# Growth and reproduction of *Cyclopina kasignete* and its application as a potential live food for fish larvae

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## Abstract

Copepods are common natural food for most fish larvae in the ocean, and fish larvae require live feed with adequate nutrition to be successfully reared in a hatchery. This thesis studied the reproductive biology, population growth and nutritional requirement of the cyclopoid copepod *Cyclopina kasignete* and further tested its use as a live food for fish larvae. Four experiments were performed to investigate (1) the improvements of algal nutritional quality by manipulations of nitrogen and phosphorus in the medium, (2) the beneficial use of dietary algae in modifying the nutritional content and digestive enzymes in copepods, (3) the potential use of enriched algae to improve the population growth and reproduction of copepods, and (4) the use of copepod as live food for feeding larval fish, the Asian seabass *Lates calcarifer*.

In experiment 1, algae cultivated with N:P ratios of 20:1 and 30:1 showed favorable algal growth and increase in protein content, while the high N:P ratio (120:1) reduced algal growth and protein content but increased lipid contents in algae. The N:P ratio of 20:1 increased the EPA content in *Nannochloropsis oculata* while the N:P ratio of 30:1 increased the DHA content in *Tisochrysis lutea*. The knowledge of using appropriate N:P ratios for algal culture in this study can be applied in a hatchery to produce algae with superior quality which in turn enhances nutritional profiles in copepods.

In experiment 2, the highest protease and trypsin enzymes in copepods were detected when fed with dried *Melosira* sp. and fresh *T. lutea*. The amounts

of fatty acids and digestive enzymes in copepods can be modified by feeding the copepod with different dietary algae because even with low contents of EPA, DHA and ARA in the dried *Melosira* sp. and mixed dry algae, *C. kasignete* showed higher EPA, DHA and ARA when fed these corresponding diets than other diets.

In experiment 3, the diatom *Melosira* sp. was used as either a single or a mixture with other algae to sustain the growth and reproduction of *C. kasignete*. The superior diets (dried *Melosira* sp., fresh *N. oculata*, and fresh *T. lutea*) were tested as a single or in a combination diet in the copepod culture. The *C. kasignete* produced maximum growth and offspring production when fed the monoalgal diet of dried *Melosira* sp. or the binary diets of dried *Melosira* sp. and fresh *T. lutea*. Experiments 2 and 3 showed that *C. kasignete* contained high EPA, DHA, protease and trypsin after feeding on enriched algae and these nutritional components are essential for fast growth and reproduction in copepods.

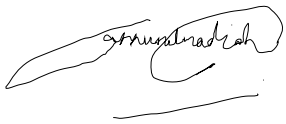
These findings on copepods led to the study of experiment 4 where *Lates calcarifer* larvae were fed with conventional live foods rotifers and *Artemia* along with *C. kasignete*. Larval fish showed better growth and survival when fed with live foods with copepod supplementation, suggesting that the improved nutritional profiles in copepods enhanced the growth and survival of fish larvae. Experiment 4 clearly indicates that the use of this copepod for Asian seabass culture can improve fish growth, survival and fatty acid contents of fish larvae.

In summary, the manipulation of nitrogen and phosphorus ratios in the

culture media can improve the quality of algae as a live food for copepods, which in turn can improve the fatty acids and digestive enzymes in copepods. Copepods can then provide the necessary nutrients to larval fish to improve the growth and survival of fish larvae.

# Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for degree or diploma in any university; and that to the best of my knowledge and belief it does not contain material previously published or written by another person except where due reference is made in the text.



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# Chapter 1

# General Introduction

## 1.1. Importance of copepods as live feed for fish larvae

Copepods are live food and contain a high level of fatty acids required by marine fish larvae. It is superior in terms of nutritional value compared to traditional live food species such as *Artemia* nauplii and rotifers, which are known to be nutritionally inadequate for some species of marine fish larvae (Rasdi & Qin 2014; Ajiboye *et al.* 2011; Conceicao *et al.* 2010). In addition, copepods also contain essential digestive enzymes required by fish larvae for digestion (Zaleha *et al.* 2012). Rotifers and *Artemia* have been commonly used as prey during this early but critical period in life history of fish, but do not always support optimal growth and survival in fish larvae (Saboor 2012). In the wild, copepods constitute as the major linkage in the nutrient pathway from primary producers to marine fish larvae (Ohs *et al.* 2010). The suitable nutritional profile in copepods contributes to growth, health and survival of marine fish larvae (Alajmi & Zeng 2015).

Larval rearing is a key step for the development of industrial marine aquaculture in most fish species such as Asian seabass *Lates calcarifer* (Rajkumar & Kumaraguruvasagam 2006), groupers *Epinephelus septemfasciatus* and *E. marginatus* (Russo *et al.* 2009; Sakakura *et al.* 2007), yellowtail kingfish *Seriola lalandi* (Chen *et al.* 2006) and mutton snapper *Lutjanus analis* (Benetti *et al.* 2002). The main issue faced by marine fish hatcheries is high mortality due to the fragility of fish larvae in the early stage of their development aggravated by insufficient nutrients in diets provided for them (Barroso *et al.* 2013). Fish larvae undergo dramatic morpho-physiological changes (Chen *et al.* 2006; Yúfera & Darias 2007) in its early stages to facilitate the capture of prey and assimilation of nutrients (Rasdi & Qin

2014). It is well documented that live feed organisms are more preferential than artificial feeds in larvae and early post larvae of various species of fish and shellfish (Das *et al.* 2007; Barroso *et al.* 2013). Rotifers, *Artemia* and cladocerans such as *Moina* sp. and *Daphnia* sp. are nutritionally incomplete and these live foods could cause high mortality in larval rearing of some fish species (Alvarez *et al.* 2002).

The use of copepods as live feed for marine fish larvae has been reported to improve growth, survival and pigment development compared with conventional live food, e.g., rotifers and *Artemia* (Lee *et al.* 2013; Mahjoub *et al.* 2013). Consequently, several studies have been carried out on copepod mass culture (Schipp *et al.* 1999; Payne & Rippingale 2001; Ajiboye *et al.* 2011). In the wild, copepods are usually the most frequent prey for marine fish larvae (Turner 2004; Ajiboye *et al.* 2011). Based on a few documented cases on rearing southern flounder *Paralichthys lethostigma* (Wilcox *et al.* 2006*),* halibut *Hippoglossus hippoglossus* (Shields *et al.* 1999) and pipefish *Stigmatopora argus* larvae (Payne *et al.* 1998), hatchery operators have largely depended on *Artemia* nauplii and rotifers as live food but these diets may result in low fish growth and poor survival in the early stage of larval fish culture. On the other hand, the introduction and provision of copepods as a live food can increase survival in fish larvae such as *Amphiprion clarkii*, *Scophtalmus maximus*, *Pagrus auratus*, *Centropomus parallelus*, and the results are shown in Figure 1 (Witt *et al.* 1984; Payne *et al.* 2001; Olivotto *et al.* 2008; Barroso *et al.* 2013). The major problem with intensive production of copepods is the long generation time and difficulty for mass culture (Conceicao *et al.* 2010). Species of copepods with a shorter

generation time and high tolerance to temperature and salinity changes are ideal for mass culture (Payne & Rippingale 2000; Stottrup 2006).

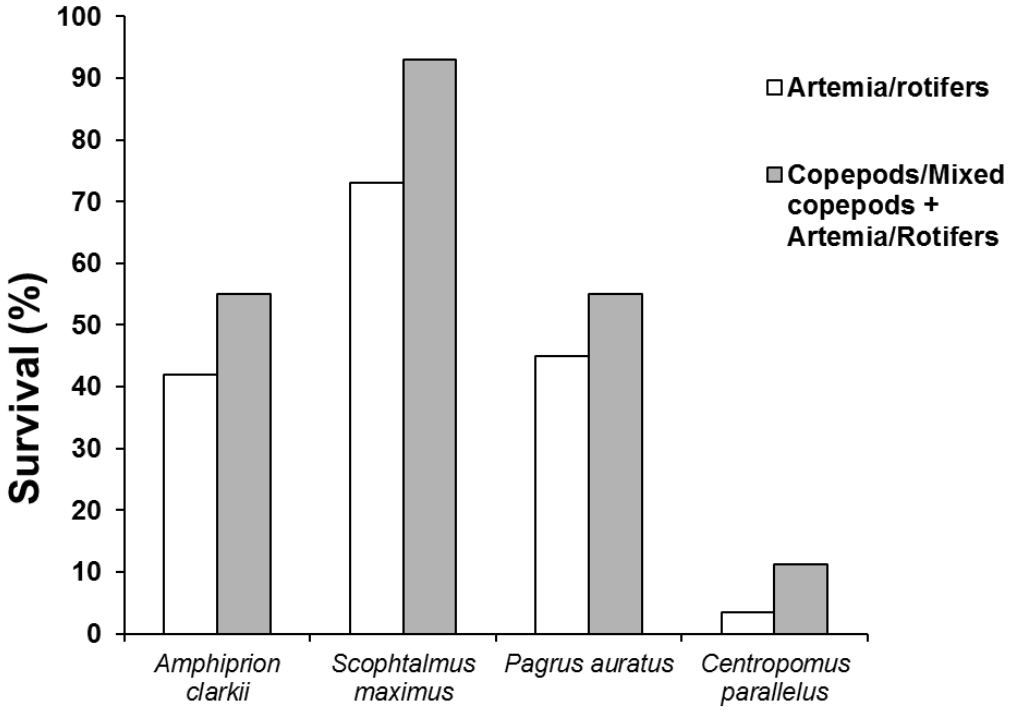


**Fig. 1** The survival of some fish species (*Amphiprion clarkii, Scophtalmus maximus, Pagrus auratus* and *Centropomus parallelus*) fed on conventional diets of rotifers and *Artemia* compared to the diet with copepod inclusion (Witt *et al.* 1984; Payne *et al.* 2001; Olivotto *et al.* 2008; Barroso *et al.* 2013).

## 1.2 Improvement of copepod nutrition

*Algae as the primary food for copepods*

One of the major questions in the advancement of copepod mass cultivation is the wide-ranging dietary requirements of different copepod species (Buttino *et al.* 2009). In general, copepods feed on live microalgae and yeast in most mass culture

conditions (Zaleha *et al.* 2012; Rasdi & Qin 2014). However, the selection of suitable microalgae species is important, as the nutritional profile differs between algal species. In fish hatcheries, the culture of copepods relies on a variety of algal species such as *Tetraselmis suecica*, *Tisochrysis lutea* (old name *Isochrysis* affinis *galbana*, or T-ISO), *Rhodomonas salina*, *Chaetoceros muelleri* and many others (Lee *et al.* 2006; Milione & Zeng 2007; Calbet *et al.* 2001). Copepods feeding on yeast usually result in low polyunsaturated fatty acid (PUFA) contents compared to feeding on fresh algae, thus making yeast an unfavorable diet for copepods (Hazel *et al.* 2011).

