than those of all other treatments ($p$<0.05). Interestingly, the lowest SL, BW and DW were found from the treatments in which *S. splendidus* were fed enriched *Artemia* containing the lowest (E-1: 6.29 mm, 2.52 mm and 1.99 mg) and the highest level of DHA (E-5: 6.92 mm, 2.66 mm and 2.04 mg), respectively. The E-2 treatment had intermediate SL, BW and DW (7.44 mm, 2.90 mm and 2.84 mg), of which DW was significantly heavier than both that of E-1 and E-5, but SL and BW was only significantly higher than those of E-1 ($p$<0.05)(Tab. 7.4). Despite significant differences on both BL and BW among treatments, the BW/BL ratios of *S. splendidus* at the end of experiment were not significantly

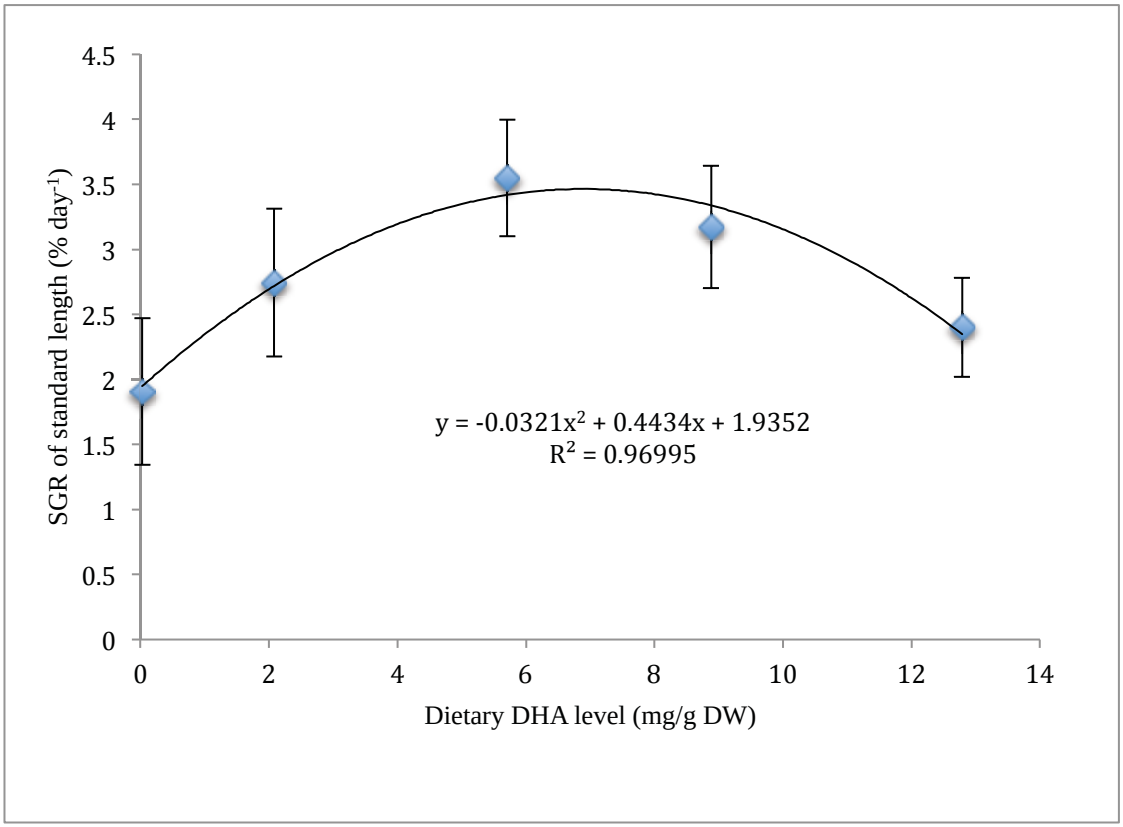
different among all the DHA treatments (ranged from 0.38 to 0.40) and were also similar to the ratio of 25 DPH larvae at the beginning of the experiment. The specific growth rates (SGR) calculated based on SL, BW and DW of the 45 DPH *S. splendidus* were the highest from E-3 treatment (3.55%, 3.48% and 6.96% $day^{-1}$, respectively), while the lowest SGR were found in both E-1 (1.91%, 2.00% and 2.91% $day^{-1}$, respectively) and E-5 treatments (2.40%, 2.29% and 3.04% $day^{-1}$, respectively) (Tab. 7.4).

**Table 7.4** The standard length (SL), body width (BW), dry weight (DW) and specific growth rate (SGR) of 45 DPH S. *splendidus* (mean ± S.D.; n=3) fed *Artemia* enriched with five emulsions (E-1 to E5: 0%, 19%, 38%, 57% and 77%) with graduated DHA concentrations from 25 DPH. Different superscript letters within the same row indicate significant differences ($p$<0.05).

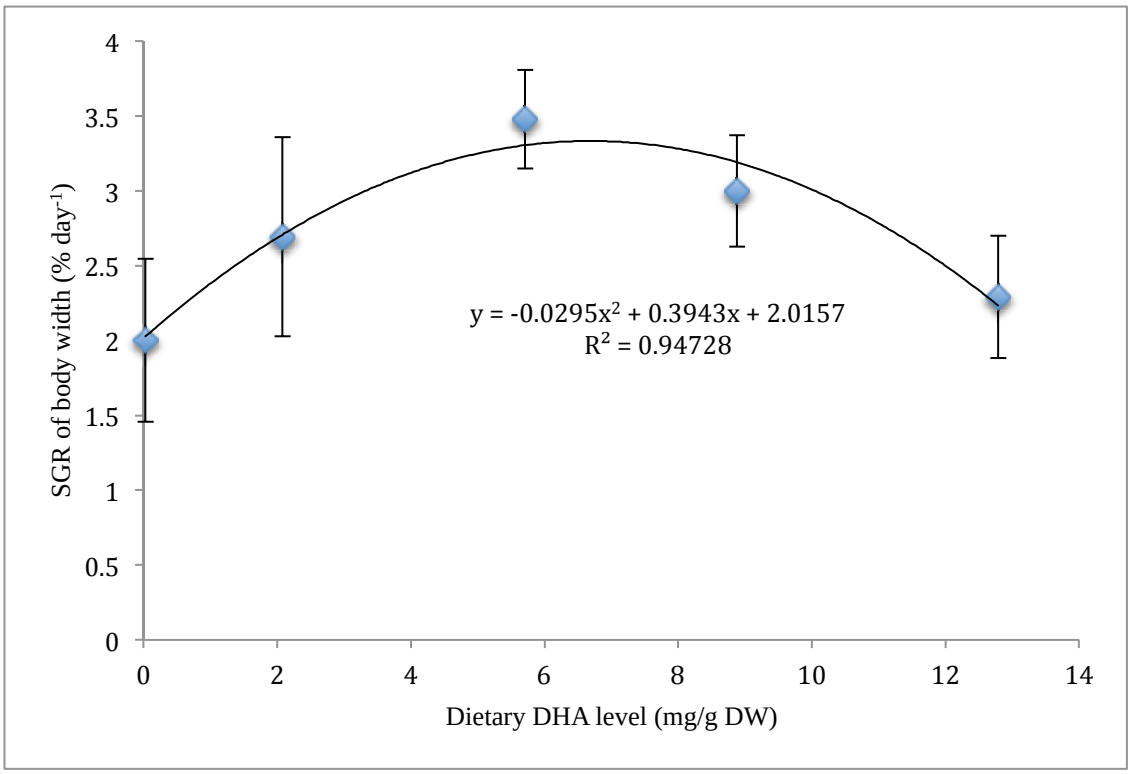
| Treatments | 25 DPH | 45 DPH | | | | |
|---|---|---|---|---|---|---|
| | | E-1 | E-2 | E-3 | E-4 | E-5 |
| SL (mm) | $4.27\pm0.45^{d}$ | $6.29\pm0.71^{a}$ | $7.44\pm0.84^{b}$ | $8.72\pm0.78^{c}$ | $8.09\pm0.74^{bc}$ | $6.92\pm0.52^{ab}$ |
| BW (mm) | $1.68\pm0.24^{d}$ | $2.52\pm0.27^{a}$ | $2.90\pm0.38^{b}$ | $3.37\pm0.22^{c}$ | $3.07\pm0.22^{bc}$ | $2.66\pm0.21^{ab}$ |
| DW (mg) | $1.11\pm0.12^{e}$ | $1.99\pm0.21^{a}$ | $2.84\pm0.31^{b}$ | $4.48\pm0.26^{c}$ | $3.61\pm0.34^{d}$ | $2.04\pm0.27^{a}$ |
| BW/SL | $0.39\pm0.02^{a}$ | $0.40\pm0.02^{a}$ | $0.39\pm0.01^{a}$ | $0.39\pm0.01^{a}$ | $0.38\pm0.01^{a}$ | $0.38\pm0.02^{a}$ |
| SGR | | | | | | |
| SL (% $day^{-1}$) | | $1.91\pm0.53^{a}$ | $2.74\pm0.57^{b}$ | $3.55\pm0.45^{c}$ | $3.17\pm0.47^{bc}$ | $2.40\pm0.38^{ab}$ |
| BW (% $day^{-1}$) | | $2.00\pm0.55^{a}$ | $2.69\pm0.67^{b}$ | $3.48\pm0.33^{c}$ | $3.00\pm0.37^{bc}$ | $2.29\pm0.41^{ab}$ |
| DW (% $day^{-1}$) | | $2.91\pm0.16^{a}$ | $4.64\pm0.43^{b}$ | $6.96\pm0.25^{c}$ | $5.92\pm0.38^{d}$ | $3.04\pm0.16^{a}$ |

The relationships between analyzed DHA content of the enriched *Artemia* and SGR of SL of the 45 DPH *S. splendidus* from the different treatments fitted into a second-order polynomial regression: $y= - 0.0321x^2 + 0.4434x + 1.9352$ ($R^2$=0.96995; x= dietary DHA level; y=SGR on SL; n=5, $p$<0.05)(Fig. 7.2A). Based on the regression, 6.91 mg DHA/g DW was identified as the dietary DHA level for maximum growth of SL for post-settlement *S. splendidus*. Similarly, the relationship between dietary DHA level and SGR of BW was regressed as: y= -

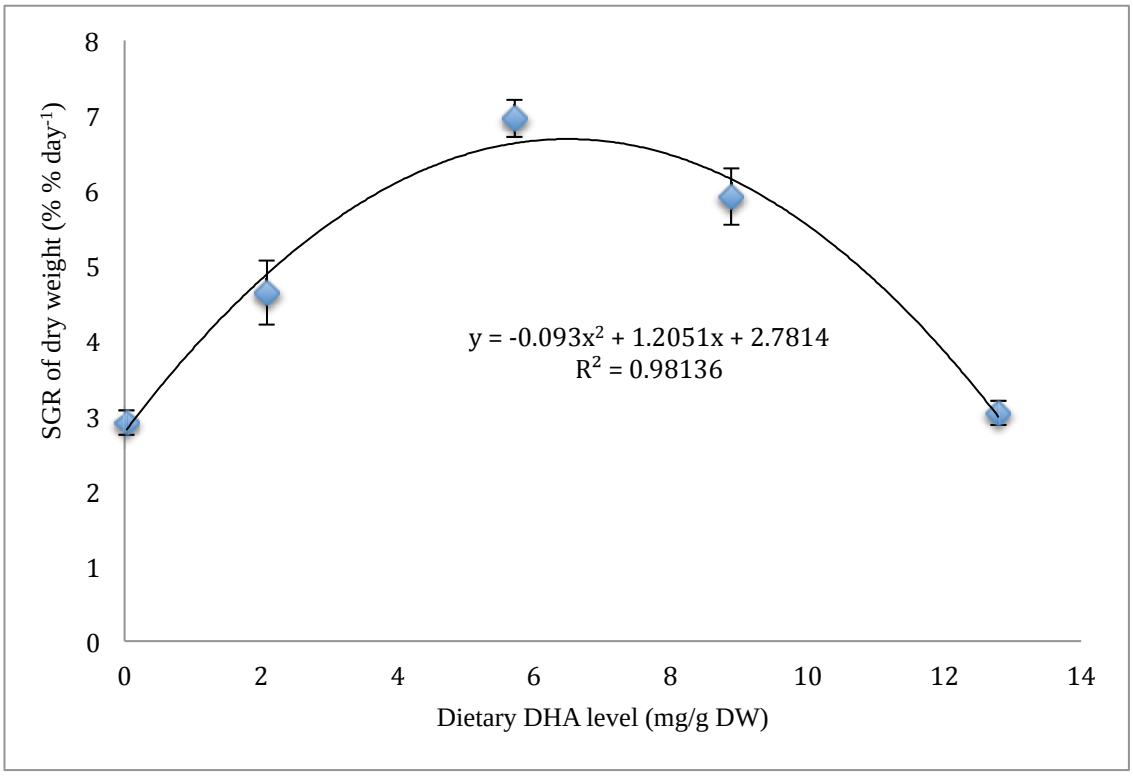
$0.0295x^2 + 0.3943x + 2.0157$ ($R^2$=0.94728; x= dietary DHA level; y=SGR on BW; n=5, $p$<0.05) with the optimal DHA for maximum growth in BW identified as 6.68 mg DHA/g DW (Fig. 7.2B). Finally, the dietary DHA level regressed against SGR of DW resulted in the equation: $y = -0.093x^2 + 1.2051x + 2.7814$ ($R^2$=0.98136; x= dietary DHA level; y= SGR on DW; n=5, $p$<0.01) and 6.48 mg DHA/g DW was recommended for the maximum growth in DW (Fig. 7.2C).




(A)


SGR of body width (% day-1)
y = -0.0295x2 + 0.3943x + 2.0157
R2 = 0.94728
Dietary DHA level (mg/g DW)

(B)


SGR of dry weight (% % day-1)
y = -0.093x2 + 1.2051x + 2.7814
R2 = 0.98136
Dietary DHA level (mg/g DW)

(C)

**Figure 7.2** Regressions of analyzed DHA levels in *Artemia* enriched by five emulsions with graded DHA concentrations and the SGR (% $day^{-1}$) based on standard length (A), body width (B) and dry weight (C) of the 45 DPH *S. splendidus* fed on these *Artemia* from 25 DPH.

### *7.3.3 Fatty acid composition of post-settlement S. splendidus*

The whole body fatty acid profiles of the 45 DPH *S. splendidus* at the end of the experiment from different treatments are shown in Table 7.5. After 20 days of feeding on *Artemia* enriched with five emulsions of graded DHA concentrations, the fatty acids compositions of the 45 DPH *S. splendidus* generally reflected those of their diets, however interesting deviations, particularly those from the E-1 treatment, were also noticed (Tab. 7.2, 7.3 and 7.5).

**Table 7.5** The fatty acid profiles (% total fatty acids; mean ± SD; n=3) of the 45 DPH whole green mandarin *S. splendidus* fed *Artemia* enriched with five emulsions (E-1 to E5: 0%, 19%, 38%, 57% and 77%) with graduated DHA concentrations from 25 DPH. Different superscript letters within the same row indicate significant differences ($p$<0.05).

| | 25 DPH post-larvae * | E-1 | E-2 | E-3 | E-4 | E-5 | *p* |
|---|---|---|---|---|---|---|---|
| Fatty acids | | | | | | | |
| 16:0 | 18.40 | 16.68±0.95$^{a}$ | 16.07±0.60$^{a}$ | 18.34±0.76$^{ab}$ | 19.42±1.15$^{b}$ | 22.04±1.34$^{c}$ | <0.001 |
| 18:0 | 19.38 | 13.00±1.51$^{a}$ | 10.60±1.49$^{ab}$ | 9.27±0.33$^{b}$ | 9.64±0.46$^{b}$ | 11.34±0.67$^{a}$ | <0.01 |
| ∑SFA | 38.21 | 30.11±2.35$^{a}$ | 27.22±0.90$^{a}$ | 28.15±0.40$^{a}$ | 29.71±0.74$^{b}$ | 34.10±2.01$^{c}$ | <0.001 |
| 16:1n-7 | 2.35 | 1.95±0.20 | 1.88±0.14 | 1.70±0.11 | 1.95±0.12 | 1.99±0.15 | ns |
| 18:1n-9(OA) | 18.07 | 30.68±2.38$^{a}$ | 35.51±1.72$^{b}$ | 35.27±1.77$^{b}$ | 28.85±1.67$^{a}$ | 21.12±2.55$^{c}$ | <0.001 |
| 18:1n-7 | 3.44 | 4.33±0.79 | 4.60±0.55 | 4.80±0.40 | 5.05±0.25 | 5.03±0.28 | ns |
| ∑MUFA | 28.62 | 37.56±1.34$^{a}$ | 42.09±2.20$^{b}$ | 42.60±1.14$^{b}$ | 36.70±2.24$^{a}$ | 30.02±2.32$^{c}$ | <0.001 |
| 18:2n-6(LOA) | 9.08 | 5.50±0.53$^{a}$ | 6.99±0.44$^{b}$ | 6.24±0.31$^{b}$ | 6.98±0.58$^{b}$ | 7.10±0.46$^{b}$ | <0.01 |
| 18:3n-3(ALA) | 8.34 | 5.68±0.35$^{a}$ | 7.92±0.40$^{b}$ | 6.52±0.30$^{c}$ | 7.97±0.57$^{b}$ | 9.81±0.95$^{d}$ | <0.01 |
| 20:4n-6(ARA) | 1.28 | 4.89±0.31$^{a}$ | 3.17±0.42$^{b}$ | 3.20±0.36$^{b}$ | 3.28±0.30$^{b}$ | 3.18±0.30$^{b}$ | <0.001 |
| 20:5n-3(EPA) | 2.48 | 3.03±0.25$^{a}$ | 1.63±0.16$^{b}$ | 1.49±0.14$^{b}$ | 1.66±0.27$^{b}$ | 2.28±0.23$^{c}$ | <0.01 |
| 22:6n-3(DHA) | 3.90 | 3.31±0.30$^{a}$ | 2.65±0.33$^{b}$ | 3.84±0.44$^{a}$ | 4.88±0.45$^{c}$ | 5.94±0.43$^{d}$ | <0.001 |
| ∑PUFA | 25.31 | 22.80±0.37$^{a}$ | 22.72±0.17$^{a}$ | 21.62±0.25$^{b}$ | 25.04±0.09$^{c}$ | 28.63±0.47$^{d}$ | <0.001 |
| ∑LC-PUFA | 7.66 | 11.39±0.27$^{a}$ | 7.58±0.20$^{b}$ | 8.67±0.24$^{c}$ | 9.93±1.03$^{c}$ | 11.53±0.91$^{a}$ | <0.001 |
| ∑n-3PUFA | 14.72 | 12.02±0.40$^{a}$ | 12.20±0.21$^{a}$ | 11.85±0.87$^{a}$ | 14.49±0.15$^{b}$ | 18.02±0.42$^{c}$ | <0.001 |
| ∑n-6PUFA | 10.36 | 10.55±0.74 | 10.35±0.09 | 9.61±0.64 | 10.43±0.26 | 10.46±0.72 | ns |
| n-3/n-6 | 1.42 | 1.15±0.12$^{a}$ | 1.18±0.03$^{a}$ | 1.24±0.17$^{a}$ | 1.39±0.05$^{ab}$ | 1.73±0.16$^{c}$ | <0.01 |
| DHA/EPA | 1.57 | 1.10±0.19$^{a}$ | 1.65±0.37$^{a}$ | 2.58±0.06$^{b}$ | 2.97±0.24$^{b}$ | 2.62±0.12$^{b}$ | <0.001 |
| ARA/EPA | 0.52 | 1.62±0.04$^{a}$ | 1.94±0.13$^{b}$ | 2.18±0.43$^{b}$ | 2.00±0.16$^{b}$ | 1.40±0.02$^{c}$ | <0.001 |

* 25 DPH post-larvae were not included in the statistical analysis. SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; LC-PUFA: long-chain polyunsaturated fatty acid; ns: no significance; Totals (∑) include some minor components not shown.

Firstly, except the E-1 treatment, C16:0, OA and DHA of the 45 DPH *S. splendidus* as percentage of total fatty acids (TFA) appeared to most closely reflect those of their diets. For example, the main constituent of SFA, C16:0 (> 60% of SFA), as well as total SFA were both increased in concomitant with their

dietary levels (Tab. 7.2, 7.3 and 7.5). Among MUFA, while C18:1n-7 and C16:1n-7 remained relative stable among treatments, OA (> 70% of MUFA) and total MUFA showed the trend reflecting their dietary levels. Since this study was specially designed to assess the effects of dietary DHA levels on post-settlement *S. splendidus*, it should be highlighted that except E-1 treatment, DHA of the 45 DPH *S. splendidus* closely reflected those in their diets, showing a clearly increasing trend with increasing dietary levels of DHA from treatment E-2 to E-5 (Tab. 7.2, 7.3 and 7.5). Of other PUFAs, it is worth noting that levels of ALA were generally much lower than those in their diets for all treatments. Finally, the ratio of n-3 PUFA/n-6 PUFA of 45 DPH *S. splendidus* showed a slight increasing trend concomitant with dietary DHA levels (Tab. 7.5).

Interestingly, fatty acid profile of the 45 DPH *S. splendidus* from the E-1 treatment, in which fish were fed *Artemia* containing the lowest DHA, deviated substantially from the general trend in other treatments which largely reflected their dietary fatty acid levels (Tab. 7.2, 7.3 and 7.5). In fact, *S. splendidus* of the E-1 treatment were fed enriched *Artemia* containing DHA at 0.01% TFA or 0.03 mg DHA/g DW, however their DHA level at the end of the experiment reached 3.31% TFA, which was even higher than that of E-2 treatment (2.65% TFA) in which *Artemia* with significantly higher level of DHA (0.97% TFA or 2.08 mg DHA/g DW) were fed to the fish. Moreover, the 45 DPH *S. splendidus* from the E-1 treatment also had significantly higher levels of EPA and ARA but significantly lower OA, LOA and ALA as compared to those from E-2 treatment (Tab. 7.5).

## 7.4. Discussion

In the present study, survival of post-settlement *S. splendidus* was not influenced by dietary DHA level as no mortality was found in any treatments. Similar results were reported in various past studies. For examples, Bransden et al. (2005) found that survival of the stripped trumpeter, *Latris lineata,* by 36 DPH was not significantly influenced by dietary DHA. Villalta et al. (2005) also reported that the survival of the Senegal sole, *Solea senegalensis*, up to 36 DPH averaged 81% and was not significantly affected by dietary DHA.

Interestingly, it was shown that *S. splendidus* were able to survive without an single mortality, as well as grow albeit substantially slower when fed exclusively on *Artemia* metanauplii almost absent of DHA (containing only 0.03 mg/g DW DHA; treatment E-1) up to 45 DPH. Such results suggested that the post-settlement *S. splendidus* might have a relatively very low dietary DHA requirement. In fact, similar results have also been reported in larvae of other necto-benthic fish species, including the Japanese flounder, *Paralichthys olivaceus* (Izquierdo et al., 1992), the plaice, *Pleuronectes platessa* (Dickey-Collas and Geffen, 1992), the California halibut, *Paralichthys californicus* (Vizcaíno-Ochoa et al., 2010), and the Senegal sole, *S. senegalensis* (Morais et al., 2004; Villalta et al., 2005). It has been shown that dietary DHA was not so crucial to them if adequate EPA or other n-3 LC-PUFAs were presented in their diets, which is very similar to the present results on *S. splendidus*.

While there is little information existing regarding the natural diets of wild *S. splendidus* larvae, in the wild, pelagic carnivorous fish larvae generally have good

access to abundant dietary DHA sources through the pelagic food chain (Ackman, 1982). In fact, the early pelagic *S. splendidus* larvae prior to metamorphosis (i.e. 2 to 20 DPH larvae, Chapters 3, 4 and 6) were shown to very selective on feeding on copepods, which are well known to contain rich n-3 PUFA, particularly DHA (Sadovy et al., 2001). After metamorphosis and settlement, *S. splendidus* become mainly benthic dwelling and are expected to be exposed to an abundance of diatoms diets that are rich in EPA and 16:0 fatty acids; they are likely fed on small benthic animals, such as amphipods and polychaetes, who also contain rich EPA (De Silva and Anderson, 1995). Hence post-settlement *S. splendidus* probably is well adapted to utilizing EPA as the main source of n-3 PUFA, which if true, should explain the relatively low DHA requirement in this study in which normal dietary EPA supply was maintained.

Both DHA and EPA are important n-3 LC-PUFA for marine fish larvae (Sargent et al., 1999; Izquierdo et al., 2000). They are well known to play important roles in various physiological functions of the fish larvae, including functions of visual and neural systems, bone development, pigmentation and stress resistance, thus are essential for normal growth and development of marine fish larvae (Sargent et al., 1997). Various nutritional studies have shown that higher contents of DHA in the diets generally improved larval growth and development (Watanabe, 1989; Bransden et al., 2005; Glencross, 2009; Hossain et al., 2012; Matsunari et al., 2013). Similarly, in the present study, post-settlement *S. splendidus* showed significantly enhanced growth performance when they were fed enriched *Artemia* with increasing levels of DHA from E-1 treatment (0.01% TFA or 0.03 mg DHA/g DW) to E-3 treatment (2.53% TFA or 5.71 mg DHA/g DW). However, interestingly such trend appeared to reverse as those *S.*

*splendidus* fed enriched *Artemia* with a higher DHA (i.e. E-4 treatment: 3.98% TFA or 8.88 mg DHA/g DW) showed slower growth, particularly DW was significantly lower than that of E-3 treatment. The same trend continued for the E-5 treatment in which *S. splendidus* were fed *Artemia* with the highest DHA level (5.69% TFA or 12.79 mg DHA/g DW) and all growth parameters, including SL, BW and DW, were significantly lower than those of the E-3 treatment.

While it is well known and widely accepted that DHA deficiency can negatively impact on fish growth, our results showed that excessive dietary DHA could also lead to depressed growth in the post-settlement *S. splendidus*. Similar situation was also reported for the Senegalese sole *S. senegalensis*, which demonstrated high dietary DHA levels were not always beneficial for growth and survival of Senegalese sole larvae (Morais et al., 2004; Villalta et al., 2005). It was hypothesized that it was a result of oxidative stress under excessive supply of dietary LC-PUFAs, since LC-PUFAs are highly susceptible to peroxidation (Kjær et al., 2008; Saera-Vila et al., 2009). Another hypothesis being put forward to explain the situation was that excessive dietary LC-PUFAs level reduced available dietary space for other fatty acids served as important energy substrates (Navarro-Guillén et al., 2014). LC-PUFAs are known to relatively poorly catabolized, DHA in particular is not easily catabolized via β-oxidation; saturated and monounsaturated fatty acids, especially C16:0, OA, C20:1n-9 and C22:1n-11, on the other hand are relatively easy catabolized by fish to produce energy (Rainuzzo et al., 1997; Sargent et al., 2002). Hence an excessive high level of dietary DHA associated with a substantially reduced monounsaturated fatty acids, particularly OA, in the enriched *Artemia* of the E-5 treatment as compared to the other treatments observed in the present study may have led to reduced energy

availability and subsequently slower growth of the post-settlement *S. splendidus*.