Many types of algae have been offered to copepods as food, but there is a need to identify algae with a high content of essential nutrients such as fatty acids. PUFAs are indispensable nutrients that are directly involved in many key processes of marine copepods such as development and reproduction (Chen *et al.* 2012). Providing good nutrition to copepods is an effective way to transfer nutrients to larval fish and can coincidentally improve fish survival and growth. Another concern with fresh algal diets is the difficulty in maintaining an adequate supply due to algal culture crash (Mostary *et al.* 2007). Algae need to be carefully maintained in order for it to reach substantial biomass to feed copepods. Therefore, there is a need to further consider the use of dried algae in the place of fresh algae especially during the time of algae culture crashes. In order to consider dried algae for copepod feeding, however, further study is needed to assess its impact on copepod nutrition. Once this information is gathered, we can then decide whether dried algae can be used as an efficient substitution for fresh algae to feed copepods.

*Enrichment of microalgae*

Several enrichment procedures such as oil emulsion have been applied on rotifers and *Artemia* nauplii to improve the nutritional value of these live feeds (Haché *et al.* 2011). However, the enriched rotifers and *Artemia* nauplii still do not meet the nutritional demand of some fish larvae and could not challenge the quality of nutrition provided by copepods (Hamre *et al.* 2008). Since provision of copepods as food can increase larval fish survival, there is a need to enrich copepods to make it as a superior live food to improve larval fish performance. However, unlike *Artemia* and rotifers, copepods could not be enriched using chemical formulas as copepods will induce rejection towards any form of chemical stimuli in the medium. Therefore, the existing enrichment techniques on most other live foods could not be applied to copepod enrichment (Kleppel 1993). Thus the best solution for copepod enrichment is by changing copepod nutrition through manipulation of its food, i.e., algae. Copepods use chemical and mechanical receptors on their feeding appendages to identify potential prey and to determine whether to ingest or reject the captured particles (Paffenhofer & Van Sant 1985; Price *et al.* 1983). The intensity of a particular stimulus may depend, not upon the total food density, but rather upon the density of specific elements that elicit a feeding response (Poulet & Marsot 1982). For example, copepods can differentiate between nutritionally enriched and unenriched microcapsules (Poulet & Marsot 1978) and between nutritionally distinct forms of a single algal species (Cowles *et al.* 1988).

The key to enrich copepods is through enriching its primary food source which is the microalgae. The nutrition content of copepods needs to be enhanced first

before feeding to fish larvae. Nutritional manipulation on algal diets is necessary to ensure that copepods are enriched with important PUFAs that are vital to connect the nutritional link between copepods and fish larvae. Algal nutritional composition is essential for mass culture of copepods (Plath & Boersma 2001). The quality of food available to copepods varies with the composition of phytoplankton community and its nutritional composition (Tirelli & Mayzaud 2005; Mitra & Flynn 2005). Understanding the method to enhance copepod reproduction and fecundity through modification of algal quality is necessary to improve copepod production.

Nitrogen and phosphorus are known to alter the biochemical composition and particularly the lipid content of algae (Ahlgren & Hyenstrand 2003; Sharma *et al.* 2012). In some algae, nitrogen limitation can increase the lipid content of microalgae (Sharma *et al.* 2012). However, most studies have only emphasised the change of overall lipid production through nutrient manipulation, but not specifically on the change of PUFA composition in algae. Since *Tisochrysis lutea* and *Nannochloropsis* sp. contain high EPA and DHA, respectively (Rasdi & Qin 2015), further study is needed to test the effects of different nutrient ratios on fatty acid levels of microalgae, particularly the EPA and DHA ratio, and to test whether *Tisochrysis lutea* and *Nannochloropsis* sp. have the ability to produce more EPA and DHA in the cells at different nitrogen and phosphorus supplies. Development of the method of N:P ratios is expected to produce algae containing desired nutritional values as food for copepod production.

*Influence of nutrition on growth and reproduction of copepods*

Previous studies have found that copepods are difficult to be mass cultured due to several factors related to food quality and environmental conditions that are prerequisite for copepod growth and reproduction. Food quality is the key factor regulating copepod nutrition and production (Kleppel & Burkart 1995). Previous studies have supported the notion that copepod production is influenced by attributes associated with the types of food eaten, and significantly influenced by the diet that can meet the nutritional needs of copepods (Kleppel & Burkart 1995).

The production of zooplankton depends on the biochemical composition of microalgae fed to them and the type of diet will affect its population growth and reproductive capacity (Khatoon *et al.* 2013). The long generation time of some copepod species coupled with poor understanding of its nutritional requirements renders many copepod species inappropriate for a large scale mariculture (Conceicao *et al.* 2010). Presently, exploration for copepod culture has been experimented on several species such as *Acartia* sp. (Broglio *et al.* 2003), *Euterpina acutiferons* (Da Costa *et al.* 2005), *Paracyclopina nana* (Lee *et al.* 2012) and *Parvocalanus crassirostris* (Alajmi & Zeng 2015) and the results are promising for fish larviculture applications. Currently, the search continues for copepods with favourable features for mass culture that are also high in food quality for mariculture applications. Exploitations for additional copepod species with short generation time, small body size, and flexible food requirements with ability to bioconvert essential fatty acids from food are needed for utilization in fish mariculture. The copepod *Cyclopina kasignete* is a common species along the coast of Arno Bay in South Australia, which

has been caught from the wild for larval fish rearing in a hatchery. In this thesis, *C. kasignete* was studied to understand its nutritional requirement and explore its potential as a live food source for fish larvae. The taxon details are:

Phylum: Arthropoda

Subphylum: Crustacea

Superclass: Multicrustacea

Subclass: Copepoda

Infraclass: Neocopepoda

Superorder: Podoplea

Order: Cyclopoida

Family: Cyclopinidae

Genus: *Cyclopina*

Species: *C. kasignete*

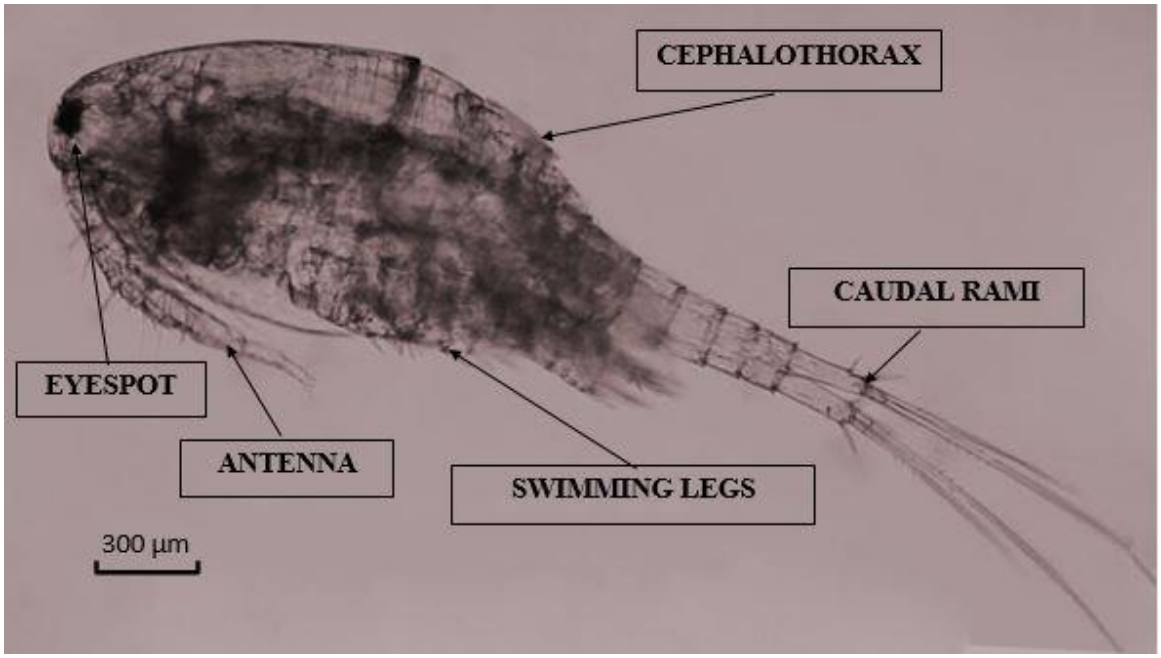


Fig. 2. Illustration of the copepods *Cyclopina kasignete* (length: 0.3 mm)

### 1.3 Digestive enzymes in copepods

Copepods contains essential digestive enzymes (Zaleha *et al.* 2012) that contribute to improving growth and nutrient assimilation in larval fish (Kolkovski 2001). However, studies investigating the level of digestive enzyme secretion by copepods remain scarce with only several reports on traditional live food species such as rotifers and *Artemia* (Naz 2008; Naz & Yufera 2012). Information on the excretion of enzymes in response to the food composition in copepods is insufficient. Previous studies on turbot *Scophtalmus maximus* and Atlantic herring *Clupea harengus* larvae indicate that copepods not only contribute their digestive enzymes (protease and trypsin) but also activate zymogens in the gut of fish larvae (Pedersen & Hjelmeland 1988; Munilla-Moran *et al.* 1990; Sun *et al.* 2013). Fish larvae utilize digestive

enzymes from their live prey to facilitate the process of digestion until the larval alimentary system is functionally developed (Kolkovski 2001). It is vital to examine the response of protease and trypsin in copepods towards enriched algae to assess the significance of using algal diets with high nutritional values for practicality in intensifying copepod nutrition. In such a feeding strategy, fish can utilize digestive enzymes from live feed to aid digestion of formulated feed (Kolkovski *et al.* 1997; Kolkovski 2001; Zhao *et al.* 2013). Fish larvae use enzymes from live prey to assist the digestive activity until the larval alimentary system is entirely functional and able to independently provide digestive enzymes to digest formulated feed (Kurokawa *et al.* 1998; Lazo *et al.* 2000; Jancarik 1964). It is remarkable that the enzyme activities in copepods are influenced by the types of algae in the food and copepod species (Freese *et al.* 2012). Since the growth and reproduction of copepods rely on food quality (Freese *et al.* 2012), it is necessary to scrutinize the effects of nutritional compositions of microalgae on fatty acids and digestive enzymes in copepods.