Past lipid nutrition studies in marine fish larvae have focused on supplying supplemental essential fatty acids (EFAs) to meet requirements for optimal larval growth and development (Izquierdo et al., 1992; Gapasin and Duray, 2001; Bransden et al., 2005; Lund et al., 2007). However, it should not be forgotten that other components of lipids, particularly saturated and monounsaturated fatty acids, provide the "fuels" for fish growth. Therefore, to strike a balance between LC-PUFAs that are required for key functions, such as functional integrity of cell membranes, and other less unsaturated fatty acids required for energy production, is vitally important (Sargent et al., 2002). This may be particularly important for species with lower LC-PUFA requirements, such as necto-benthic Senegalese sole (Navarro-Guillen et al., 2014) and green mandarin fish. In fact the deleterious effects of excessive supply of dietary DHA found in this study suggested that it might have led to insufficient supply of energy substrates. Clearly, more attention should probably be given to identify the right balance between LC-PUFA and other energy-yielding fatty acids in future fish larval nutrition studies.

The regressions of SGRs of the post-settlement *S. splendidus* and their dietary DHA levels identified dietary DHA level at 6.91, 6.68 and 6.48 mg DHA/g DW for the optimal growth of SL, BW and DW respectively. Such an optimum dietary DHA is substantially lower than 15.2, 17 and 20.8 mg DHA/g DW reported for early juvenile of the silver pomfret, *Pampus argenteus* (Hossain et al., 2012), striped jack, *Pseudocaranx dentex* (Takeuchi et al., 1992) and the striped trumpeter, *Latris lineata* (Bransden et al., 2005), respectively. However it is higher than 0.1-4.9 DHA mg/g DW reported for Senegal sole *S. senegalensis*

larvae, which is also a necto-benthic fish (Morais et al., 2004).

In the present study, a dietary n-3 LC-PUFA level of 12.75 mg/g DW (including both DHA and EPA) resulted in the best growth of the post-settlement *S. splendidus*. This is generally similar to the optimum n-3 LC-PUFA levels reported for larvae of the Asian seabass *Lates calcerifer* (10-17 mg/g DW)(Boonyaratpalin and Williams, 2002) and larvae and juveniles of the gilthead bream *Sparus aurata* (10-15 mg/g DW)(Rodriguez et al., 1997), but is substantially lower than 27.5 mg/g DW reported for early juvenile *P. argenteu* (Hossain et al., 2012). Past research have further suggested that for dietary n-3 LC-PUFA requirement, not only the total amounts are important, but the DHA to EPA ratio also play an important role (Rodriguez et al., 1997; Sargent et al., 1999; Seoka et al., 2008). The dietary DHA:EPA ratio in the present study ranged from 0.01 to 1.72 from E-1 to E-5 treatment, among them the E-3 treatment with a DHA:EPA ratio of 0.88 resulted in the best growth. This value is lower than the optimal ratio reported for larvae of the gilthead sea bream, *S. aurata* (1.5)(Rodriguez et al., 1997), the milkfish, *Chanos chanos* (>1)(Gaspaspin and Duray, 2001), the starry flounder juvenile, *Platichthys stellatus* (1.24)(Ma et al., 2014) and the grouper juvenile, *Epinephelus malabaricus* (1.4)(Wu et al., 2002). The optimal DHA:EPA ratios for larvae of the above mentioned fish were all higher than 1, implying DHA content was relatively more important than EPA in promoting larval growth in these species. In contrast, an optimal DHA:EPA ratio of only 0.88 found in this study appeared to suggest that post-settlement *S. splendidus* might require more EPA than DHA in their diets. In fact, Villalta et al. (2005) also hypothesised that the post-larvae of Senegal sole *S. senegalensis* required negligible amounts of dietary DHA but higher EPA content. Morais et al.

(2012) explained that such a result may relate to the fact that *S. senegalensis* as a necto-benthic species since after switching to benthic life following settlement, their natural diets generally contain low lipids and with substantially higher EPA than DHA, which are differed substantially from commonly cultivated carnivorous nekton species.

The fatty acid composition of fish tissue is the momentary net result of complex dynamic interrelationships of a range of factors, which are so far not fully understood (Tocher, 2003). It is believed that the major factors include dietary fatty acid intakes; rates of oxidative catabolism of the fatty acids; kinetics of desaturation and elongation reactions; as well as competitive incorporation and retro-conversions among fatty acids (Rodriguez et al., 1998). The fatty acid proportions of the 45 DPH *S. splendidus* from the E-2, E-3, E-4 and E-5 treatments all closely reflected those in the diets, which is consistent with the findings from the majority of past studies (Caballero et al., 2002; Bransden et al., 2005; Villalta et al., 2005; Hossain et al., 2012). However, it is interesting to find that the result from the E-1 treatment deviated from such a general pattern. It was in fact unexpected to find that the DHA level of the fish from the E-1 treatment, in which a diet almost devoid of DHA (0.01% TFA or 0.03 mg DHA/g DW) was fed to them, to have higher tissue DHA than that of the E-2 treatment, in which a diet contained much higher DHA (0.97% TFA or 2.08 mg DHA/g DW) was provided to the post-settlement *S. splendidus*. At the same time, the fish from the E-1 treatment also showed much lower levels of OA, LOA and ALA but higher ARA than expected based on their dietary fatty acid composition. Clearly, such a result can not be simply explained by higher absorption or retention of certain fatty acids from the diet, rather than implying that post-settlement *S. splendidus*

might have certain capacity to biosynthesize LC-PUFAs from LOA or ALA as was reported in some marine fish, such as the Atlantic bluefin tuna *Thunnus thynnus*, the gilthead seabream *S. aurata* and the European sea bass *Dicentrarchus labrax* (Morais et al., 2012; Vagner & Santigosa, 2011). It is of particular interesting that recently Navarro-Guillen et al. (2014) reported that larvae of the Senegalese sole appeared to be capable of biosynthesizing DHA from ALA, and ARA from LOA, particularly under the condition with low dietary supply of DHA and high availability of the C18 precursors. It was reported that the process started from an initial Δ 6 desaturation of ALA or LOA, followed by a chain elongation of C18 to C20 and a further Δ 5 desaturation of ARA or EPA, eventually a chain elongation from C20 to C22 with a Δ 4 desaturation to produce DHA (Navarro-Guillen et al., 2014). If post-settlement *S. splendidus* also possess similar capacity of biosynthesizing LC-PUFAs, then the result of the E-1 treatment could be reasonably explained.

It is generally established that saturated and monounsaturated fatty acids are the preferential substrates for mitochondrial and peroxisomal β-oxidation in both marine and freshwater fish (Sargent et al., 2002). In this study, post-settlement *S. splendidus* from the E-3 treatment, which showed the faster growth, had significant higher levels of saturated and monounsaturated fatty acids, particularly OA, than those fish from the E-4 and E-5 treatments. This might suggest that *S. splendidus* of the E-3 treatment had more readily available substrates to generate metabolic energy by fatty acid mitochondrial β-oxidation, which may partially contribute to their high growth rate. In a previous study, 4 DPH *S. splendidus* larvae fed rotifers were shown to maintain tissue DHA at 12.37% TFA, while 4-12 DPH larvae grown on copepods also maintained tissue DHA between 13.27

and 18.10% TFA (Chapter 5). These values closely matched the DHA found in newly-spawned eggs of *S. splendidus* (15.35% TFA)(Chapter 5). In the present study, DHA in 45 DPH *S. splendidus* from different treatments ranged from the lowest 2.68 % TFA of the E-2 treatment to the highest 5.94 % TFA of the E-5 treatment, all were substantially lower than that found in the earlier larvae. For most marine fish, larval requirement for n-3 LC-PUFA is generally higher than those of juveniles and pre-adults (Sargent et al., 2002), hence it could also be possible that DHA requirement of early planktonic larvae differ from the relatively low DHA requirement found in the post-settlement *S. splendidus* in this study.

In summary, investigation of the effects of dietary DHA on post-settlement *S. splendidus* through a dose-response design showed a clear pattern between dietary DHA and larval growth between 25 DPH to 45 DPH; that is, both deficient and excessive levels of DHA in their diets could negatively impact their growth. The extrapolation of the regression curve based on SGR of SL suggested that the dietary DHA level that likely to obtain maximum growth is 6.91 mg DHA/g DW and DHA/EPA ratio about 0.88. Such a result is similar to other necto-benthic fish with relatively low DHA requirements during post-metamorphosis phase, which may be related to their natural habitats in which diets from the benthos are relative abundance in EPA than DHA. Finally, the surprisingly relatively high level DHA detected in the *S. splendidus* of the E-1 treatment in which fish were fed a diet almost devoid of DHA suggests that post-settlement *S. splendidus* might have certain capacity to biosynthesize LC-PUFA from C18 precursors, particularly under the condition of very low dietary supply of DHA, which warrants further investigation.

# Chapter 8

## The effects of graded concentrations of dietary ARA (20:4n-6) on growth, survival and tissue fatty acid profile of the post-settlement green mandarin fish, *Synchiropus splendidus*

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### 8.1. Introduction

Lipids are an indispensable class of nutrients during larval development of marine fish, serving as sources of metabolic energy, precursors to both biologically active and structural molecules, and as reservoirs of essential fatty acids (EFAs) (Watanabe and Kiron 1994; Rainuzzo et al., 1997; Sargent et al., 1999). Long-chain polyunsaturated fatty acids (LC-PUFAs) are known as EFAs for marine fish due to the negligible activity of delta-5 desaturase, the principal desaturating enzyme responsible for LC-PUFAs formation from shorter-chained precursors, in marine fish (Tocher et al., 1989; Linares and Henderson, 1991; Tocher, 2003). An increasing body of evidence has confirmed the important roles n-3 LC-PUFAs, i.e. docosahexaenoic acid (22:6n-3; DHA) and eicosapentaenoic acid (20:5n-3; EPA), play on survival, growth and pigmentation of marine fish larvae, such as the formation of cell membranes and the development of vision and neural systems (Izquierdo et al., 2000; Morais et al., 2004; Bransden et al., 2005a). It has been well documented that dietary deficiency or imbalances of n-3 LC-PUFAs could lead to mortality or delay in growth of fish larvae (Izquierdo, 1996; Lee and Ostrowski, 2001; Velu and Munuswamy, 2003; Olivotto et al., 2003; Vagelli, 2004; Avella et al., 2007). In a previous study, the DHA requirement for post-settlement green mandarin fish, *Synchiropus splendidus*, was

found to be comparatively lower than other marine fish species, and it was also indicated that diets rich in EPA is nutritionally more significant for the post-larvae of *S. splendidus* (Chapter 7).

Among LC-PUFAs, despite numerous research into larval requirements for n-3 LC-PUFAs, very limited research efforts have been placed on the requirements of n-6 LC-PUFAs, i.e. arachidonic acid (20:4n-6, ARA) (Bell and Sargent, 2003). The importance of ARA in fish nutrition has often been overlooked when compared to DHA and EPA probably due to the predominance of the two n-3 LC-PUFAs in fish tissues. However, it is well known that as an EFA, ARA generally serves as the preferred precursor for biosynthesis of eicosanoids compared to EPA (Bell and Sargent, 2003). The eicosanoids, such as prostaglandin E2 ($PGE_2$) and prostaglandin F2α (PGF2α), are a group of highly diverse and complex array of physiologically highly active, hormone-like compounds who play important roles in the modulation of growth, metamorphosis and stress resistance in fish larvae (Bell and Sargent, 2003; Van Anholt et al., 2004). Furthermore, ARA-derived eicosanoid are believed to be generally the most abundant and bioactive whereas those produced from EPA are of lower efficacy (Tocher, 2003). Hence, ARA/EPA ratio could influence the production of eicosanoids and appears playing an important role in fish larval development (Bell and Sargent, 2003).

So far, ARA has been reported to be highly conserved during starvation of larvae of several fish species (Rainuzzo et al., 1997; Izquierdo, 1996). Increasing dietary ARA has also been shown to improve larval survival and growth in some marine fish (Castell et al., 1994; Bell et al., 1985; Bessonart et al., 1999).

Moreover, ARA has been found to improve resistance to handling stress in the gilthead seabream, *Sparus aurata* (Koven et al., 2001) and to maximize stress resistance to hypersaline challenge in larval summer flounder *Paralichthys dentatus* (Willey et al., 2003). On the other hand, both positive and negative effects of increasing dietary ARA on benthic dwelling marine fishes have been demonstrated (Bell and Sargent, 2003). For example, ARA has also been associated with mal-pigmentation problems in flatfish (Bell et al., 2003; Villalta et al., 2005; Lund et al., 2007), which was believed via a biochemically induced stress and overproduction of eicosanoids (Sargent et al., 1999). However, no information is available on the effects of dietary ARA level on the performance of post-settlement *S. splendidus*, who is also a benthic species and pigmentation as a key feature in ornamental trade.

In a previous pilot study, *Artemia* have been identified as an appropriate diet for post-settlement *S. splendidus* while various formulated diets were shown to lead to poor survival. Although *Artemia* generally lack LC-PUFAs, enrichment is a commonly adopted technique to improve their LC-PUFA contents (Sorgeloos et al., 2001). *Artemia* enrichment has also often been used in nutrition studies of fish and crustacean larvae since many marine larvae did not accept formulated diets well (Copeman et al., 2002; Bransden et al., 2005; Villalta et al., 2007; Hamre and Harboe, 2008). Recently utilizing enriched *Artemia* metanauplii with graduated concentrations, the optimal concentration of dietary DHA for post-settlement *S. splendidus* has been identified (Chapter 7). The present study aimed at further understanding lipid nutrition during early life stage of green mandarin fish by assessing the effects of dietary ARA on survival, growth and fatty acid profiles of post-settlement *S. splendidus* via feeding them enriched *Artemia* with graduated

ARA levels; and on the basis, identifying the optimal dietary ARA requirement for the fish.

## 8.2. Materials and Methods

### *8.2.1 Broodstock maintenance*

Broodstock source and husbandry were described in Chapter 2 (Section 2.1).

### *8.2.2 Live prey production and Artemia enrichment*

Rotifers and copepods culture and harvest methods were described in Chapter 2 (Section 2.2 and 2.3).