## 1.4 Study objectives

Fish larvae require live feed with adequate nutrition to be successfully reared in a hatchery and therefore it is vital to produce live food with high nutritional quality to feed fish larvae. The nutritional requirements of the copepods have to be fully understood to sustain their growth if they are to be produced on a massive scale. Therefore, understanding the mechanism controlling egg production in copepods based on its nutritional requirement allows better selection for algal species in aquaculture and their optimal density for copepod culture. This thesis aims to improve copepod nutrition by modifying algal quality as food for copepods, which in

turn can improve the nutritional value and digestive enzymes. Copepods with superior quality in nutrition are prerequisite for larval fish feeding, and improved copepod nutrition will be essential for larval growth and survival in hatcheries.

In a separate review paper (Chapter 2), I have reviewed the recent literature on the nutritional requirement of larval fish and live food enrichment, nutritional comparison between copepods and other conventional live food organisms, current culture practices in copepods, and alteration of nutrient composition of copepods through manipulation of copepod food such as microalgae enrichment before they are fed to fish larvae. Based on the information in my literature review and background information provided in this chapter, the specific objectives of this thesis are stated as follows:

1. To identify whether algae nutritional profiles are affected by the nitrogen to phosphorus (N:P) ratio in the culture medium;
2. To identify suitable algal types to improve fatty acids and digestive enzymes in copepods;
3. To identify whether the growth and productivity of copepods are affected by the mono or binary algal species in diets;
4. To identify the potential use of copepods as live food supplementation to improve the performance of larval fish.

## 1.5 Research approach and thesis organization

In this study, four trials were conducted to achieve the above aims. Specifically, these four experiments include:

1. Effect of N:P ratio on growth and chemical composition of *Nannochloropsis oculata* and *Tisochrysis lutea*, which addressed aim 1 and is presented in chapter 3.

2. Effects of dietary microalgae on fatty acids and digestive enzymes in the copepod *Cyclopina kasignete*, a potential live food for fish larvae, which addressed aim 2, and is presented in chapter 4.

3. Impact of food type on growth, survival and reproduction of the cyclopoid copepod *Cyclopina kasignete* as a potential live food in aquaculture, which addressed aim 3 and is presented in chapter 5.

4. Copepod supplementation as live food improved growth and survival of Asian sea bass *Lates calcarifer* larvae. This trial addressed aim 4 and is presented in chapter 6.

The thesis is presented in seven chapters including a general introduction, a literature review, four data chapters and general discussion.

Chapter 1 is the general introduction to the thesis outlining the need of using copepods to feed fish larvae, mechanisms to enhance copepod nutrition through algal enrichment, and the way how nutrition can positively influence the growth and reproduction of copepods. In addition, the introduction outlines the significance of using digestive enzymes in copepods for larval fish digestion. A more in-depth examination of these issues is covered by a literature review in Chapter 2.

Chapter 2 is a literature review on the basic principle of nutritional requirement, feeding biology of copepods and the use of copepods for larval fish rearing in aquaculture hatcheries. This chapter has been published in Aquaculture Research (Rasdi & Qin 2014). This chapter outlines the knowledge on the nutritional

requirements of copepods and potential enrichment procedures for algae as well as evaluation on the nutritional supply of algae to copepods. In addition, this chapter illustrates the current copepod culture practices and demonstrates the current live food enrichment methods in aquaculture. Based on this information and the knowledge gaps identified in the literature review, this thesis focused on improving copepod nutrition for supplementation as live feed for fish larvae.

Chapter 3 studies the impacts of N:P ratios on the growth, elements, lipid, fatty acids and protein contents of *Tisochryris lutea* and *Nannochloropsis oculata.* This chapter has been published in Journal of Applied Phycology (Rasdi & Qin 2015). This chapter adressesed aim 1, based on data from Trial 1. The purpose of this chapter was to identify the optimal N:P ratios for cultivation of *N. oculata* and *T. lutea* and improve biochemical composition in these two algae.

Chapter 4 studies the responses of fatty acids and digestive enzymes in copepods to different dietary algae. This chapter has been published in Aquaculture Research (Rasdi *et al.* 2015). This chapter addresses aim 2, based on the data from Trial 2. The algae cultivated from the optimized nutrition regime in Chapter 3 (Trial 1) was further tested as live food to feed copepods in Chapter 4.

Chapter 5 studies the effects of food type on growth, survival, and reproduction of the copepod *Cylcopina kasignete*. This chapter has been submitted to Aquaculture Research and is currently under review. This chapter is a continuation of Trial 2 in Chapter 4, examining aim 3 based on the data from Trial 3. The copepods produced high quantity of essential fatty acids and digestive enzymes after feeding on dry *Melosira* sp., fresh *N. oculata*, and fresh *T. lutea* as indicated in Chapter 4.

Therefore, a follow up trial was conducted using these algae either as a combination or as a single diet to further evaluate the growth and reproductive performance of the copepod *Cyclopina kasignete*.

Chapter 6 examines the potential use of the copepod *Cyclopina kasignete* as a supplement to improve larval fish performance where the Asian sea bass larvae were used as the test fish. This chapter is now ready for submission to Aquaculture Research. This chapter addresses aim 4, based on data from Trial 4.

Chapter 7 is the general discussion, which compiles and summarizes all the major research outcomes. This chapter outlines the substantial and practical outcomes and implementation for the copepod culture that has arisen from this baseline nutritional research. Recommendations are provided to improve copepod nutrition, which is essential towards building a more sustainable and economically efficient protocol for cultivating copepods in aquaculture hatcheries. Further research is also suggested to address issues that have not been resolved in this thesis.

Four independent studies form four data chapters (3, 4, 5 and 6) and each one is presented as an independent manuscript suitable for publication in different journals. Consequently, some repetition of background and methods may be found among chapters. Although independent objectives are specified in each chapter, they are all complementary to each other towards the overall thesis objectives stated in this introduction chapter. All of the chapters were performed by myself as the author of the present thesis under the supervision of my principal supervisor. However, my supervisors Prof. Jian Qin and Dr. Yan Li are also listed as co-authors in published papers to indicate their substantial contribution to my PhD research.

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# Chapter 2

## Improvement of copepod nutritional quality as live food for aquaculture: a review

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**Abstract**

In hatchery, an adequate supply of live food for first feeding fish larvae is essential and nutritional quality of live food organisms can be improved through nutrient enrichment. The use of live food organisms, especially at first feeding, is a requisite for most marine fish larvae. In ocean, marine fish larvae primarily feed on copepods, but the production protocols of copepods as live food is underdeveloped in hatchery. As the food ingestion and the digestive system of copepods are different from other live food organisms (e.g., rotifers), the nutrition enrichment procedures with emulsion oil used in rotifers is not effective on copepods. This review focuses on alteration of nutrient composition of copepods through manipulation of copepod food before they are fed to fish larvae. Specifically, we discuss the relationship between the changes of fatty acid compositions in dietary algae and in copepods. The review links nutrient supply to copepods and the change of nutrition in copepods and suggests ways to improve copepod nutrition in hatcheries.

**Keywords**; copepods; fatty acids, copper, selenium; vitamins; algae; enrichment; fish larvae.

## Introduction

As the expansion of mariculture has become increasingly important as a means to meet the protein supply for human consumption, the development of novel technologies for fish farming is urgently needed (Camus & Zeng 2009; Chullasorn *et al.* 2011). In aquaculture hatcheries, the use of live food is essential for successful larval fish rearing. Conventional live foods, such as rotifers (*Brachionus* spp.) and brine shrimps (*Artemia* spp.) provide sufficient nutrients for finfish and crustacean larvae in most cases (Jepsen *et al.* 2007). However, the commercially available live food organisms may not be suitable for the size and nutrition required by certain fish species such as bluefin tuna (De Lima *et al.* 2013; Solgaard *et al.* 2007) as these fish larvae primarily feed on planktonic copepods that are not usually available as commercial live food in most hatcheries (VanderLugt & Lenz 2008). The use of copepods as supplemental live food can improve the survival for a variety of fish larvae (Drillet *et al.* 2006; Rajkumar & Kumaraguruvasagam 2006; Sipaúba-Tavares *et al.* 2008; Camus & Zeng 2009; Jobling 2016). Copepods, therefore, have been considered a promising candidate as live food for the culture of marine fish species in hatchery (Qin 2013).

In the early life stage of most fishes, live food is essential for fish larvae as the digestive system of fish larvae is functionally immature to process inert diets (Chen *et al*. 2006b). In larval fish rearing tank, the movement of the live food organisms can stimulate fish feeding behaviour (Kolkovski 2001) and the high water content in various sized live food organisms makes them easy to be digested by marine fish larvae (Wittenrich *et al.* 2007; Conceicao *et al.* 2010). Since fish

larvae directly consume live zooplankton in the wild, live food organisms have been routinely used in aquaculture to feed fish larvae. To date, the production of high quality fish larvae is constrained by a bottleneck of poor fingerling quality in many commercial marine hatcheries (Zaleha *et al.* 2012). More specifically, inadequate nutritional supply has caused low survival and high deformity in fish larvae (Rajkumar & Kumaraguruvasagam 2006). In intensive-rearing systems, first-feeding marine fish larvae are most commonly fed on rotifers *Brachionus* spp. with a range in lorica size from 100 to 340 μm (Theilacker & McMaster 1971; Støttrup 2000), followed by larger sized *Artemia* nauplii in a later developmental stage. The brine shrimp cysts from a large variety of strains vary in size, energy content and nutritional quality and the newly hatched *Artemia* nauplii measure 422 - 517 μm in length (Sorgeloos *et al*. 2001a). However, rotifers and *Artemia* do not fulfil all the nutritional requirements by some marine fish larvae even after nutrient enrichment on live food.

The importance of copepods in aquaculture has long been recognized, especially in the larval rearing of many marine fishes (Lee *et al.* 2006). However, to develop a suitable live food species in aquaculture, it is necessary to understand the biology of the live food organism. Particularly, as a potential live food, the biological aspects of copepods in relation to nutritional needs should be clearly understood to ensure successful culture and boost live food production (Pinto *et al.* 2001). In copepod culture, the mass production skill offers the possibility to produce a large number of individuals under a given condition of temperature, food supply, and water quality (Farhadian *et al.* 2008). To maintain a sustainable

copepod population, it is essential to identify the prominent factors regulating the population dynamics of copepods, particularly the nutritional requirements of copepods. The high contents of eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), and some digestive enzymes in copepods are the important properties to make copepods as a superior live food to *Artemia* and rotifers (Shields *et al.* 1999; Drillet *et al.* 2006; Drillet *et al.* 2011). Since most attempts of copepod culture are focussed on the quantity of mass production, the articulation of nutritional requirement of copepod remains less known (Drillet *et al.* 2011). Due to species variation of algae as a food for copepods, the nutritional value of copepods is less predictable (Støttrup 2000). Fortunately, the nutritional composition of copepods can be manipulated through feeding them with different algal species to meet the nutritional demand of a particular fish species (Hernández Molejón & Alvarez-Lajonchère 2003).