*Artemia* cysts (Great Salt Lake strain, INVE Aquaculture, Belgium) were hatched daily at 26-28 °C and salinity 28-30 ‰ to produce newly hatched nauplii for the experiment. After 24 h of hatching, newly hatched nauplii (430-520 μm) were harvested for subsequent enrichment. Five *Artemia* enrichment emulsions containing different ARA levels were formulated by incorporating 0% (E-1), 8% (E-2), 16% (E-3), 24% (E-4) and 32% (E-5) of ARA-rich oil (Bulk Nutrients TAS, Australia; containing 40% ARA) respectively into the emulsion. In addition to ARA oil, the formulations of the emulsions included identical amounts of a concentrated n-3 LC-PUFA oil (Obsidian Research Ltd, U.K) and different amounts of corn oil (rich in 18:2n-6) and macadamia oil (rich in 18:1n-9) to adjust the 18:2n-6 content in order to keep it similar for all emulsions (Bransden et al., 2005b). Finally, soy lecithin was added at 10% as emulsifier while 2% of α-tocopherol acetate was added as antioxidants for all emulsions (Estévez et al.,

2001; Villalta et al., 2005)(for detailed formulations of the 5 emulsion oils, please see Table 8.1). Each emulsion oil was then added an equal amount of distilled water and emulsified by using an Ultra-turrax T25 homogenizer (IKA works inc, Wilmington, North Carolina, USA) operating at 10,000 rpm for 60 seconds. They were subsequently stored in five labeled bottles and kept at 4 °C until used.

**Table 8.1.** The formulation (mg/g) of five experimental emulsion oils used for *Artemia* enrichment.

| Emulsion Oil | 0% | 8% | 16% | 24% | 32% |
|---|---|---|---|---|---|
| ARA oil[a] | 0 | 80 | 160 | 240 | 320 |
| Macadamia oil[b] | 440 | 370 | 300 | 230 | 160 |
| Corn oil[c] | 40 | 30 | 20 | 10 | 0 |
| n-3 LC-PUFA oil[d] | 400 | 400 | 400 | 400 | 400 |
| Soy lecithin[c] | 100 | 100 | 100 | 100 | 100 |
| α-tocopherol[e] | 20 | 20 | 20 | 20 | 20 |

[a] Bulk Nutrients, Australia
[b] Macadamias Ltd, Gympie, Australia
[c] Sigma-Aldrich Pty Ltd, Castle hill, Australia
[d] Obsidian Research Ltd, U.K
[e] Blackmors Ltd, Warriwood, Australia

The *Artemia* enrichment was conducted in 500 mL beakers with a density of 200-250 *Artemia* $mL^{-1}$ with the addition of 0.6 g $L^{-1}$ of each of the five emulsion oils (Sui et al., 2007). During enrichment, the temperature and salinity was maintained at 26-28 °C and 30 ‰, respectively. Following enrichment, enriched *Artemia* metanauplii were harvested next morning and thoroughly rinsed with freshwater to remove traces of the emulsion before they were estimated for density and ready for feeding them to the post-settlement *S. splendidus*.

*8.2.3 Experimental design and setup*

The incubation and experimental system were described in Chapter 2 (Section 2.5). The newly hatched larvae were left in the incubation buckets until 2 DPH when healthy larvae were transferred to a 50 L black rectangular polypropylene tank for culture. Based on larval feeding regime established in this lab, the larvae were initially fed copepod *P. crassirostris* at a density of 1 $mL^{-1}$ until 18 days post hatching (DPH), they were then fed 5 *Artemia* nauplii $mL^{-1}$ from 19 to 24 DPH. The experiment started with 25 DPH larvae, which were sourced from the communally culture in the 50 L tank as described above. A total of 225 *S. splendidus* on their 25 DPH of development were randomly selected from the 50 L tank and evenly transferred into fifteen 3 L purposely designed cylindrical rearing vessels (i.e. 15 each). They were then divided into five groups of 3 vessels (replicates) and fed 5 ind. $mL^{-1}$ *Artemia* metanauplii enriched with one of the five enrichment emulsions with graded concentrations of ARA. An additional twenty 25 DPH *S. splendidus* were sampled from the 50 L tank for the measuring initial standard length, body width and dry weight. The experiment terminated on 45 DPH. Throughout the experiment, the major physical parameters were maintained as follows: temperature 26-29 °C, salinity 34-38 ‰, pH 8.0-8.2, $NH_3$, $NO_2$ < 0.02 ppm, and $NO_3$ < 0.5 ppm.

*8.2.4 Sampling and data collection*

The procedure of sampling and data collection were described in Chapter 7 (Section 7.2.4).

*8.2.5 Total lipid and fatty acid analysis*

The methods of lipid and fatty acid analysis were described in Chapter 7 (Section 7.2.5).

*8.2.6 Data analysis*

Homogeneity of variance of all data was first tested with Levene's test. Where necessary, arcsine-square root or logarithmic transformation was performed prior to analysis. One-way ANOVA was used to determine significant differences among treatments. If any significant difference was detected, Tukey's multiple range test was used as the means separation procedure. Pairwise t-test was used to determine statistically significant of relationship between dietary ARA level and different growth parameters measured. The relationships between dietary ARA level and different growth parameters were further analyzed with second-order polynomial regression. When a normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Kruskal-Wallis H non-parametric test followed by the Games-Howell non-parametric multiple comparison test. $p<0.05$ was regarded as the statistically significant level. All statistics were performed using SPSS package 23 (Statsoft$^{TM}$, Inc.).

## 8.3. Results

*8.3.1 Total lipid and fatty acid profiles of Artemia enriched with graded ARA emulsions*

The total lipid and fatty acid composition of *Artemia* metanauplii enriched with five emulsion oils containing different levels of ARA are presented in Table

8.2. Total lipid contents of the enriched *Artemia* were very similar among five treatments, ranging from 20.97 (E-2) to 22.99 % (E-3) dry weight. Close ranges of C16:0 (10.22-11.72 % total fatty acids, TFA) and C18:0 (5.63-6.18% TFA), as well as total saturated fatty acid (SFA; 18.07-19.87% TFA) were also detected among *Artemia* enriched with the five emulsion oils. However, a clear decreasing trend in C18:1n-9 (OA; decreased from 31.97% to 25.06% TFA) and total monounsaturated fatty acids (MUFA; decreased from 47.26% to 39.77% TFA) was apparent in the *Artemia* enriched with the emulsions from E-1 to E-5 (Tab. 8.2).

**Table 8.2** Total lipid contents (% dry weight) and fatty acid composition (% total fatty acids) of *Artemia* metanauplii enriched with five emulsions (E1 to E5: 0%, 8%, 16%, 24% and 32%) with graduated ARA concentrations.

| | *Artemia* enriched with E-1 | *Artemia* enriched with E-2 | *Artemia* enriched with E-3 | *Artemia* enriched with E-4 | *Artemia* enriched with E-5 |
|---|---|---|---|---|---|
| Total lipid (%) | 21.90 | 20.97 | 22.99 | 21.86 | 21.08 |
| Fatty acids | | | | | |
| 14:0 | 1.11 | 0.98 | 0.83 | 0.79 | 0.85 |
| 16:0 | 11.41 | 11.72 | 10.72 | 10.45 | 10.22 |
| 18:0 | 5.80 | 6.18 | 5.63 | 5.84 | 5.68 |
| ∑SFA | 19.17 | 19.87 | 18.10 | 18.17 | 18.07 |
| 16:1n-7 | 0.88 | 1.03 | 1.04 | 1.10 | 1.09 |
| 16:1n-5 | 4.24 | 3.66 | 3.56 | 3.29 | 3.30 |
| 18:1n-9(OA) | 31.97 | 30.58 | 29.78 | 28.21 | 25.06 |
| 18:1n-7 | 7.89 | 8.38 | 8.24 | 8.46 | 7.97 |
| 20:1n-9 | 1.30 | 1.21 | 1.27 | 1.27 | 1.23 |
| ∑MUFA | 47.26 | 45.98 | 45.03 | 42.48 | 39.77 |
| 18:2n-6(LOA) | 7.52 | 7.64 | 7.51 | 7.24 | 7.25 |
| 18:3n-3(ALA) | 15.38 | 15.26 | 15.87 | 16.31 | 16.89 |
| 18:3n-6 | 2.27 | 2.62 | 2.48 | 2.70 | 2.78 |
| 20:4n-6(ARA) | 0.62 | 1.63 | 2.81 | 3.78 | 5.70 |
| 20:5n-3(EPA) | 2.95 | 2.72 | 3.22 | 3.28 | 2.92 |
| 22:6n-3(DHA) | 2.62 | 2.33 | 2.44 | 2.38 | 2.32 |
| ∑PUFA | 32.59 | 33.68 | 36.12 | 37.82 | 40.27 |
| ∑LC-PUFA | 6.63 | 7.18 | 9.24 | 10.37 | 12.09 |
| ∑n-3PUFA | 20.99 | 20.43 | 21.80 | 22.34 | 22.67 |
| ∑n-6PUFA | 8.63 | 9.87 | 11.03 | 11.84 | 13.77 |
| n-3/n-6 | 2.43 | 2.07 | 1.98 | 1.89 | 1.65 |
| DHA/EPA | 0.89 | 0.86 | 0.76 | 0.72 | 0.80 |
| ARA/EPA | 0.21 | 0.60 | 0.87 | 1.15 | 1.95 |

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; LC-PUFA: long-chain polyunsaturated fatty acid; Totals (∑) included some minor components not shown.

Among the polyunsaturated fatty acids (PUFA), *Artemia* enriched with different emulsions generally contained close ranges of C18:2n-6 (LOA,

7.24-7.64% TFA), C18:3n-3 (ALA; 15.26-16.89% TFA), C20:5n-3 (EPA; 2.72-3.28% TFA) and C22:6n-3 (DHA; 2.32-2.62% TFA). However, as expected, the enrichment of *Artemia* using five emulsion oils with increased ARA contents from E-1 to E-5 resulted in a sharp increase in ARA level (from 0.62% to 5.70% TFA, which was of a magnitude of 9.19 times). Concurrently, the ratio of n-3 PUFA/n-6 PUFA decreased from 2.43 to 1.65 E-5 while the ARA/EPA ratio increased from 0.21 to 1.95. A significant positive correlation was detected between ARA contents of the enriched *Artemia* and incorporation levels of ARA-rich oil of the experimental emulsions (Fig. 8.1, n=5, r=0.99, $p < 0.01$).

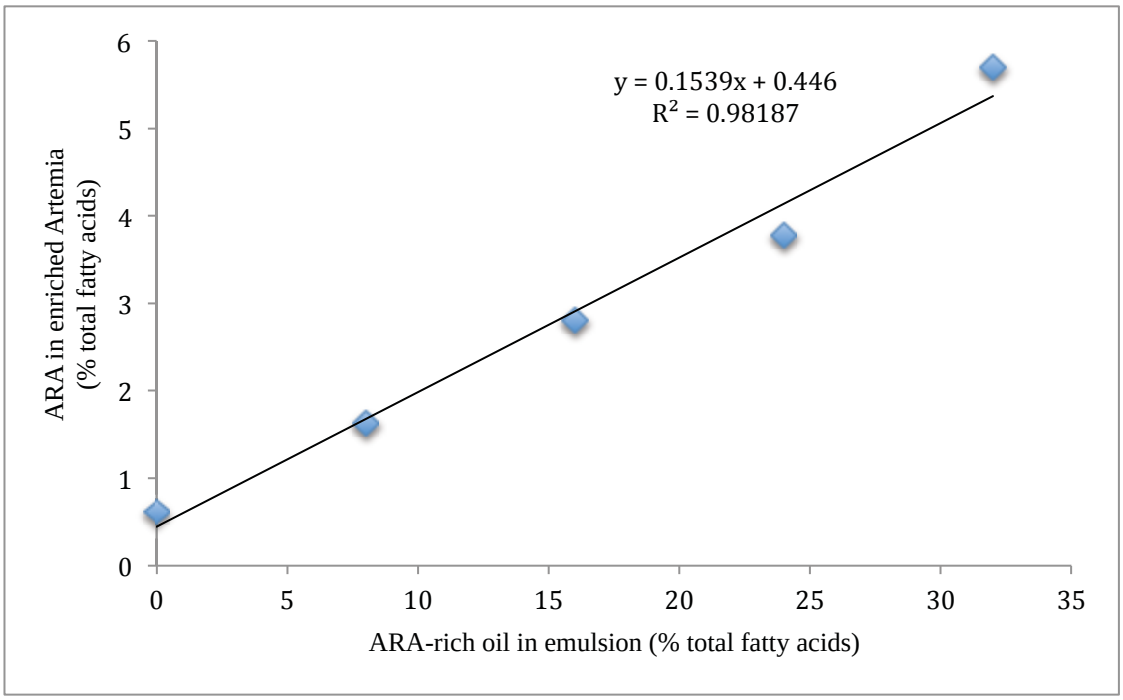



**Figure 8.1** The relationship between ARA contents of enriched *Artemia* and the incorporated levels (%) of the ARA-rich oil in the five emulsion oils.

Table 8.3 presents the absolute contents (mg/g DW) of principal fatty acids of the *Artemia* enriched with the five emulsions. Similarly, while the TFA

contents remained very similar among all treatments (ranging between 210.02-220.65 mg/g DW), the ARA content increased more than nine times from 1.31 mg/g DW in the *Artemia* enriched with E-1 emulsion to 12.56 mg/g DW in those enriched with E-5 emulsion (Tab. 8.3).