Recent studies have focused on substitution of live food by formulated diets (Cahu & Zambonino Infante 2001; Carneiro *et al.* 2003; Chen *et al.* 2006), but low digestibility and nutritional quality of formulated diets are not likely to be a stand-alone starter food for fish larvae (Carneiro *et al.* 2003). The composition of highly unsaturated fatty acids in live foods such as rotifers and *Artemia* can be changed by artificial enrichment to pack the live food with nutrients required by fish larvae (Levine & Sulkin 1984; Chen *et al.* 2000; Zhou *et al.* 2008). However, little information to date is available on protocols to enrich copepods with nutritional and trace elements to meet the requirement for growth and health of fish and crustacean larvae (Sun *et al.* 2013).

The aim of this review is to provide further understanding into the nutritional requirements of copepods using different algal diets to improve the nutritional composition of copepods and make them nutritionally suitable for larval fish. Furthermore, the interaction between the alteration of nutrient composition of algae and the changes of fatty acids in live food organisms is also reviewed. This review focuses on the basic principle of nutritional requirement, the feeding biology of copepods and the application of copepods for larval fish rearing in hatchery. Specifically, we (i) review the nutritional requirement of larval fish and live food enrichment, (ii) discuss the nutritional composition of copepods, (iii) evaluate the nutritional requirements of copepods and nutritional supply of algae to copepods, (iv) illustrate the current culture practices in copepods, and (v) demonstrate the current live food enrichment methods in aquaculture.

## 2.1 Nutritional requirement of larval fish and live food enrichment

Marine fish larvae in intensive culture are typically fed on traditional live foods such as rotifers and *Artemia* nauplii (Soroy 2012) but unfortunately these organisms are naturally deficient in some HUFAs (Rajkumar & Kumaraguruvasagam 2006), and the growth and survival responses to enriched live food depend on fish species (Ma & Qin 2012). Furthermore, as the potential shortage of *Artemia* cysts may affect hatchery production and threat sustainability of fish fingerling supply for further grow-out, there is a strategic need to diversify live food species to reduce the heavy reliance on *Artemia* nauplii in hatchery

(Lavens & Sorgeloos 2000). In larval fish rearing, copepods have been tried as a live food (Qin 2008), but low production of copepods (Støttrup 2000) has been a major concern in copepod mass culture (McKinnon *et al.* 2003; Gopakumar *et al.* 2009). Studies have shown that the addition of copepod as a live food can enhance growth and survival of first feeding fish larvae compared to the diets consisting solely of rotifers and *Artemia* (Watanabe *et al.* 1983; Stottrup *et al.* 1999; Shields *et al.* 1999; Payne & Rippingale 2001). Furthermore, the high natural ω-3 fatty acid profile in copepods compared to other live food organisms has driven researchers' motivation on copepod culture in recent years (Evjemo *et al.* 2003). Copepod is one of the most abundant and important components of aquatic invertebrates that link the primary producers and consumers in many marine and freshwater ecosystems. As copepods have coevolved with fish larvae in the ocean, the use of copepods for marine larvae is likely to meet fish nutritional requirements.

Because of small size and a poorly developed digestive system, most marine fish larvae are unable to ingest or digest pelleted or microencapsulated diets at first feeding (Segner *et al.* 1994; Czerniawski *et al.* 2015). The common live-prey species in aquaculture are rotifers (*Brachionus plicatilis*) and brine shrimp nauplii (*Artemia* spp.), but these organisms contained limited types of long-chain HUFA, especially DHA (Bell *et al.* 1995). These live foods must be enriched with specific fatty acids to meet the nutritional requirement of marine fish larvae. Several enrichment techniques have been developed, including oil-based emulsions and microencapsulated preparations (Barclay & Zeller 1996;

Shields *et al.* 1999; Sorgeloos *et al.* 2001b). However, most commercially available enrichment products are unable to provide the appropriate DHA/EPA ratios required by different fish species (Shields *et al.* 1999; Navarro 1999; (Zhang *et al.* 2015). Thus, the efficacy of using live food to deliver sufficient DHA to fish larvae remains a matter of concern.

Nutritional imbalance may impede normal morphogenesis and skeletogenesis at early stages and deficiency of some essential nutrients can affect fish normal development (Cahu & Zambonino Infante 2001; Lall & Lewis-McCrea 2007; Boglino *et al.* 2012). Dietary lipids are particularly important for early development of marine finfish larvae (Bell & Sargent 2003) because they represent the main energy source for larvae (Izquierdo *et al.* 2000; Cahu *et al.* 2003; Lall & Lewis-McCrea 2007). Moreover, the intake of HUFA from the diet is the only way for marine fish to obtain EFA from the n−3 fatty acid series, such as EPA, DHA and ARA (arachidonic acid, 20:4n−6) since fish larvae are unable to synthesize these fatty acids from their precursors 18:3n−3 (linolenic acid) and 18:2n−6 (linoleic acid) (Bell *et al.* 2002).

The improvement of pigmentation success in turbot larvae (*Scophthalmus maximus*) is positively correlated with the increasing dietary and tissue ratio of DHA/EPA (Reitan *et al.* 1994; Kjørsvik *et al.* 2003) . The ratios of DHA/EPA in copepod nauplii and copepodites are significantly greater than in enriched *Artemia* nauplii. Consequently, the DHA/EPA ratios in the eye, brain and liver of halibut larvae fed copepods are much greater than in those fed *Artemia* (Reitan *et al.* 1994). In addition to the benefit of maintaining a high DHA/EPA ratio in live-

prey and larval tissues, there exists the potential importance of dietary phospholipid and vitamin A in preventing pigmentation abnormalities in fish larvae (Estevez & Kanazawa 1995). For instance, the naupliar and copepodite stages of *Eurytemora velox* contain an excess of phospholipids over triacylglycerols, whereas the opposite composition occurs in enriched *Artemia* (Shields *et al.* 1999). Evidence suggests that larval fish can digest phospholipids more effective than triacylglycerols and the presence of phospholipids facilitates digestion of other lipids in the rudimentary digestive tract of fish larvae (Koven *et al.* 2001; Sargent *et al.* 2002).

Enrichments of rotifers and *Artemia* have been done to enhance the nutritional value, but the results for the culture of certain marine fish larvae such as southern bluefin tuna are not encouraging (Drillet *et al.* 2011; Mæhre *et al.* 2012). Previously, live foods are enriched using the oil emulsion method where live food containing various amounts of DHA and/or EPA is usually prepared using emulsified oils including DHA ethyl ester, EPA ethyl ester, and corn oil (Satoh & Takeuchi 2009; Satoh *et al.* 2009; Li *et al.* 2015). Commercial enrichment products and methodologies have been formulated for *Artemia* nauplii and rotifers (Agh & Sorgeloos 2005; Loh *et al.* 2012), but this traditional enrichment technique does not work well on copepods since copepods can induce rejection, avoidance and morbidity when exposed to the chemical substances in the enrichment products due to the presence of sensory chemoreceptors at the mouthparts (Huntley *et al.* 1986; Kleppel 1993).

## 2.2 Nutritional comparison between copepods and other live food organisms

Copepods offer nutritional advantage over *Artemia* nauplii as live prey for marine fish larvae, although it has been a concern to culture a large quantity of copepods to coincide the need for the production of marine fish larvae. Copepods are the natural food for most marine fish larvae in the wild and contain rich long-chain HUFA with DHA/EPA ratios >1 during their naupliar and early copepodite stages (Gronkjaer *et al.* 1995; McEvoy *et al.* 1998; Shields *et al.* 1999; Hamre *et al.* 2002; Gladyshev *et al.* 2015) . Furthermore, first feeding larvae require fatty acids and amino acids as the major sources of energy (Park *et al.* 2006). Newly hatched yolk-sac fish larvae have high levels of energy reserve, but the internal nutrition is quickly depleted after hatch (Buentello *et al.* 2011). Therefore, first-feeding fish larvae need live food that offers sufficient levels of energy supply (Støttrup 2000). Marine fish larvae require n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) such as EPA and DHA as EFA for survival, growth, and development (Pinto *et al.* 2012). DHA and EPA in copepods can promote the development of larval fish as the consumption of these essential fatty acids by fish larvae can reduce the incidence of morphological abnormalities (Satoh *et al.* 2009). Since *Artemia* and rotifers do not contain adequate essential fatty acids and elemental contents, researchers have endeavoured to find other suitable live food species such as copepods that have superior nutritional values for the need of fish larvae (Drillet *et al.* 2011; Abate *et al.* 2015).

In the ocean, most fish larvae feed on copepods rather than *Artemia* nauplii and rotifers, thus logically making copepods a live food for marine fish larvae in aquaculture (Evjemo *et al.* 2003). In addition to DHA and other PUFA, copepods also contain a high level of exogenous digestive enzymes that contribute to larval fish digestion (Zaleha *et al.* 2012). Moreover, according to Schipp *et al.*(1999), copepods have a high proportion of polar lipids that are more biologically available to fish larvae than triacylglycerols. The copepod nauplii are small (< 100 μm) and easily digestible, and are also a good source of antioxidants, astaxanthin and vitamins C and E (Barroso *et al.* 2013). Another important advantage of copepods is their jerking (i.e. zigzag) swimming motion, providing a visual stimulus for the larvae of pelagic fish, which are recognised by visual feeding predators (Barroso *et al.* 2013). A major problem with copepod intensive production is the long generation time showed by some species and difficulty for mass culture (Conceicao *et al.* 2010). Species of copepods with shorter generation times and high tolerance to temperature and salinity changes are ideal for mass culture (Payne & Rippingale 2000; Stottrup 2006). Therefore, exploration has been tried on several species but with varying success (Table 2.1). Results from some sustainable intensive cultures have been reported and are very encouraging (Sun & Fleeger 1995; Schipp *et al.* 1999; Payne & Rippingale 2001).