**Table 8.3** Principal fatty acid contents (mg/g DW) of *Artemia* metanauplii enriched with five emulsions (E1 to E5: 0%, 8%, 16%, 24% and 32%) with graduated ARA concentrations.

| | *Artemia* enriched with E-1 | *Artemia* enriched with E-2 | *Artemia* enriched with E-3 | *Artemia* enriched with E-4 | *Artemia* enriched with E-5 |
|---|---|---|---|---|---|
| Fatty acids | | | | | |
| 16:0 | 24.19 | 25.99 | 23.69 | 22.37 | 22.53 |
| 18:0 | 12.31 | 13.69 | 12.44 | 12.49 | 12.52 |
| ∑SFA | 40.65 | 44.06 | 39.99 | 38.87 | 39.82 |
| 16:1n-5 | 8.99 | 8.11 | 7.85 | 7.05 | 7.27 |
| 18:1n-9 (OA) | 67.81 | 67.79 | 65.78 | 60.36 | 55.24 |
| 18:1n-7 | 16.74 | 18.57 | 18.21 | 18.11 | 17.58 |
| ∑MUFA | 100.24 | 101.92 | 99.48 | 93.05 | 87.68 |
| 18:2n-6(LOA) | 15.96 | 16.94 | 16.58 | 15.50 | 15.98 |
| 18:3n-3 (ALA) | 32.62 | 33.83 | 35.06 | 34.90 | 37.23 |
| 18:3n-6 | 4.80 | 5.81 | 5.47 | 5.78 | 6.13 |
| 20:4n-6(ARA) | 1.31 | 3.61 | 6.21 | 8.09 | 12.56 |
| 20:5n-3(EPA) | 6.26 | 6.04 | 7.11 | 7.03 | 6.43 |
| 22:6n-3(DHA) | 5.56 | 5.16 | 5.40 | 5.09 | 5.12 |
| ∑PUFA | 69.13 | 74.66 | 79.79 | 80.93 | 88.77 |
| ∑LC-PUFA | 14.07 | 15.92 | 20.40 | 22.18 | 26.64 |
| ∑n-3PUFA | 44.52 | 45.29 | 48.16 | 47.80 | 49.97 |
| ∑n-6PUFA | 18.30 | 21.88 | 24.36 | 25.34 | 30.35 |
| Total fatty acids | 210.02 | 220.65 | 219.25 | 212.85 | 216.27 |

DW: dry weight; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; LC-PUFA: long-chain polyunsaturated fatty acid; Totals (∑) include some minor components not shown.

*8.3.2 Survival and growth performance of S. splendidus*

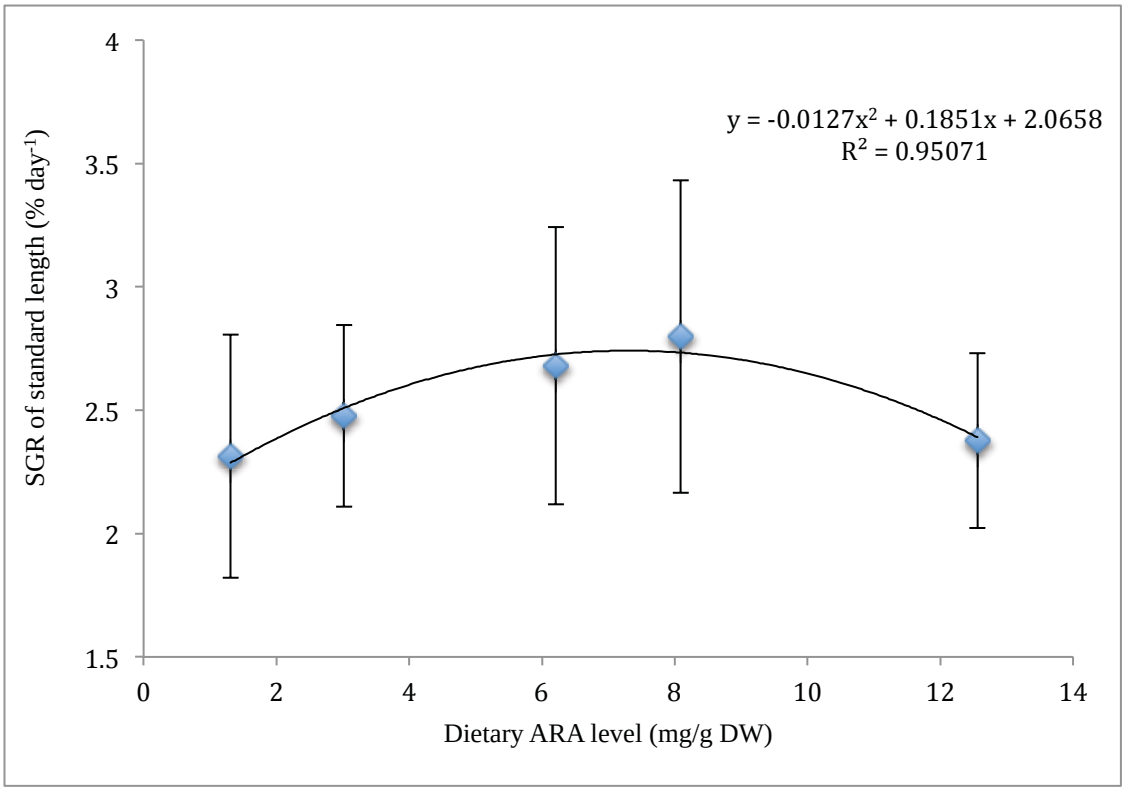
Throughout the experiment duration, no mortality was observed in any of the treatments. Hence, the survival of the post-settlement *S. splendidus* was not affected by the dietary ARA levels.

At the end of the experiment, no significant difference was detected for the standard length (SL) of the 45 DPH *S. splendidus* among different treatments (ranging between 6.82-7.53 mm) ($p<0.05$; Tab. 8.4). Of the five treatments, *S. splendidus* fed *Artemia* enriched with E-4 emulsion had the highest body width (BW) and dry weight (DW) (3.16 mm and 3.26 mg, respectively), which was followed by E-3 emulsion (3.04 mm and 2.61 mg, respectively). While the BW of these two treatments was not significantly different ($p>0.05$), DW of the E-4 treatment was significantly heavier than that of E-3 ($p<0.05$). Meanwhile, the BW of the E3 treatment was significantly higher than that of E-1 and E-5 although for DW, it was only significantly heavier than that of E-1. The BW and DW of E-1, E-2 and E-5 were not significantly different (ranging from 2.65 to 2.74 mm for BW; 2.08 to 2.27 mg for DW). The specific growth rates (SGR) calculated based on SL of the 45 DPH *S. splendidus* was the highest from the E-4 treatment but was not significant different among all treatments (ranging from 2.31 of E-1 to 2.80% $day^{-1}$ of E-4). E-4 treatment also had the highest SGR based on BW and DW (3.15% and 5.37% $day^{-1}$, respectively) while the lowest SGR of DW (3.11% $day^{-1}$) and BW (2.27% $day^{-1}$) was from the E-1 and E-5 treatment respectively (Tab. 8.4).

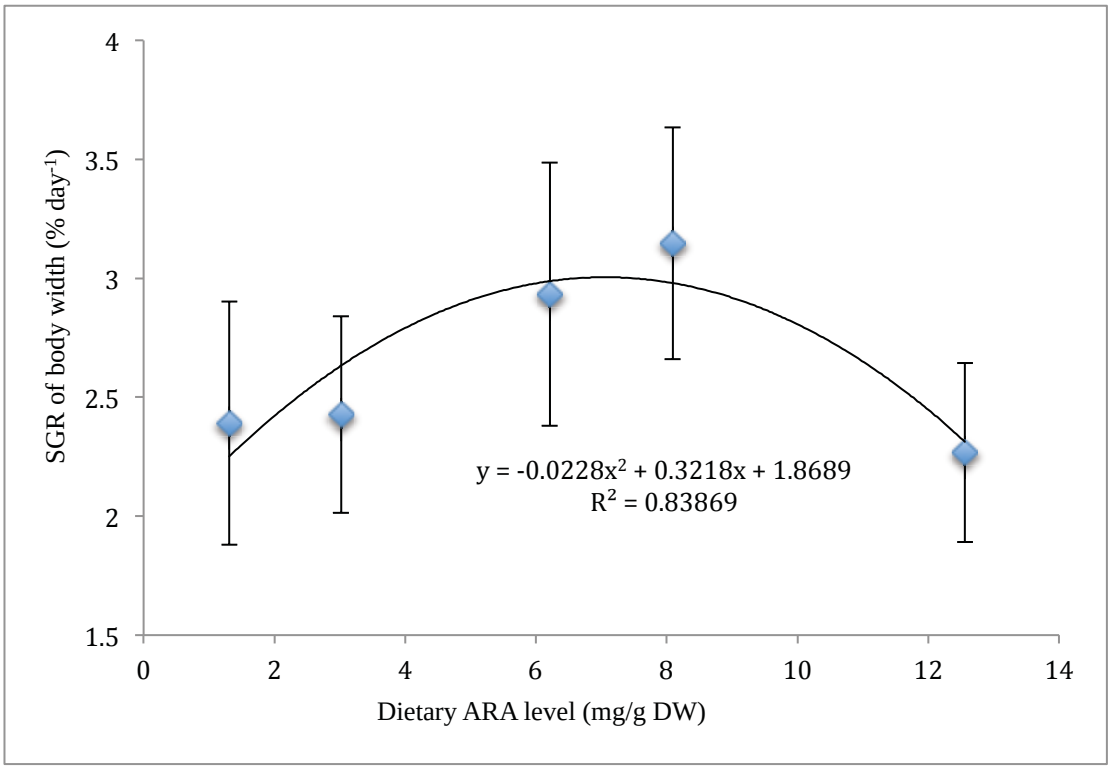
**Table 8.4** The standard length (SL), body width (BW), dry weight (DW) and specific growth rate (SGR) of 45 DPH *S. splendidus* (mean ± SD; n=3) fed *Artemia* enriched with five emulsions (E-1 to E5: 0%, 8%, 16%, 24% and 32%) with graduated ARA concentrations for 20 days. Different superscript letters within the same row indicate significant differences ($p$<0.05).

| Treatments | E-1 | E-2 | E-3 | E-4 | E-5 |
|---|---|---|---|---|---|
| SL (mm) | 6.82±0.66$^{a}$ | 7.03±0.51$^{a}$ | 7.35±0.79$^{a}$ | 7.53±0.93$^{a}$ | 6.89±0.48$^{a}$ |
| BW (mm) | 2.72±0.28$^{a}$ | 2.74±0.23$^{ab}$ | 3.04±0.33$^{bc}$ | 3.16±0.30$^{c}$ | 2.65±0.19$^{a}$ |
| DW (mg) | 2.08±0.22$^{a}$ | 2.27±0.23$^{ab}$ | 2.61±0.10$^{b}$ | 3.26±0.22$^{c}$ | 2.12±0.13$^{ab}$ |
| SGR | | | | | |
| SL (% day$^{-1}$) | 2.31±0.49$^{a}$ | 2.48±0.37$^{a}$ | 2.68±0.56$^{a}$ | 2.80±0.63$^{a}$ | 2.38±0.36$^{a}$ |
| BW (% day$^{-1}$) | 2.39±0.51$^{a}$ | 2.43±0.41$^{ab}$ | 2.93±0.55$^{bc}$ | 3.15±0.49$^{c}$ | 2.27±0.38$^{a}$ |
| DW (% day$^{-1}$) | 3.11±0.53$^{a}$ | 3.54±0.49$^{ab}$ | 4.27±0.19$^{b}$ | 5.37±0.34$^{c}$ | 3.21±0.32$^{a}$ |

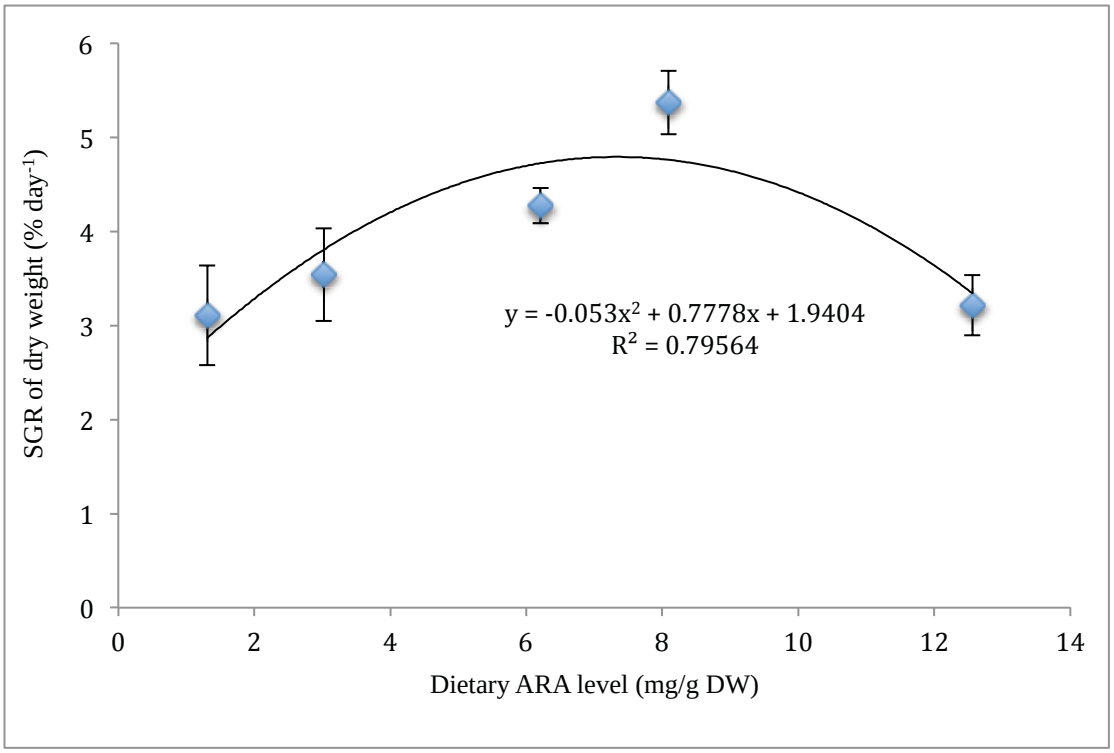
The relationships between analyzed ARA content of the enriched *Artemia* and SGR of SL of the 45 DPH *S. splendidus* from different treatment fitted into a second-order polynomial regression: $y = -0.0127x^2 + 0.1851x + 2.0658$ ($R^2$=0.95071; x= dietary ARA level; y= SGR on SL; n=5, $p$>0.05)(Fig. 8.2A). Based on the regression, 7.29 mg ARA/g DW was identified as the dietary ARA level for the maximum growth of SL for the post-settlement *S. splendidus.* Similarly, the relationship between dietary ARA level and SGR of BW was regressed as: $y = -0.0228x^2 + 0.3218x + 1.8689$ ($R^2$=0.83869; x= dietary ARA level; y= SGR on BW; n=5, $p$<0.05) with the optimal ARA for maximum growth in BW identified as 7.06 mg ARA/g DW (Fig. 8.2B). Finally, the dietary ARA level regressed against SGR of DW resulted in the equation: $y = -0.053x^2 + 0.7778x + 1.9404$ ($R^2$=0.79564; x= dietary ARA level; y= SGR on DW; n=5, $p$<0.01) and 7.34 mg ARA/g DW was recommended for the maximum growth in DW (Fig. 8.2C).