**Table 2.1**. Potential copepod species explored for mass culture.

| **Copepod species** | **Temperature** (ºC) | **Salinity** (ppt) | **Light** | **Food** | **Authors** |
|---|---|---|---|---|---|
| *Acartia grani* | 19 | 38 | 12L:12D | *Rhodomonas salina* | (Calbet *et al.* 2007) |
| *Acartia sinjiensis* | 27–30 | 30 –35 | 18L:6D | *Tetraselmis chuii* and *Isochrysis tahitians* | (Milione & Zeng 2007) |
| *Acartia southwelli* | 25–30 | 15–20 | 12L:12D | *Isochrysis galbana* | (Vengadeshperumal *et al.* 2010) |
| *Acartia tonsa* | 17 | 30 | 12L:12D | *Rhodomonas salina* | (Broglio *et al.* 2003) |
| *Apocyclops royi* | 25–30 | 15–20 | 12L:12D | *Isochrysis galbana* | (Su *et al.* 1997) |
| *Centropages typicus* | 19–21 | 38 | 12L:12D | *Prorocentrum minimum*/ *Isochrysis galbana/Tetraselmis suecica* | (Bonnet & Carlotti 2001) |
| *Eurytemora affinis* | 10–15 | 15 | 12L:12D | *Rhodomonas marina* | (Devreker *et al.* 2009) |
| *Euterpina acutifrons* | 19 | 38 | 12L:12D | *Rhodomonas salina* | (Da Costa *et al.* 2005) |
| *Gladioferens imparipes* | 23–27 | 18 | 24D | *Isochrysis tahitians* and *Chaetoceros muelleri* | (Payne & Rippingale 2001) |
| *Temora stylifera, Centropages typicus* | 20±1 | 36 | 12L:12D | *Prorocentrum minimum, Isochrysis galbana,* | (Buttino *et al.* 2012) |

| | | | | | |
|---|---|---|---|---|---|
| | | | | *Tetraselmis suecica* | |
| *Parvocalanus crassirostris* | 26 ± 1 | 36 ± 1 | 16L: 8D | *Isochrysis* sp., *Chaetoceros muelleri* | (Alajmi & Zeng 2015) |
| *Pseudodiaptomus euryhalinus,Tisbe monozota* | 27 | 35 | 12L:12D | *Isochrysis* sp. | (Puello *et al.* 2013) |
| *Oithona davisae* | 20 | 30 | 12L:12D | *Oxhyrris marina* | (Kiørboe 2007) |
| *Pseudodiaptomus annandalei* | 25–30 | 15–20 | 12L:12D | *Isochrysis galbana* | (Dhanker *et al.* 2012) |
| *Paracyclopina nana* | 28 | 15 | 12L:12D | *Tetraselmis suecica/Isochrysis galbana* | (Lee *et al.* 2006) |
| *Robertsonia knoxi* | 25 | 27 | 12L:12D | baker's yeast | (Zaleha *et al.*2012) |
| *Acartia erythraea, Oithona revicornis* | 32 | 30-34 | 12L:12D | *Isochrysis galbana* & mixed algal diets | (Rajkumar & Rahman 2016) |

**Table 2.2**. Fatty acid changes in algae under different nitrogen and phosphorus limitations

| **Algal species** | **Limiting Nutrients** | **PUFA** | **Authors** |
|---|---|---|---|
| *Synechococcus* sp. and *Scenedesmus* sp. | N | decreased | (Ahlgren & Hyenstrand 2003) |
| *Isochrysis galbana* | N | increased | (Flynn *et al.* 1994) |
| *Stephanodiscus hantzschii* and *Scenedesmus quadricauda* | P | increased | (Ahlgren *et al.* 1998) |
| *Chlamydomonas reinhardtii* | P | increased | (Weers *et al.* 1997) |
| *Scenedesmus acutus* | P | increased | (Navarra 1995) |
| *Rhodomonas minuta* | P | decreased | (Urabe *et al.* 1997) |
| *Scenedesmus acutus* | P | decreased | (Plath & Boersma 2001) |
| *Scenedesmus spinosus* | P | decreased | (Park *et al.* 2002) |

## 2.3 Nutritional requirement of copepods

### *2.3.1 Microalgae and their nutritional value*

Algal nutritional composition is essential for mass culture of copepods (Plath & Boersma 2001). The quality of food available to copepods varies with the composition of phytoplankton community and algal nutritional composition (Tirelli & Mayzaud 2005; Mitra & Flynn 2005). Understanding the method to enhance copepod reproduction and fecundity is necessary to improve copepod production. Optimal conditions supporting copepod egg production determine

population growth and culture scales, and female fecundity can be used as an index for production potential in copepod culture. The primary biotic factors regulating egg production in copepods are the quality and quantity of food (Gusmão & McKinnon 2009). An increase in food density can promote egg production in a sigmoidal function up to the level of food saturation (Finiguerra *et al.* 2013). This saturation level of egg production is critical for copepod culture since it allows better coupling and optimization of microalgae and copepod production. Most of the evidence on food saturation levels for copepod egg production originates from field observations where there is little or no control over the quality of food available to the animals, and there is little information on food density-dependent growth of copepods in aquaculture. Therefore, understanding the mechanism controlling egg production in copepods based on its nutritional requirement allows better selection for algal species to be used in aquaculture and their optimal density for copepod culture. However, before establishing a mass culture protocol of copepod, there is a critical need to understand the nutritional requirements to sustain the growth and reproduction of copepods.

Microalgae have been fed to many aquatic organisms and particularly for copepods. A number of recent studies have demonstrated correlations between zooplankton production and some amino acids and fatty acids in dietary algae (Anderson & Pond 2000). Marked relationships have been found between zooplankton population growth, egg production and consumption of algae with

polyunsaturated fatty acids (EPA and DHA) (Demott & Muller-Navarra 1997; Mueller-Navarra *et al.* 2000). In one of the studies on rotifer, after ingesting the green algae, *Tetraselmis* sp., the PUFA content of rotifers increases, and in a few hours these rotifers are ready to feed fish and crustacean larvae (Rainuzzo *et al.* 1997). In aquaculture, the PUFA content is critical for maintaining fast growth, high survival and reproduction, and food conversion efficiencies for a variety of marine and freshwater organisms. The study on the biomass accrual of copepod grazing on phytoplankton shows a strong correlation between the HUFA contents in algae and in herbivorous zooplankton (Brett *et al.* 2009).

Marine algae are major producers of omega 3-PUFA's, while freshwater algae predominantly produce saturated or monosaturated fatty acids. However, the amount of EPA and DHA differs among algal species. EPA levels in *Tetraselmis* sp. is only 4.8 mg $g^{-1}$ while EPA levels in *Nannochloropsis* sp. is 23.4 mg $g^{-1}$ and DHA levels in *Tetraselmis* sp. is 0.2 mg $g^{-1}$ while DHA levels in *Isochrysis* sp. is 15.8 mg $g^{-1}$ (Patil *et al.* 2007). *Isochrysis* sp. and *Nannochloropsis* sp. have been widely used as a mariculture food due to their high content of long chain polyunsaturated fatty acids (Dunstan *et al.* 1993). Marine microalgae such as *Isochrysis* sp. and *Nannochloropsis* sp. have received increasing interest because of their ability to produce polyunsaturated fatty acids (Liu & Lin 2001; Patil *et al.* 2007). There is an interaction at the phytoplankton-copepod interface that affects the transfer of long-chain (ω3) PUFAs to higher trophic levels. Copepods contain high HUFA-PUFA constituents after consuming microalgae

with high PUFA and become the essential food for vital development of larval fish and crustaceans (Evjemo *et al.* 2003).

In some algae, nitrogen limitation can increase the lipid content of microalgae (Sharma *et al.* 2012; Adams *et al.* 2013). However, most studies only emphasised the overall lipid induction through nutrient limitation, but not specifically on the change of PUFA in algae. Since *Isochrysis* sp. and *Nannochloropsis* sp. contain high EPA and DHA, further studies need to test the effects of different nutrient ratios on fatty acid levels of microalgae, particularly the EPA and DHA ratio to test whether *Isochrysis* sp. and *Nannochloropsis* sp. have the ability to produce more of EPA and DHA in the cells at different nitrogen and phosphorus supplies. Development of the method of N:P ratios is expected to produce algae containing desired nutritional values as food for copepod production.

### *2.3.2 Impact of medium nutrients on fatty acids in algae*

The effect of the algal biochemistry on primary consumers has received considerable attention, but some fundamental questions still remain unanswered. Growth and egg production of copepods are correlated with the bulk protein content of algal food (Breteler *et al.* 2005) and the lipid composition of seston (Kleppel 1993; Jónasdóttir & Kiørboe 1996; Jonasdottir *et al.* 2009). Phosphorus (P) and nitrogen (N) are the two elements mostly referred to when the effects of nutrient limited autotrophic growth on herbivores are evaluated (Sterner *et al.* 1992; Nakanishi & Kawabata 1995; Sterner *et al.* 2008). This is based upon the roles that N and P play in the tissue construction of organisms, plants as well as animals. Furthermore, nitrogen and phosphorus have known to alter the

biochemical composition and particularly the lipid content of algae (Ahlgren & Hyenstrand 2003; Sharma *et al.* 2012).

Algal growth is related to nitrogen and phosphorus supply in the culture medium (Beardall, Young & Roberts 2001; Ahlgren & Hyenstrand 2003; Zhang & Hu 2011) and the availability of nutrients affects algal quality for grazers (Mitchell *et al.* 1992; Kilham *et al.* 1998; Ahlgren & Hyenstrand 2003). For example, analyses of 18 freshwater and 11 marine algal species showed that in most cases N limitation can cause an increase of lipid content by 2-3 times higher than in the culture with replete N (Shifrin & Chisholm 1981). The amount of saturated lipids seems to increase, whereas phospholipids and polyunsaturated fatty acids (PUFA) decrease under N limitation (Parrish & Wangersky 1990).

The lipid and fatty acid (FA) compositions of green algae can be affected by N supply (Piorreck *et al.* 1984). Ahlgren & Hyenstrand (2003) studied the influence of different amount of nitrogen on biochemical compositions of algae particularly on fatty acid profiles of *Scenedesmus quadricauda*, cyanobacterium, and *Synechococcus* sp. In their study, the most important ω3 fatty acid, α-linolenic acid (ALA: 18:3n-3) increased with the algal growth rate under N limitation (549 µM) and N replete (5496 µM). However ALA decreased under N limitation in *S. quadricauda*, and the ω6 PUFA, linoleic acid (LA, 18:2n-6) showed much lower levels with a decreasing tendency as algae grew fast (Ahlgren & Hyenstrand 2003). N limitation affected the fatty acid content differently between the two algal species. *Scenedesmus* sp. did not follow the same pattern of saturated fatty acid at N limitation as observed in *Synechococcus* sp. In *Scenedesmus*, ω3 PUFA

(mainly ALA) reduced at N limitation, whereas no influence was detected in ω6 PUFA (mainly LA) (Ahlgren & Hyenstrand 2003).