y = -0.0127x² + 0.1851x + 2.0658
R² = 0.95071
SGR of standard length (% day-1)
Dietary ARA level (mg/g DW)
4
3.5
3
2.5
2
1.5
0
2
4
6
8
10
12
14

**(A)**


y = -0.0228x² + 0.3218x + 1.8689
R² = 0.83869
SGR of body width (% day-1)
Dietary ARA level (mg/g DW)
4
3.5
3
2.5
2
1.5
0
2
4
6
8
10
12
14

**(B)**




**(C)**

**Figure 8.2** Regressions of analyzed ARA levels in *Artemia* enriched by five emulsions with graded ARA concentrations and the SGR (% day$^{-1}$) based on standard length (A), body width (B) and dry weight (C) of the 45 DPH *S. splendidus* fed on those *Artemia* from 25 DPH.

*8.3.3 Fatty acid profile of the 45 DPH S. splendidus*

The whole body fatty acid profiles of 45 DPH *S. splendidus* from different treatments are shown in Table 8.5. After 20 days of feeding on *Artemia* enriched with five emulsions of graded ARA concentrations, the fatty acids compositions of the 45 DPH *S. splendidus* generally reflected those in their diets, however some deviations were also noticed, particularly for LOA and EPA (Tab 8.2, 8.3 and 8.5). Overall, C16:0 and C18:0 were the main constituents of SFAs and remained relative stable among all treatments although the levels of C18:0 were generally

lower than those found in their respective diets. Among MUFAs, while C18:1n-7 and C16:1n-7 were similar among treatments, OA (representing >70% of MUFA) and total MUFA showed a decreasing trend from E-1 to E-5, largely reflecting their dietary levels. Of PUFAs, as expected, with increasing dietary ARA level from E-1 to E-5, ARA of the 45 DPH *S. splendidus* showed a clear increasing trend from 1.15 to 6.31% TFA. Conversely, EPA showed a decreasing trend despite dietary levels remained relatively stable (Tab. 8.2, 8.3 and 8.5). Although LOA levels of enriched *Artemia* were within a close range among five treatments, significantly lower levels were found in the 45 DPH *S. splendidus* from the E-4 and E-5 treatments. Finally, concomitant with the increasing dietary ARA levels from E-1 to E-5, the ratio of ARA/EPA of 45 DPH *S. splendidus* also showed a clear trend of increasing from 0.31 to 2.99 (Tab. 8.5).

**Table 8.5** The fatty acid profiles (% total fatty acids; mean ± SD; n=3) of the 45 DPH *S. splendidus* fed *Artemia* enriched with five emulsions (E-1 to E-5: 0%, 8%, 16%, 24% and 32%) with graduated ARA concentrations from 25 DPH. Different superscript letters within the same row indicate significant differences ($p$<0.05).

| Treatment | E-1 | E-2 | E-3 | E-4 | E-5 | *P* |
|---|---|---|---|---|---|---|
| Fatty acids | | | | | | |
| 16:0 | 15.96±0.35$^{a}$ | 15.86±0.42$^{a}$ | 16.83±0.74$^{ab}$ | 17.69±1.51$^{bc}$ | 18.72±0.95$^{c}$ | <0.01 |
| 18:0 | 13.10±1.10$^{c}$ | 10.83±0.48$^{ab}$ | 10.05±0.26$^{a}$ | 11.27±0.95$^{bc}$ | 12.03±0.55$^{c}$ | <0.01 |
| ∑SFA | 29.69±0.96$^{b}$ | 27.31±0.91$^{a}$ | 27.55±0.98$^{a}$ | 29.70±2.38$^{ab}$ | 31.49±1.32$^{b}$ | <0.01 |
| 16:1n-7 | 2.66±0.23 | 3.02±0.29 | 2.94±0.10 | 2.83±0.15 | 2.89±0.06 | ns |
| 18:1n-9(OA) | 27.47±2.15$^{a}$ | 26.75±0.57$^{a}$ | 26.57±1.23$^{a}$ | 26.37±1.97$^{a}$ | 21.69±1.08$^{b}$ | <0.001 |
| 18:1n-7 | 5.45±0.16 | 5.65±0.44 | 5.82±0.27 | 6.06±0.33 | 5.94±0.60 | ns |
| ∑MUFA | 36.72±2.37$^{a}$ | 36.53±0.65$^{a}$ | 36.51±1.68$^{a}$ | 36.45±1.83$^{a}$ | 31.77±1.14$^{b}$ | <0.001 |
| 18:2n-6(LOA) | 8.23±0.12a | 8.36±0.28a | 8.05±0.18a | 6.34±0.60b | 6.54±0.88b | <0.001 |
| 18:3n-3(ALA) | 10.76±0.61 | 11.28±0.82 | 11.50±0.91 | 11.41±1.11 | 10.99±0.22 | ns |
| 20:4n-6(ARA) | 1.15±0.10$^{a}$ | 2.95±0.39$^{b}$ | 4.73±0.43$^{c}$ | 5.38±0.24$^{d}$ | 6.31±0.72$^{e}$ | <0.001 |
| 20:5n-3(EPA) | 3.71±0.19$^{a}$ | 2.91±0.28$^{b}$ | 2.83±0.31$^{b}$ | 2.27±0.17$^{c}$ | 2.11±0.28$^{c}$ | <0.001 |
| 22:6n-3(DHA) | 2.46±0.39 | 2.37±0.16 | 2.34±0.22 | 2.26±0.09 | 2.13±0.08 | ns |
| ∑PUFA | 26.71±0.87 | 28.40±1.08 | 30.09±1.91 | 28.38±1.22 | 28.87±2.08 | ns |
| ∑LC-PUFA | 7.38±0.59$^{a}$ | 8.36±0.11$^{b}$ | 10.13±0.93$^{c}$ | 10.18±0.44$^{c}$ | 10.82±1.10$^{c}$ | <0.001 |
| ∑n-3PUFA | 16.93±0.65 | 16.56±0.66 | 16.67±1.38 | 15.94±1.05 | 15.23±0.52 | ns |
| ∑n-6PUFA | 9.55±0.18$^{a}$ | 11.60±0.62$^{b}$ | 13.18±0.55$^{b}$ | 12.17±0.41$^{b}$ | 13.35±1.61$^{b}$ | <0.01 |
| n-3/n-6 | 1.77±0.04$^{a}$ | 1.43±0.08$^{b}$ | 1.26±0.06$^{b}$ | 1.31±0.09$^{b}$ | 1.15±0.11$^{b}$ | <0.01 |
| DHA/EPA | 0.66±0.09 | 0.82±0.12 | 0.83±0.06 | 1.00±0.04 | 1.02±0.11 | ns |
| ARA/EPA | 0.31±0.01$^{a}$ | 1.02±0.21$^{b}$ | 1.68±0.09$^{c}$ | 2.37±0.07$^{d}$ | 2.99±0.06$^{e}$ | <0.001 |

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; LC-PUFA: long-chain polyunsaturated fatty acid; ns-no significance; Totals (∑) include some minor components not shown.

Table 8.6 showed the relative retention ratio of major PUFAs for the 45 DPH *S. splendidus*. From E-1 to E-5 treatment, decreasing trends of the retention ratios were found for LOA, ARA and EPA while ALA and DHA remained relatively stable. Among the five key PUFAs, ALA had the lowest retention ratio (0.65-0.74) while ARA was the highest (1.11-1.85).

**Table 8.6.** The relative retention ratios of PUFA of 45 DPH *S. splendidus* fed enriched *Artemia* containing different levels of ARA. Relative retention ratio = percentage of a fatty acid detected in *S. splendidus* / percentage of corresponding fatty acid in their diet.

| Treatment | E-1 | E-2 | E-3 | E-4 | E-5 |
|---|---|---|---|---|---|
| C18:2n-6(LOA) | 1.09 | 1.09 | 1.07 | 0.88 | 0.90 |
| C18:3n-3(ALA) | 0.70 | 0.74 | 0.72 | 0.70 | 0.65 |
| C20:4n-6(ARA) | 1.85 | 1.81 | 1.68 | 1.42 | 1.11 |
| C20:5n-3(EPA) | 1.26 | 1.07 | 0.88 | 0.69 | 0.72 |
| C22:6n-3(DHA) | 0.94 | 1.02 | 0.96 | 0.95 | 0.92 |

## 8.4. Discussion

Studies on effect of n-6 LC-PUFAs, ARA on survival and growth of marine fish larvae are relatively few as compared to the n-3 LC-PUFAs, DHA and EPA. The present study investigated the effect of dietary ARA level on survival and growth performance of post-settlement *S. splendidus* using a dose-response design. Pilot trials has shown that the inert formulated diets were not able to support good survival and normal development of 25 DPH post-settlement *S. splendidus*, hence using formulated diets for such study is not feasible. Enriched *Artemia* were subsequently trialed and it was shown that ARA in homemade lipid enrichment emulsions was readily absorbed/assimilated by *Artemia* during enrichment. Therefore, by careful manipulating ARA levels in the lipid emulsions, graded ARA levels in enriched *Artemia* were achieved. The enriched *Artemia* were then used to feed *S. splendidus* to study their dietary ARA requirements.

The results showed that survival of the 25 to 45 DPH post-settlement *S. splendidus* was not affected by dietary ARA level as no mortality was found in any treatments with dietary ARA ranging from a very low level of only 0.62% TFA to a high level of 5.70% TFA. Such a result might be explained by the fact

that this stage *S. splendidus* are capable of preferentially absorbed and deposited low level of dietary ARA in their tissue to support normal functions, which is supported by the fact that the 45 DPH *S. splendidus* from the E-1 treatment with the lowest dietary ARA level (0.62% TFA) had the highest relative retention ratio of ARA among all five treatments (Tab. 8.6). In fact, past research has shown that dietary ARA levels had no significant effects on larval survival of various marine fish, particularly benthic species, including the common sole *Solea solea* (Lund et al., 2007), the striped trumpeter, *Latris lineata* (Bransden et al., 2004), the summer flounder, *Paralichthys dentatus* (Willey et al., 2003), the yellowtail flounder, *Limanda ferruginea* (Copeman et al., 2002), the turbot, *Scophthalmus maximus* (Estévez et al., 1999) and the halibut, *Hippoglossus hippoglossus* (McEvoy et al., 1998).

However, a number of other studies have demonstrated that appropriate dietary ARA level could improve survival (Bell et al., 1985; Castell et al., 1994; Bessonart et al., 1999) and resistance to handling stress (Koven et al., 2001) of marine fish larvae and early juveniles. For example, dietary deficiencies in ARA have reportedly resulting in decreased survival and obvious pathology in juvenile turbot *S. maximus* (Bell et al., 1985) and Castell et al. (1994) reported a dietary ARA levels of 0.5-1.0% DW as optimal for their survival. Similarly Bessonart et al. (1999) found that an increase in dietary ARA from 0.1 to 1.8% DW improved larval survival of 17 to 31 DPH gilthead seabream *Sparus aurata*, and ARA was more effective in improving larval survival under the condition of high DHA/EPA ratios. In another study, although Koven et al. (2001) found that dietary ARA level of 0.17- 0.53% fed to 5 to 19 DPH larvae of the gilthead seabream did not result in marked differences on their survival; under the acute stress of tank

transfer, treatments with elevated ARA markedly improved larval survival. On the other hand, excess dietary ARA has also been reported to have negative impacts on larval survival, for example, Zheng et al. (1996) reported that larval cod *Gadus macrocephalus* fed enriched *Artemia* with high ARA levels (3.7– 7.6% DW) led to poorer survival than those fed *Artemia* with lower ARA of 0.5% DW. Similarly, Ishizaki et al. (1998) also found a clear negative effect of high ARA level (4% DW) on survival of the Japanese amberjack, *Seriola quinqueradiata,* larvae and juveniles. Such obvious discrepancies from literature likely reflect species-specific ARA requirements during early development of marine fish and highlight the need to conduct ARA requirement study on each of targeted aquaculture species.