Many cyanobacteria contain low levels of fatty acids, particularly PUFA. N limitation clearly influences the fatty acid composition and content by increasing monounsaturated fatty acids and decreasing PUFAs. For instance, Lynn *et al.* (2000) showed that N limitation induced high triglycerides and low phospholipids. In another study, N limitation also increased the level of triglycerides in the prymnesiophyte *Isochrysis galbana* (Flynn *et al.* 1992). However, the corresponding reduction of ω3 PUFA in *Scenedesmus* was not notable in *Isochrysis* where, instead, both ω3 and ω6 PUFA increased when N was less than 100 μM (Ahlgren & Hyenstrand 2003; Flynn *et al.* 1992). Carbon content was stable in the green alga *Scenedesmus quadricauda* and the cyanobacterium *Synechococcus* sp. under replete and N-limited conditions. However, nutrient limitation seems to affect the fatty acid content differently in the above algal species. For instance, in *Scenedesmus* ω3 PUFA was reduced whereas ω6 PUFA was stable (Ahlgren & Hyenstrand 2003). Furthermore, the low food quality of P-deficient algae might be due to an indirect effect via alterations in biochemical composition, such as reduced algal EPA or linolenic acids (Ahlgren & Hyenstrand 2003).

P-limitation can reduce ω3 PUFA in the diatom species *Stephanodiscus hantzschii* and green algae such as *S. quadricauda* (Ahlgren *et al.* 1998) and *Chlamydomonas reinhardtii* (Weers & Gulati 1997), while ω3 PUFA increases under P-limitation in *S. acutus* (Navarra 1995). However, Park *et al.* (2002)

reported that the ω3 PUFA content was not decreased by P limitation in three algal species *Rhodomonas minuta, Scenedesmus acutus* and *Synechococcus* sp. The same results were also found by other researchers who emphasize that P-limitation is real and not an indirect effect of changes in the algal fatty acid composition (Urabe *et al.* 1997; Weers & Gulati 1997; Boersma *et al.* 2001). This is contrary with the results by Ferrão-Filho, Fileto, Lopes & Arcifa (2003) in the green algae *Scenedesmus spinosus* where two important PUFAs (linoleic and linolenic acids) are increased under P deficiency. Therefore, further research is needed to examine the fluctuation trends on biochemical composition of algae (Table 2.2) since algal taxonomy largely determines the results of fatty acid levels under different nutrient manipulations (Park *et al.* 2002).

### *2.3.3 Feeding copepods with N or P manipulated microalgae*

Algal diet quality has been the research focus to improve copepod reproduction and population growth through nutritional supply (Saba *et al.* 2009). Nitrogen is important in amino acid and protein synthesis while phosphorus is an important component of phospholipids in energy metabolism and in nucleic acid synthesis for algal growth. Consumption of different food types by zooplankton affects their growth and reproduction. High levels of P in cladocerans are associated with high content of ribonucleic acid (RNA) (Ferrão-Filho *et al.* 2003). Thus, fast-growing copepod species may have a high demand for P and are likely to be more affected by P-deficient algae than by slow-growing species (Main *et al.* 1997; Sterner & Schulz 1998). Difference in the elemental ratios between

zooplankton and their foods suggests that zooplankton have the capacity to accumulate different amounts of N or P in body tissue through feeding and metabolic processes (Urabe & Watanabe 1992).

Copepods can easily obtain the necessary amount of N, P, and C, if N:C or P:C ratios in food are appropriate (Anderson & Hessen 1995). However, under N-limitation, algae tend to decrease essential PUFA which may limit the production of copepods that feed nutrient-deplete algae (Piorreck *et al.* 1984; Harrison *et al.* 1990; Anderson & Pond 2000; Breteler *et al.* 2005). As a result, the slow growth of copepods fed P and N-deficient algae might be due to the change of dietary algal biochemical composition as reported on a calanoid copepod *Acartia tonsa* (Jones *et al.* 2002b). For instance, the *Calanus finmarchicus* is capable of producing eggs by catabolizing N-rich alga, and egg production is limited by the quantity and quality of available food (Mayor *et al.* 2009). The egg productions of the copepods *Calanus finmarchicus* and *Acartia tonsa* are also directly proportional to the amount of N in food (Checkley Jr 1980; Jones *et al.* 2002a; Mayor *et al.* 2009).

*2.3.4. Nutritional composition of copepods*

Food quality and quantity are the regulating factors for developmental time in copepods (Peterson 2001; Suchy *et al.* 2013). Naupliar development appears to be less sensitive to food quantity but the quality of the food is an important factor for copepod development (Malin *et al.* 2011). The biochemical composition of the algae affects naupliar development of copepods due to either the presence of inhibitory compounds (Carotenuto *et al.* 2002; Ianora *et al.* 2004)

or the lack of certain nutritional components necessary for copepod development (Breteler *et al.* 2005). PUFAs, in particular, the long-chained omega-3 fatty acids EPA and DHA are produced exclusively by marine algae, and play a vital role in reproduction, growth and metabolism in marine copepods (Daase *et al.* 2011; Vidhya *et al.* 2014). The usefulness of omega-3 fatty acids for copepod egg production, egg hatching success and copepod growth has been well reported in the field (Jónasdóttir *et al.* 2005; Dutz *et al.* 2008) and small-scaled experiments (Dutz *et al.* 2005; Jónasdóttir *et al.* 2009).

Herbivorous consumers primarily have higher mass-specific N and P contents than their algal food, and thus their growth rate may be limited by a shortage of N and P (Hessen 1993). Algae with high food quality are thus usually characterized by an elevated PUFA content as well as low C:N and C:P ratios (Demott & Muller-Navarra 1997; Dutz *et al.* 2005; Malin *et al.* 2011). Polyunsaturated fatty acids particularly EPA and DHA together with nitrogen seem to be the most important factors in controlling growth and reproduction of copepods (Saba *et al.* 2009). Nutrient imbalance leads to less efficient C-fixation and poorer growth in algae that again leads to lower PUFA content in algae (Dutz *et al.* 2005). Essential elements other than lipids in algae may also be affected by nutrient limitation. Egg production of copepods depends on the amino acid composition of the algal diet (Guisande *et al.* 2002). Hence, it is possible that copepod development and growth are also limited by the content of specific amino acids in food. Free amino acid pools of algae may rapidly change due to N availability (Flynn *et al.* 1992). Nevertheless, development of copepods under

different nutrient constituents is actually dependent on the HUFA and PUFA contents of algae. Copepods can enhance reproductive success by high availability of dietary HUFA and PUFA and thus the increase of PUFA content is recommended in algae prior to feeding to the animal (Anderson & Pond 2000).

## 2.4 Copepod culture systems

Copepods as live food have been used for larval fish rearing in hatchery (Drillet *et al.* 2011; Mahjoub *et al.* 2013; Kline & Laidley 2015) and showed success in a number of fish species (Støttrup 2000; Payne & Rippingale 2001; Janakiraman & Altaff 2015). It is possible to improve efficiency of larval fish rearing by using copepods as supplement to traditional live prey species (Drillet *et al.* 2006). Considerable success of copepods cultures have already been accomplished with high quality copepods being used in aquaculture industries and aquarium trade (Evjemo *et al.* 2003; Rajkumar & Kumaraguruvasagam 2006; Sørensen *et al.* 2007; Olivotto *et al.* 2008; Barroso *et al.* 2013). Amarasinghe *et al.* (1997) assessed vital rates of copepods and indicated that marine copepod culture techniques are more advanced than freshwater ones. McLaren *et al.* (1969) were among the first who developed pioneer culture systems for copepods, followed by others who enhanced and modified the system (Sun & Fleeger 1995; Schipp *et al*. 1999; Payne & Rippingale 2001). Despite technical advances in recent studies, further improvements are needed to meet the vast demand of copepod production in aquaculture industry. Wild or semi-wild copepods are used

occasionally to raise fish larvae but the wide variations of nutrition exist in wild copepods (McEvoy *et al.* 1998; Peck & Holste 2006; Sørensen *et al.* 2007).

In semi-extensive tank culture systems, isolated copepod populations from a natural environment are used directly to feed fish larvae or kept in tanks where phytoplankton blooms are stimulated by the addition of nutrients to the water. Semi-extensive systems require low maintenance and allow production of large amounts of copepods, eggs and nauplii (Stottrup 2006; Drillet *et al.* 2011). However, due to the open nature of extensive culture, the inoculated copepods are prone to be infected or contaminated by other copepod species, other zooplankton and parasites or viruses that either infect the copepods or use them as intermediate parasite hosts infecting fish larvae (Rajkumar & Kumaraguruvasagam 2006; Drillet *et al.* 2011). Recent studies on production and biochemical composition of copepods all year round in outdoor culture system are highly valuable for larval fish rearing, regardless of risks of contamination (Sørensen *et al.* 2007; Van der Meeren *et al.* 2008). Thus, improvement and sustainability enhancing procedures are achievable and should be optimized through future research.

Density of copepod individuals is crucially important to copepod culture. Some copepods are much more sensitive to high density (hundreds per liter) (Ban & Minoda 1994) than other live prey species like rotifers (millions ind $L^{-1}$) (Treece & Davis 2000). Thus, copepods have been used only occasionally in fish hatcheries. The density issue is of central importance because production costs are directly related to potential densities of copepod cultures. Calanoid copepods are considered poor candidates for mass cultivation simply because they do not

survive well at high densities, but harpacticoid and cyclopoid copepods can be raised at densities of a few thousands ind $L^{-1}$ or more (Lubzens *et al.* 2003; Steenfeldt 2008; Ki *et al.* 2009).

Culture density is also a limiting factor, with many calanoid species suffering from reduction of fecundity due to overcrowding (Lee *et al.* 2012) while harpacticoid copepods seem to have the ability to be cultured at high densities of over 100,000 ind $L^{-1}$ (Stottrup 2006). For example, the cyclopoid copepod *Paracyclopina nana* has been successfully cultured up to 119,000 ind $L^{-1}$ (Lee *et al.* 2006). Intensive copepod cultures have been used nowadays due to the advantages of closely controlled conditions that will enhance consistency and sustained production by utilizing less space than extensive culture systems. These criteria have become the important features of intensive copepod systems for both calanoids and harpacticoids (Lee *et al.* 2012). Payne & Rippingale (2001) used three intensive culture techniques for the temperate water estuarine calanoid copepod *Gladioferens imparipes*. These techniques comprise 60- and 500-L batch cultures and 1,000-L semicontinuous cultures. Five hundred-litre cultures are part of a recirculating system that features automated nauplius collection and water treatment. Standardized nauplius production, expressed as number of nauplii produced per litre of the culture vessel per day, is given for each technique. Within 420 days of culture, 878 ± 46 nauplii $L^{-1}$ culture /vessel/day was achieved for *G. imparipes* cultured in 500-L vessels (Schipp *et al.* 1999).

## 2.5 Live food enrichment

### *2.5.1 Current enrichment practice in aquaculture*

Several commercial products have been used to enrich *Artemia* and rotifers such as Easy Selco© (INVE, Belgium, fish oil based-emulsion), Red Pepper© (Bernaqua, Belgium, encapsulated fish oil-based emulsion), Aquagrow Gold© (Advanced BioNutrition, ABN, USA, *Schizochytrium* sp. based spray dried product), Aquagrow DHA© (ABN, USA, *Crypthecodinium cohnii*-based spray dried product) and Multigain© (Bio- Mar, Denmark, single cell marine organism-based spray dried product) (Cavalin & Weirich 2009; Haché & Plante 2011; Boglino *et al.* 2012). Currently, AquagrowGold© and Aquagrow DHA© products are not manufactured anymore, but the biochemical composition of *Schizochytrium* sp. spray-dried Algamac 3050© (Bio-marine Inc., Aquafauna, USA) is the closest product to the Aquagrow Gold© (Boglino *et al.* 2012).

Many commercial enrichment products are formulated to contain high amounts of essential nutrients. However, among all these products there are large differences in physical forms (emulsions, pastes, and spray-dried powders), ingredients (fish oils, vegetal oils, heterotrophic or phototrophic organisms) and nutritional compositions, such as lipids, fatty acids, amino acids, minerals and vitamins (Ribeiro *et al.* 2011a). These enriching products are currently used in most hatcheries to improve the nutritional value of live food for marine fish larvae (Boglino *et al.* 2012; Li & Olsen 2015).

Zooplankton can feed on microalgae and yeast under mass cultured conditions (Farhadian *et al.* 2008), but in live food culture, algae are a prominent

food in cultivation of most zooplankton species. *Artemia*, rotifers, and copepods are the major live food organisms that have been used in fish hatchery (Drillet *et al.* 2011). In natural conditions, zooplankton feed on a wide range of algal species and bacteria, but copepods prefer to feed algae (Payne & Rippingale 2000). Since *Artemia* and rotifers have low levels of essential macro and micronutrients in their tissues, copepods have recently been used to partially substitute these low nutritional foods to boost production of larval fish and crustacean in hatcheries. As copepods cannot be easily enriched with the enriching methods for rotifers and *Artemia* nauplii, some microalgae species are commonly used to enhance the nutritional quality of copepods, which, in turn, are fed to fish larvae (Shields *et al.* 1999; Payne & Rippingale 2000; Zaleha *et al.* 2012). In the following sections, we provide the methods for algae enrichment with micronutrients and the nutrient-enhanced algae can be subsequently fed to copepods and other marine larvae.

## 2.6 Algal enrichment with micronutrients

### *2.6.1 Selenium*

Studies have shown that enrichment of micronutrients in live food has been done on several species of zooplankton for aquaculture purposes (Table 2.3). Selenium (Se) is an essential trace element for animal nutrition and health, thyroid hormone production, and fish development (Ribeiro *et al.* 2011a; Ribeiro *et al.* 2012). Consequently, if this element is limited in the environment, it must be added to the fish diet. Selenium deficiency has been reported to reduce growth,

productivity and immunity and even cause death in fish (Doucha *et al.* 2009). This element is transferred to zooplankton and larval fish by food chain (Surai 2006).

**Table 2.3**. Enrichment of live food with micronutrients in aquaculture

| Algae | Zooplankton | Micronutrients | Impacts | Sources |
|---|---|---|---|---|
| *Euglena gracilis, Dunaliella salina Tetraselmis suecica* | *Artemia* | Vitamin E | Higher contents of vitamin E in *Artemia* | (Vismara *et al.* 2003) |
| *Ankistrodesmus gracilis* | Copepods | Vitamin C | Enhancing offspring production, lipids and carbohydrates in copepods | (Helena Sipaúba-Tavares *et al.* 2001) |
| *Isochrysis galbana* | *Artemia* | Vitamin C | High amount of vitamin C in *Artemia* | (Olsen *et al.* 2000) |
| *Pavlova* sp. and *Isochrysis* sp. | Rotifers | Selenium | Enhancing selenium and thyroid hormones in fish larvae | (Hamre *et al.* 2008) |

Due to deficiency of selenium in most fishes during development, recently selenium has been incorporated in algal cultivation by mixing nutrient solution with sodium selenite (Doucha *et al.* 2009). When *Chlorella* sp. are exposed to Se in the form of selenite in the environment, the algae are able to incorporate this element and make it available to grazers at the top trophic level (Bottino *et al.* 1984; Bennett *et al.* 1986; Knauer & Hemond 2000; Li *et al.* 2003; Doucha *et al.*

2009). For instance, selenium is rapidly absorbed within a few minutes on the algal cell (*Chlorella* sp.) surface where it is irreversibly fixed (Doucha *et al.* 2009). After 24–48 h, about 40% of the total fixed Se is moved to the inside of *Chlorella* cells in an organic-bound form (De Alcantara *et al.* 1998). Furthermore, it is reported that *Spirulina platensis* is able to accumulate Se efficiently during cultivation, and the accumulated Se increases with the concentration of Se in the culture media, which is a good indicator that algae incorporated with selenium through the culture media can improve the algal quality as a food for zooplankton nutrition (Chen *et al.* 2006; Doucha *et al.* 2009). Since copepods feed on microalgae as their prominent food (Shields *et al.* 1999; Wyckmans *et al.* 2007; Ajiboye *et al.* 2011), further research on producing Se-enriched algae is needed to improve the selenium content in copepods.

In addition to algae, selenium can be enriched in yeast through addition of selenium to the culture media (Yin *et al.* 2009). It has been found that the water soluble selenium salt ($Na_2SeO_3$) can be easily absorbed to by yeast (Suhajda *et al.* 2000). In the course of this process, the inorganic selenite (low bioavailability and potentially toxic) is converted to safer highly bioactive species with improved nutritional properties (Yin *et al.* 2010). Previously, selenium-enriched yeast have been used to boost the Se in rotifers (Hamre *et al.* 2008; Penglase *et al.* 2011b) and the Se concentration is enhanced in fish larvae after feeding on these Se enriched rotifers (Hamre *et al.* 2008). It is known that copepod has a sensory perception for feeding (Bonnet & Carlotti 2001; Saage *et al.* 2009), therefore it is important to incorporate selenium into the type of food that are ingested by

copepods such as microalgae and yeast (Palmer & Edmands 2000; Santos 2015). Since previous trials on rotifers fed Se-yeast have produced higher survival rates of rotifers and the use of selenium is also recommended in rotifer's enrichment formula (Hamre *et al.* 2008; Ribeiro *et al.* 2011b; Penglase *et al.* 2011a). Hence, it is suggested to feed copepods with Se-enriched yeast to produce Se-enriched copepods that are nutritionally beneficial for the culture of marine fish larvae.

### *2.6.2 Copper*

Copper (Cu) is one of the most important trace metals essential to enzyme, immunity and metabolic processes in fish (Kim & Kang 2004). However, supplementation of Cu to the food of aquatic animals needs to consider the balance between fulfilling the Cu requirement and avoiding Cu toxicity (Tan *et al.* 2011). Copepods are currently used for mass production of fish and crustacean larvae (Hagiwara *et al.* 2001; Sui *et al.* 2011), as fish and crustacean larvae feed mainly on copepods in the wild (Sun *et al.* 2013; Støttrup 2000). In mass culture of copepods, efforts have been made to improve trace element contents to maintain nutritional superiority of copepods to *Artemia* and rotifers (Lee *et al.* 2012). Among minerals that are responsible for skeletal formation, colloidal system maintenance, acid–base balance and enzyme activities (Lall 2002), copper has become increasingly important in larval fish nutrition because it is a key micronutrient for growth and development (Clearwater *et al.* 2002) Most crustacean species rely on haemocyanin to carry oxygen in blood (Lall 2002) and Cu is structurally important in haemocyanin molecules (Sun *et al.* 2013a). Furthermore, copper plays critical functions in haematopoiesis and in Cu-

dependent enzymes including lysyl oxidase, cytochrome oxidase, ferroxidase, tyrosinase and superoxide dismutase (Lall 2002).

The Cu-enriched algae have been tested to feed rotifers and we believe that this concept of enrichment is applicable to copepods. Cu-enriched algae have proven to be effective for rotifer enrichment in the feeding of Chinese mitten crab *Eriocheir sinensis* zoea larvae (Sun *et al.* 2013). Microalgae can be used as a copper capsules to transfer copper to rotifers through dietary enrichment because algal cell walls are porous and allow free passage of molecules and ions in aqueous solutions (Al-Rub *et al.* 2006; Sun *et al.* 2013). The constituents of algal cell wall provide an array of ligands with different functional groups capable of binding various heavy metals (Sheng *et al.* 2004). Given the fact that Cu-enriched algae are effective in rotifer enrichment and it is possible of using Cu-enriched algae to feed copepods to improve fish health and growth in hatchery. Furthermore, copper can be quickly attached to the surface of algal cells since most algal species mainly consists of polysaccharides, lipids and proteins, which have the potential to bind metal molecules (Sheng *et al.* 2004; Mehta & Gaur 2005; Sun *et al.* 2013). Thus, the algae containing high levels of copper can be possibly used to feed copepods to satisfy the copper requirement of fishes and crustaceans.

*2.6.3 Vitamin C*

Ascorbic acid (vitamin C) is recognized as a critical vitamin during early development of fish larvae (Brown & Hohmann 2002). Rapidly growing fish and prawn larvae appear to have higher requirements for vitamins than juveniles or

adults (Kanazawa 2003). It is reported that the algal growth phase has a significant effect on the percent of ascorbic acid in the culture of *Isochrysis* sp. (Brown & Hohmann 2002). However, the results need to be put into a context with standard hatchery practice for algal production, and the balance of other nutrients in microalgae (e.g., *Isochrysis* sp.) (Brown & Hohmann 2002). High dietary concentrations of ascorbic acid may also be beneficial during intensive larval fish production by conferring stress-resistance on fish larvae (Lazo *et al.* 2000).