The results of previous research on effects of dietary ARA on larval and juvenile growth of marine fish were also highly variable (Castell et al., 1994; Zheng et al., 1996; Ishizaki et al., 1998; Bransden et al., 2004; Lund et al., 2007;). For example, it has been demonstrated that larval growth was not significantly affected by dietary ARA level in the common sole *S. solea*, the striped trumpeter *L. lineata* and the turbot *S. maximus* (Lund et al., 2007; Bransden et al., 2004; Castell et al., 1994). On the contrary, Zheng et al. (1996) reported that larval cod *G. macrocephalus* fed enriched *Artemia* with ARA levels between 3.7– 7.6% DW showed slower growth than those fed *Artemia* with ARA < 0.5% DW. Similarly, Ishizaki et al. (1998) found a clear negative effect of ARA at 4% DW on the growth of Japanese amberjack *S. quinqueradiata* larvae and juveniles. In an early study with juvenile turbot lasting 11 weeks, Castell et al. (1994) found a rapid growth response to dietary ARA supplementation as compared to those fed a non-ARA-supplemented diet for the first 2 weeks. However, no significant

differences in growth was observed for the remaining weeks, suggesting the juveniles was deficient in ARA at the beginning of the experiment. Experiments with gilthead sea bream larvae (16 - 37 DPH) showed that ARA increasing from 0.1% to 1.0% DW in their microdiets significantly promoted growth, but only when the experiment duration lasting at least 21 days (Bessonart et al., 1999). On the contrary, Koven et al. (2003) found the increase in ARA levels from 0.06 to 0.6% DW in enriched *Artemia* did not significantly differentiate growth of gilthead sea bream metamorphosing larvae (29 - 42 DPH) for a 12 days feeding duration. In the present study, while the SL of 45 DPH *S. splendidus* were not statistically different among all treatments, significantly enhanced BW and DW were recorded when they were fed enriched *Artemia* with increasing levels of ARA from E-1 (0.62% TFA or 0.13% DW; ARA/EPA=0.23) to E-4 treatment (3.78% TFA or 0.81% DW; ARA/EPA=1.15). However, the trend reversed with a further increase in dietary ARA level in the E-5 treatment (5.70% TFA or 1.26% DW; ARA/EPA=1.57), resulting in slower growth, particularly significant lower BW and DW as compared to that of the E-4 treatment.

The mechanisms by which dietary ARA affected growth, whether improving or inhibiting, have so far not clearly identified (Bell and Sargent, 2003). It has been suggested that between EPA and ARA, a high competitive interactive relationship in eicosanoids formation exists (Glencross, 2009). Eicosanoids, a collective term for prostaglandins, thromboxanes and leukotrienes, are a group of biologically active molecules once known as local hormones (Bell and Sargent, 2003). Eicosanoids are known to play important roles in a wide variety of localised physiological processes in fish, which include regulating fluid and electrolyte fluxes; the functioning of cardiovascular system, reproductive and the

neural system (Mustafa and Srivastava, 1989). ARA and EPA are both substrates for the formation of eicosanoids with ARA being the preferred substrate for the production of series-2 eicosanoids (e.g. prostaglandin $E_2$, $PGE_2$) of higher biological activity (Bell et al., 1994) while EPA for producing series-3 eicosanoids (e.g. prostaglandin $E_3$, $PGE_3$) of lower biological activity (Yang et al., 2002). These two series of eicosanoids have different physiological significances and an excessive level of ARA in the diet may alter an appropriate balance. A possible explanation for the observed results would be that when dietary ARA was below the optimal level, increasing ARA led to increased $PGE_2$ synthesis and concentration, hence enhanced larval development and growth. However, since there exists a competitive inhibition between conversion of ARA to $PGE_2$ and EPA to $PGE_3$ (Bell and Sargent, 2003), further increase in dietary ARA beyond the optimal level might lead to excessive $PGE_2$ being produced but lower $PGE_3$ production, resulting in an imbalanced $PGE_2/PGE_3$ ratio. An imbalanced $PGE_2/PGE_3$ ratio could result in fish experiencing a biochemically-induced stress (Sargent et al., 1999), leading to slower growth of post-settlement *S. splendidus*.

In fact, experiments on mammals have shown that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) exerted positive effects on protein synthesis while $PGE_2$ inhibited muscle fiber formation and stimulated protein degradation (Palmer and Wahle, 1987; Veliça and Bunce, 2008). It is hence possible that muscle growth in fish might be modulated by altered ratio of these two prostaglandins. The ratio of $PGF_{2\alpha}$ to $PGE_2$ might be affected by the intake of exogenous ARA (i.e. dietary or injected ARA) due to preferred bioconversion or competitive inhibition of a same enzyme system for their synthesis (Reddy et al., 2004; Veliça and Bunce, 2008). Indeed, Bransden et al. (2004) found that increasing dietary ARA elevated $PGF_{2\alpha}$ and

$PGE_2$ concentrations in 23 DPH striped trumpeter larvae, with a higher increasing rate of $PGE_2$. Therefore, the observed effects of dietary ARA on the growth of post-settlement *S. splendidus* might be regulated by the ratio of $PGF_{2\alpha}$ to $PGE_2$. In summary, the optimal dietary ARA level might represent the level that lead to the production of $PGF_{2\alpha}$ and $PGE_2$ at a most appropriate ratio, hence resulting in the greatest BW and DW.

Recent research has also shown that elevated dietary ARA levels resulted in abnormal pigmentation in several flatfish species (Estévez and Kanazawa, 1996; Estévez et al., 1999; Copeman et al., 2002; Lund et al., 2007; Villalta et al., 2005; Villalta et al., 2007) and suggested that appropriate eicosanoids level and the ARA/EPA ratio are important for normal pigmentation. For instance, in the senegal sole *S. senegalensis,* feeding larvae on *Artemia* devoid of DHA did not lead to mal-pigmented in juveniles, a high dietary ARA level produced pseudo-alibinistic juveniles. Interestingly, in the present study, at the end of the experiment, no abnormal pigmentation was observed for 45 DPH *S. splendidus* in all treatments.

The regressions of SGRs of the post-settlement *S. splendidus* and their dietary ARA levels identified dietary ARA level at 7.29, 7.06 and 7.34 mg ARA/g DW for the optimal growth of SL, BW and DW respectively. Interestingly, these levels are much higher than some of past studies and even higher than the levels that showed negative effects on growth in other marine fish (Koven et al., 2001; Bransden et al., 2004). For example, Bransden et al. (2004) demonstrated that there was no advantage in higher dietary ARA level above 1.33 mg/g DW for striped trumpeter larvae, and its optimal dietary ARA requirement might be lower

than this value. Such a significant difference may be attributed to their different geological distributions as the green mandarin is a typical tropical species while the striped trumpeter is a temperate species. Generally marine fish reside sub-tropical and tropical waters contain higher tissue ARA concentrations as compared to their temperate counterparts, hence they may require higher dietary ARA (Ackman, 1982; Ogata et al., 2004).

The fatty acid profiles of the 45 DPH *S. splendidus* of different treatments largely reflected their diets, which is consistent with the majority of past studies (Rodriguez et al., 1994; Koven et al., 2001; Bransden et al., 2004). In particular, increasing dietary ARA level resulted in a concomitant increase in tissue ARA of *S. splendidus*. However, despite very similar EPA levels in *Artemia* enriched with different emulsions, from E-1 to E-5 treatment, fish tissue EPA significantly reduced with increasing ARA in their diet and resulted in sharp increase in tissue ARA/EPA ratio from 0.31 to 2.99. Similar effects of dietary ARA level on tissue EPA content have been reported in other marine fish larvae (Estévez et al., 1999; Bessonart et al., 1999; Harel et al., 2001; Willey et al., 2003; Bransden et al., 2004) and have been attributed to the competitive cellular interaction between these two fatty acids with a higher affinity of ARA to the cell enzymatic binding site out competing EPA (Bell et al., 1994). Similar decreasing trend of tissue LOA from E-1 to E-5 treatment was noticed and may also be ascribe to the increasing tissue ARA level. Both LOA and ARA are n-6 PUFAs, and excessive tissue ARA probably depressed or hindered the assimilation as well as accumulation of LOA (Bell and Sargent, 2003; Bransden et al., 2004). In opposite to tissue ARA level, the relative retention ratio of ARA showed clear decreasing trend and dropped sharply from 1.85 to 1.11 with increasing dietary ARA levels from E-1 to E-5

treatment (Tab. 8.6). This suggested that post-settlement *S. splendidus* were able to preferentially assimilate and accumulate ARA from their diets when their diets were deficient of ARA.

# Chapter 9

## General discussion and conclusions

### 9.1 General discussion

As reviewed in Chapter 1, with the current dependence of the marine aquarium industry on wild collected specimens, there is increasingly heightened concern that the marine ornamental industry is not sustainable unless steps are taken to limit destructive exploitation as well as to seek alternative way of supply (Olivotto et al., 2003). In order to conserve the fragile coral reef ecosystems and ensure the sustainability of the marine ornamental industry, an obvious and only option would be the development of captive breeding techniques for marine ornamental species to supply the increasing demands of the global markets (Moorhead and Zeng, 2010).

The green mandarin fish, *Synchiropus splendidus* (Herre, 1927), is among the most desirable species for marine aquarium hobbyists (Savody et al, 2001). As a pelagic-spawner, it represents a group of marine ornamental fish, including butterfly, Tang and angelfish, which are high-valued and also with high trade volume, but generally much more difficult to culture than those known as deposit spawners (e.g. clownfish, damselfish, blennies and gobies)(Moorhead and Zeng, 2010). It is hoped that *S. splendidus* could serve as a model species for marine pelagic spawning species for the development of captive breeding techniques. To this end, a series of experiments were conducted to develop an reliable and cost-effective larval feeding protocol that maximizing larvae survival and growth, and the key outcomes are summarized in Table 9.1.

**Table 9.1** Summarization of key results.

| Experimental chapter | Key results and recommendation |
|---|---|
| **Chapter 3** Insights into the importance of copepods as larval prey: effects of copepod density, rotifer co-feeding and larval ingestion rate | ● Larvae fed 10 rotifers $mL^{-1}$ alone had very low survival while copepod addition at 2 $mL^{-1}$ for co-feeding with the rotifers dramatically improved larval survival and growth. Additionally, co-feeding copepod at 1 $mL^{-1}$ could produce compatible results with those of 2 $mL^{-1}$.<br>● Co-feeding rotifers at copepod densities tested ranged from 0.1 to 1 $mL^{-1}$ did not produce significant beneficial effects on both survival and growth.<br>● The ingestion rates and electivity index calculated showed that the copepods were always positively selected over the rotifers by all larval ages under various co-feeding conditions.<br>● As co-feeding copepod at 1 $mL^{-1}$ could produce compatible results with those of 2 $mL^{-1}$ and co-feeding rotifers did not have any beneficial effect, a standard larval feeding regime of feeding copepods solely at 1 $mL^{-1}$ was recommended for *S. splendidus* early larvae culture. |
| **Chapter 4** Effects of timing for transition of prey from copepods to rotifers on larval survival, growth, development, feeding behavior and efficiency | ● If larval prey shifted from copepods to rotifers occurred earlier than 8 DPH, larvae survival and growth were significantly impacted.<br>● Despite prey switch on 12 DPH not impacting on survival and growth, larval development was significantly affected with a significantly lower percentage of larvae reached the stage of initial notochord flexion than that of the copepod feeding control on 15 DPH.<br>● Larval feeding behavior experiments showed that larvae of all ages attacked copepods more frequently than rotifers, and copepods were never rejected after being captured, whereas rejection following capture was commonly observed for rotifers. |

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| | • The feeding intervals (the time of digestive pause, which was defined as the time after a prey being ingested till the time a larva started next active searching for prey) on copepods by larvae of all ages were also significantly shorter than rotifers.<br>• Copepods should be provided for extended period for larval rearing of *S. splendidus* and possible replacement of the copepods by rotifers should only occur on 12 DPH or later. |
| **Chapter 5** Ontogenetic changes in the fatty acid composition during embryonic and larval development | • The copepods possessed a superior fatty acid profile than the rotifers as MUFA, PUFA and LC-PUFA contents as well as DHA/EPA ratio of the copepods better matched with the newly spawned eggs of *S. splendidus*.<br>• The relative conservation of EPA, but preferential utilization of DHA and ARA were observed during the embryonic development.<br>• 12 DPH *S. splendidus* larvae contained significantly lower percentage of ARA, EPA and DHA than younger larvae, indicating that dietary LC-PUFAs may become increasingly important with graduate depletion during early larval development. |
| **Chapter 6** Identifying the suitable time for introducing *Artemia* nauplii and subsequently enriched metanauplii in either live or dead form for larval rearing | • Larvae had their prey switched from copepods to *Artemia* nauplii directly on 18 and 21 DPH without a transitional rotifers feeding period had the highest survival.<br>• Introducing a rotifers feeding period too early on 12 DPH led to significant lower larval survival.<br>• Introducing live enriched *Artemia* metanauplii to replace the newly hatched *Artemia* nauplii on 25 DPH obtained the best larval survival and growth; the use of dead enriched *Artemia* instead led to significantly lower survival and growth.<br>• Based on results from previous experiments, the recommended larval feeding regimes for *S. splendidus* are: copepod feeding (1 $mL^{-1}$) between 2-17 DPH with *Artemia* nauplii introduced on 18 DPH (5 $mL^{-1}$) with live enriched *Artemia* (5 $mL^{-1}$) |

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| | introduced from 25 DPH onward. In the case shortages in copepod supply, an alternative feeding regime with a transitional rotifers feeding period (10 $mL^{-1}$) between 12 DPH to 20 DPH could be adopted. |
| **Chapter 7** The effects of graded dietary DHA (22:6n-3) levels on growth, survival and tissue fatty acid profile of the post-settlement green mandarin fish | ● Dietary DHA had no significant influence on the survival of 25-45 DPH *S. splendidus* (post-settlement) but significantly affected their growth<br>● Optimal dietary DHA level for growth of post-settlement larvae was suggested to be 6.91 mg/g DW based on SGR of standard length.<br>● It was indicated that the post-settlement *S. splendidus* might have a certain capacity to biosynthesize LC-PUFAs from precursors, particularly under the condition of a very low dietary supply of DHA. |
| **Chapter 8** The effects of graded dietary ARA (20:4n-6) levels on growth, survival and tissue fatty acid profile of the post-settlement green mandarin fish | ● The survival of 25-45 DPH *S. splendidus* was not significantly influenced by dietary ARA level.<br>● Dietary ARA did not significantly affected larval SL, but significantly affected larval body width and dry weight.<br>● Optimal dietary ARA level was suggested to be 7.29 mg/g DW for the highest growth of standard length. |

In addition to the main outcomes outlined in Table 9.1, further description of the outcomes as well as other major points/technical aspects are discussed in the following. Firstly, it was noticed that due to high sensitivity and vulnerability of *S. splendidus* pre-feeding larvae to handling and turbulence, it is recommended that they being handled with great care and only very gentle aeration being applied to minimize turbulence. In all the experiments, a small conical cup (30 mL) was used to transfer larvae, minimized the stress to the larvae. Very gentle aeration was also supplied through an air-lift inlet of the purposely-designed vessels (refer

to Chapter 2 for details), which is directed towards the water surface to minimize the negative effects of the turbulence on the fragile early larvae.

A series of feeding experiments described in Chapters 3, 4 and 6 combined to establish a complete optimal feeding protocol for *S. splendidus* larvae. Although a previous study showed significant improvements on larval survival and growth to 11 DPH by co-feeding copepods at 2 $mL^{-1}$ with rotifers (Ricketts, 2012), the production of copepods is still challenging as well as costly, it is hence necessary to determine the minimum level of the copepods needed for *S. splendidus* early larval culture. The results of the proposedly designed experiments in Chapter 3 showed that larval survival and growth of co-feeding 1 copepod $mL^{-1}$ with 9 rotifers $mL^{-1}$ were similar to the treatment of co-feeding 2 copepods $mL^{-1}$ with 8 rotifers $mL^{-1}$ while both treatments had a substantially higher larval survival than the treatments in which copepods were offered at lower densities of 0.5 and 0.1 $mL^{-1}$ for co-feeding with rotifers. A further experiment showed that when the copepods were fed at 1 $mL^{-1}$, co-feeding rotifers didn't actually provide any significant benefits (Chapter 3). As a result, feeding copepods solely at 1 $mL^{-1}$ was recommended for *S. splendidus* early larvae culture up to 11 DPH.

Considering copepods are often only indispensable during the first few days after mouth opening in fish larval rearing (Gopakumar et al., 2009; Olivotto et al., 2011), and there is an increasing trend on rotifer consumption as *S. splendidus* larvae grew older (Chapter 3), another experiment was designed and conducted to determine a suitable time for substituting copepods with rotifers for feeding *S. splendidus* larvae (Chapter 4). The result showed that different from many other

fish species, copepods should be provided for an extended period for larval rearing of *S. splendidus*, and replacement of the copepods by rotifers should only occur on 12 DPH or later. With the establishment of the feeding regime for *S. splendidus* early larvae, the study moved on to identify the time for transition to *Artemia* feeding for late larvae (Chapter 6). Experiments were conducted to determine: 1) the appropriate time for switching larval prey from copepods onto newly hatched *Artemia* nauplii with or without a rotifer feeding period; 2) the best time for transition from *Artemia* nauplii feeding to larger enriched *Artemia* metanauplii, which were provided in either live or dead form. The results found that prey switch to *Artemia* nauplii occurred on 18 and 22 DPH obtained the best survival and growth and including a rotifers feeding period from 12 DPH led to significantly lower larval survival on 25 DPH. Live enriched *Artemia* gave a superior results as compared to dead ones, and the introduction of live enriched *Artemia* metanauplii on 25 DPH gave a best performance in both survival and growth (Chapter 6).

Based on the results from above mentioned experiments, a complete larval feeding regime for *S. splendidus* was established: newly hatched larvae should be fed copepods at > 1 $mL^{-1}$ initially between 2-17 DPH; *Artemia* nauplii (5 $mL^{-1}$) can then be introduced to replace copepods from 18 DPH onward while live enriched *Artemia* metanauplii (5 $mL^{-1}$) can be introduced from 25 DPH onward. In the case of shortage in copepod supply, an alternative feeding regime with a transitional rotifers feeding period (10 $mL^{-1}$) between 12 DPH to 17 DPH could be adopted, which will result in lower but still acceptable survival.

As the superior quality of the copepod *P. crassirostris* as larval prey

compared to the rotifers became clear, it is important to gain insights into why the copepods performed better. Two prey selection experiments were conducted to investigate the larval feeding behavior and ingestion rate under different feeding conditions (Chapters 3 and 4). Rotifers have slower swimming velocity compared to copepods and generally do not show strong evasive response to predators (Checkly Jr, 1982; Meng and Orsi, 1991), which means early larvae with limited foraging ability are likely to be more efficient in capturing them. The feeding behavior experiment confirmed this and found that the capture rate (successful captures as % of attacks) on rotifers by *S. splendidus* early larvae ranged from 91.7% to 97%, which were substantially higher than that on copepods (31.3 to 56.2%). However, larval attack rate (attacks as % of targetings) on rotifers were dramatically lower than on copepods at all ages tested (6, 8, 10 and 12 DPH), suggesting larval positive selection on copepods over rotifers (Chapter 4). A similar result was obtained of the ingestion rate experiment (Chapter 3), in which electivity index calculated based on ingestion rate clearly elucidated that *S. splendidus* larvae of various ages (4, 6, 8 and 10 DPH) positively selected copepods over rotifers, including under the condition that rotifer density was almost 100 times higher than copepods (Chapter 3). The clear foraging preference and significantly higher ingestion rates on copepods by larvae help explain the superior performance of copepods over rotifers in *S. splendidus* larval culture.

As visual predators, better developed vision of *S. splendidus* larvae clearly will improve capture success. The nervous and sensory systems of fish contain high levels of DHA, and dietary DHA supply during the early life stages is considered highly important for the development of cognitive and sensory functions (Sastry, 1985). For example, Bell et al. (1995) reported a linear

relationship between dietary DHA and the number of rods in the retina of the herring, *Clupea harengus*. It has also been suggested that a reduced visual response in the halibut, *Hippoglossus hippoglossus,* larvae after being fed diets deficient in DHA (Hamre and Harboe, 2008). O'Brien-MacDonald et al. (2006) found that larval cod *Gadus morhua* fed rotifers with high-lipid contents exhibited higher activity level and attack rate on prey than those fed rotifers with low-lipid content and such difference was ultimately attributed to their dietary DHA level difference. On this basis, the totally lipid and fatty acid profiles of both the rotifer, *Brachionus rotundiformis* and the copepods, *P. crassirostris* used in larval feeding experiments were analyzed and compared (Chapter 5). The results showed that although total lipids of the two prey were similar, fatty acid profiles, especially essential fatty acids (EFAs), were substantially different. Among EFAs, the most striking difference was that the copepods contained almost 16 times higher DHA (22.32%) than that of the rotifers (1.41%) (Chapter 5). The high DHA level in the copepod likely contributed to a better developed vision and neural system of *S. splendidus* larvae and their performance.

Generally, lipid composition of marine fish eggs provides good clues to dietary lipid requirements for early larvae and it has been suggested that the larval diets should have a lipid composition similar to that of fish eggs (Sargent et al., 1999b). However, with the eggs and larvae of *S. splendidus* among the smallest of marine fish, and that *S. splendidus* broodstock generally produced only a few hundred eggs daily on average, it proved not an easy task to collected enough egg samples to perform lipid and fatty acid analyses, in fact it took a full two months of collection to amass enough egg samples (0.6 g DW) for the analysis. Similarly, it took another 70 days to collect enough samples of the pre-feeding larvae to

enable similar analyses. The fatty acid analysis results showed that the fatty acid profile of the copepods better matched with that of *S. splendidus* newly spawned eggs than rotifers, particularly for MUFA, PUFA and LC-PUFA contents as well as DHA/EPA ratio (e.g. DHA/EPA ratio: copepods: 4.1; newly released eggs: 4.7 but rotifers: 0.20)(Chapter 5). This results showed that in additional to as preferred prey selected by the larvae (Chapter 3), copepods' nutritional profiles are superior to rotifers and both these factors likely to contribute to copepods' overall superior performance in *S. splendidus* larval culture.

Comparing the fatty acid profiles of pre-feeding larvae with the newly-released eggs, it was found that unlike EPA, both DHA and ARA were heavily utilized during this period of early development prior to first feeding (DHA decreased from 15.35% to 7.95% and ARA from 7.13% to 3.94%, respectively), indicating dietary DHA and ARA supply might be crucial for subsequent larval development (Chapter 5). Thus, two more experiments were designed to investigate effects of dietary DHA and ARA on growth, survival and tissue fatty acid profile of post-settlement *S. splendidus* (Chapters 7 and 8). Both studies showed that larval survival was not significantly influenced by the dietary DHA or ARA level as no mortality was found even at very low DHA or ARA levels (i.e. 0.01% for DHA and 0.62% for ARA). High survival was also found in prey transition experiment (Chapter 6) with ca. 95% survival to 42 DPH when larvae were fed *Artemia* enriched with a commercial emulsion (Selco S. presso, INVE Aquaculture). Therefore, the critical period for *S. splendidus* larvae rearing appears to be very short, as high mortality only happened mainly during the first 8 days (Chapter 3 and 4). Based on the optimal feeding regime recommended for *S. splendidus* larvae, the overall larval survival from newly hatched larvae to 45

DPH is estimated to be around 38% (calculated based on larval survival data from Chapters 3, 4, 6, 7 and 8), suggesting it is a good candidate species for commercial production.

Despite survival of post-settlement *S. splendidus* was not significantly affected by dietary DHA and ARA level, their growth, another important criteria on marine ornamental captive breeding, was significantly affected. The results showed that the DHA or ARA deficiency could negatively affect the growth, while the excessive DHA or ARA could also lead to depressed growth of post-settlement *S. splendidus*. Based on the regressions between the specific growth rates and the dietary DHA or ARA levels, the optimal dietary DHA for the highest growth of standard length was estimated to be 6.91 mg/g DW, and the optimal dietary ARA was 7.29 mg/g DW (Chapters 7 and 8).

**9.2 Future directions**

Since this thesis confirms that copepods are indispensable for larval culture of *S. splendidus*, and to achieve high larval survival, copepods should be used as prey for an extended period during larval culture; hence ensuring copepod supply is important for commercial scale *S. splendidus* culture and future research efforts on copepod production could include: 1) Development of an efficient, reliable and cost-effective large scale intensive culture system for *P. crassirostris* to improve culture density and upscale the cultivation level to meet the demand for commercial production. 2) Identifying and developing culture techniques for other candidate calanoid copepods as potential prey for *S. splendidus* larval culture. Given huge species diversity of calanoids, it is highly possible other species could be identified as suitable prey for *S. splendidus* early larvae. Finally, the 3 L larval

vessels used in this study was designed specifically for laboratory experiments, a scale up efficient large culture system need to be designed and constructed based on similar principle for commercial production of *S. splendidus*.

It was shown that the inactive feeds did not trigger active feeding responses for post-settlement *S. splendidus;* while responded well to live enriched *Artemia*, the post-settlement larvae grew rather slow, which may be attributed to the sole enriched *Artemia* lacking some key nutrients, such as high quality amino acids (e.g. Taurine) and minerals (e.g. Selenium)(Hamre et al., 2008; Mæhre et al., 2013). Therefore, trialing benthic copepod species as the feed for the settled *S. splendidus* might help solve the slow-growth problem. In fact several benthic harpacticoid copepod species have already been successfully used for ornamental culture, including *Euterpina acutifrons* (Kraul et al., 1992), *Tisbe* spp. (Stottrup and Norsker, 1997; Olivotto et al., 2008a, 2008b, 2008c), and *Trigriopus japonicus* (Fukusho, 1980). Another possible way to enhance the growth of the post-settlement *S. splendidus* could be through possible compensatory growth, which is defined as accelerated growth rate after re-feeding following a period of food deprivation, as has been found in some fish (Hayward et al., 1997; Ali and Wootton, 2001).

This study investigated the effects of absolute ARA and DHA on the growth of post-settlement *S. splendidus*. However, the competitive interactions among LC-PUFAs (e.g. the synthesis of eicosanoids between ARA and EPA; the synthesis of LC-PUFAs between n-3 and n-6 fatty acid series) suggest that research should not only focus on the absolute dietary amounts, but also their proportions. Unfortunately, post-settlement *S. splendidus* generally didn’t

response well to inert foods, and if live *Artemia* are used as vehicle for such nutrition studies, the problem that *Artemia* naturally tend to convert DHA to EPA themselves would make it difficult to achieve specific DHA/EPA or ARA/EPA ratio. Moreover, considering saturated fatty acid (SFA) and monounsaturated fatty acids (MUFA) are commonly metalized to provide the "fuels" for fish growth (Tocher, 2003), future research effort should also be made on proper balance between LC-PUFAs and other energy-yielding fatty acids (the balance of dietary SFA:MUFA:PUFA ratios) for promoting better growth.

Based on the results from Chapter 7 of dietary DHA experiment, it was hypothesized that the post-settlement *S. splendidus* might have certain capacity to biosynthesize LC-PUFAs from C18 precursors, particularly under the condition of very low dietary supply of DHA. Further studies should be conducted to confirm this by identifying its possible LC-PUFA biosynthesis pathway at molecular level through cloning and functional characterization of fatty acyl desaturase that provide enzymatic machinery required for DHA biosynthesis from C18 precursors. The results of the dietary ARA experiment (Chapter 8) have also demonstrated the importance of ARA for the growth of post-settlement *S. splendidus*, but the intrinsic mechanism is still largely unknown. Different prostaglandins (e.g. $PGE_2$, $PGE_3$, $PGF_{2\alpha}$ and $PGH_2$) have been suggested to be involved in these regulation processes. Therefore, future research could exploiting the modulation of prostaglandins on growth as well as their relationships with dietary and tissue fatty acid composition, particularly for ARA, EPA and DHA.

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