Copepods fed microalgae have shown positive results on the growth and reproduction, and biochemical composition of copepods depending on the algal nutritional content fed to copepods (Sipaúba-Tavares *et al.* 2001). Furthermore, *Artemia* fed with *Isochrysis galbana* contain a high amount of ascorbic acid when algal density increases (Olsen *et al.* 2000). The content of ascorbic acids in zooplankton varies with species and enrichment procedures, but incubation of microalgae with vitamin C can increase the vitamin C level of rotifers (Srivastava *et al.* 2006), *Artemia* (Olsen *et al.* 2000) and copepods (Sipaúba-Tavares *et al.* 2001) after grazing on vitamin C enhanced algae.

### *2.6.4 Vitamin E*

In the food chain, the nutritional value of copepods depends on both the macronutrients (proteins, lipids and carbohydrates) and micronutrients (vitamins and minerals) which can be obtained from food filtration (Vismara *et al.* 2003). Among micronutrients, vitamin E (i.e., α-tocopherols) represents one of the most

important lipid-soluble antioxidants in plants and animals, specifically for protection against lipid peroxidation of biological membranes by scavenging oxygen radical and breaking radical chain reaction (Trushenski & Kohler 2006). In aquaculture, vitamin E is used for the fortification of food to improve growth, resistance to stress and disease as well as survival of fish and shrimp (Durmaz 2007).

Most aquatic animals are unable to synthesize vitamin E and must obtain it from plant sources (Spolaore *et al.* 2006; Umdu *et al.* 2009). Vitamin E has a wide occurrence in nature in both photosynthetic (e.g. leaves) and non-photosynthetic (e.g. seedlings) tissues of higher plants and algae (Spolaore *et al.* 2006; Durmaz 2007). It is reported that *Artemia* fed higher bioavailability of microalgae (*Euglena* sp., *Dunaliella* sp., and *Tetraselmis* sp.) have high vitamin E due to the fast membrane uptake in zooplankton (Vismara *et al.* 2004). The screening of microalgae has revealed that several species produce α-tocopherol in concentrations higher than the conventional foods that are traditionally considered rich sources of this vitamin (Coutinho *et al.* 2006). Among the best α-tocopherol sources known, the freshwater microalga *Euglena gracilis* contains a high amount of vitamin E (Carballo-Cárdenas *et al.* 2003). Concerning the other microalgae, α-tocopherol in *Nannochloropsis oculata* is higher than those in *Euglena gracilis*, *Dunaliella salina* and *Tetraselmis suecica* (Durmaz 2007).

Nitrogen in the media and the algal growth phase can significant affect the α-tocopherol content in microalgae such as in *Nannochloropsis oculata* (Durmaz 2007). Therefore, manipulations of nitrogen sources in microalgal culture can be

used to produce algae with high vitamin E contents to feed copepods and has become a useful method to supplement vitamin E to fish larvae to improve fish quality and production in hatchery (Hemaiswarya *et al.* 2011).

## Conclusions

This review highlights the importance of using copepods as live food in marine hatchery and emphasizes the need of using non-traditional way to enrich copepods to meet the nutritional requirements in fish and crustacean larvae in aquaculture. Copepods contain essential fatty acids, e.g., EPA, DHA and ARA, which are required by most fish larvae for growth and development. As copepods cannot be enriched with the traditional method of nutrient enrichment for live food due to their picky palate, the alternation of nutritional composition in copepods has to be done through the change of dietary algal nutrition for copepods. The fatty acid profile of algae can be altered by environmental nutrients such as N, P and C. Particularly, the micronutrients such as selenium, copper and vitamins that are essential to marine larvae can be enriched in algae through medium absorption. Consequently, copepods can be enriched by feeding algae that are pre-enriched with various elements essential to marine larvae. This review clearly proves that any attempts towards developing a sustainable cultivation of copepods are worthwhile and effective larval rearing can be done to overcome the problem faced by aquaculturists during larval fish growth and development.

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# Chapter 3

## Effect of N:P ratio on growth and chemical composition of *Nannochloropsis oculata* and *Tisochrysis lutea*

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## 3.1 Abstract

The understanding of how nitrogen (N) to phosphorus (P) ratios regulate growth and chemical composition of algae is important to control the nutritional value of microalgae for industrial application. This study compared the impacts of N:P ratio manipulations on the growth, elements, lipid, fatty acids and protein contents of *Tisochrysis lutea* and *Nannochloropsis oculata*. F/2 medium was used as the basal formula to obtained six N:P ratios of 5:1, 10:1, 20:1, 30:1, 60:1, and 120:1 and tested on algal species in triplicate. The growth rate was similar in both algal species across all N:P ratios, and the carbon content in *T. lutea* was higher than in *N. oculata*. However, the carbon contents were high in the N:P ratios of 5:1 and 120:1 and low from 10:1 to 60:1 N:P ratios for both *T. lutea* and *N. oculata*. There were no significant differences in cellular N and P but the protein contents depended on algae species and were significantly affected by N:P ratios. The N:P ratio of 20:1 favoured algal growth and protein contents, while the N:P ratio of 120:1 reduced algal growth and protein synthesis but increased lipid in both algae. The 20:1 N:P ratio favoured EPA production in *N. oculata* and the 30:1 N:P ratio favours DHA production in *T. lutea.* This study indicates that N:P ratio manipulation is an effective strategy to change biochemical composition in algae and N or P limitation tends to lower polyunsaturated fatty acids (PUFA) contents in algae.

**Keywords:** algae, phosphorus, nitrogen, protein, lipid, N:P ratio

## 3.2 Introduction

Microalgae are photosynthetic organisms that constitute the first primary link of an aquatic food chain (Marchetti *et al.* 2012). The products of microalgae have various applications, particularly in pharmaceuticals, cosmetics, biofuel production and aquaculture (Pulz & Gross 2004; Rosenberg *et al*. 2008; Spolaore *et al.* 2006). In nature, microalgae are the primary food for mollusks, zooplankton and crustacean larvae (Borowitzka 1997; Brown & Hohmann 2002; Duerr *et al.* 1998). Among various forms of alternative diets, such as algal paste (McCausland *et al.* 1999; Robert *et al.* 2001) yeast (Nell 2002), bacteria (Douillet & Langdon 1994) or even lipid compounds such as oil emulsions (Coutteau *et al.* 1996; Knauer & Southgate 1997), live microalgae remain essential to the production of larval fish and juvenile bivalves in captivity (Robert & Trintignac 1997).

As live food, the nutritional compositions, particularly polyunsaturated fatty acids 20:5(n-3) (eicosapentaenoic acid, EPA) and 22:6(n-3) (docosahexaenoic acid, DHA) in algae are critically important because they can affect the growth and reproduction of aquatic animals (Jonasdottir *et al.* 2009; Mueller-Navarra *et al.* 2000). However, the amount of EPA and DHA in algae differs greatly among algae species and environmental conditions. For instance, the EPA content is 4.8 mg $g^{-1}$ in *Tetraselmis* sp. but is 23.4 mg $g^{-1}$ in *Nannochlopsis* sp., while the DHA content is 0.2 mg $g^{-1}$ in *Tetraselmis* sp. but is 15.8 mg $g^{-1}$ in *Isochrysis* sp. (Patil *et al.* 2007). Marine microalgae such as *Isochrysis* sp. and *Nannochloropsis* sp. have received increasing attention as live

food for aquatic animals because of their high contents of DHA and EPA (C-Pa & Lin 2001; Patil *et al*. 2007) which are the essential fatty acids in the diet of marine animals for growth, health and reproduction.

Nutrient availability in the environment can regulate the growth and biochemical composition of algae (Qin & Culver 1996; Johansson & Graneli 1999). Phosphorus (P) and nitrogen (N) are the two major elements limiting primary productivity (Villar-Argaiz & Sterner 2002), and their availability can alter the biochemical composition particularly the lipid content of algae (Ahlgren & Hyenstrand 2003; Sharma *et al*. 2012). A typical effect of nutrient limitation is a reduction of growth and changes of cellular composition in algae (Beardall *et al.* 2001b). N and P limitations usually increase the cellular carbon (C) content (Cembella *et al.* 1984; Berdalet *et al.* 1994) and C accumulation in algal cells may reduce nutrient utilisation efficiency in luxurious supply and increase the efficiency of nutrients in short supply (Sterner & Hessen 1994). Furthermore, the nutrient availability in the environment can affect macromolecular composition (e.g., protein, carbohydrate and lipid) in algae (Geider & La Roche 2002) . Molecular N:P ratios thereby can provide an integrated measure of the ability of nutrient uptake for algal cells (Beardall *et al.* 2001a; Fresnedo & Serra 1992; Qin *et al*. 2012). Thus, N limitation usually results in low protein content and high carbohydrate or lipid storage (Shifrin & Chisholm 1981; Ganf *et al.* 1986) while P limitation can also shift the relative contents of protein, lipid and carbohydrate in algal cells (Theodorou *et al.* 1991; Reitan *et al.* 1994).

Many studies on nutrient limitation have emphasized the capacity of total lipid change through nutrient manipulation (Liang *et al.* 2013; Sharma *et al.* 2012; Reitan *et al.* 1994) but little research has focused specifically on the changes of PUFA and elemental contents of algae under different N:P ratios. N: P ratios have been manipulated to simulate situations from nitrogen deficiency to phosphorus deficiency as reported in other studies (Hessen *et al.* 2002; Breteler *et al.* 2005; Rodolfi *et al.* 2009) on a variety of green and golden algal species. The N and P levels were manipulated under different proportions of N or P reduction in the F/2 media to reach the target N:P ratios. Since most previous studies have focused on manipulating the nutrients to evaluate the overall lipid compositions of algae (Kunikane *et al.* 1984; Fong *et al.* 2003; Anderson & Pond 2000; Lai *et al*. 2011; Ahlgren & Hyenstrand 2003), this study fills the knowledge gap by evaluating the effects of different N:P ratios on the biochemical composition particularly the major components of fatty acids such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), α-linolenic acid (ALA) and arachidonic acid (ARA) as well as on some elemental compositions (N, P and C), protein and lipid in two algal species with intrinsic distinctions in biochemical compositions.

The supply of a single nutrient can affect physiological, biochemical and molecular adaptation of algae (Lai *et al.* 2011; Breteler *et al.* 2005; Liu *et al.* 2013), but our knowledge on the role of N:P ratio in regulating the contents of EPA, DHA and other fatty acids in algae is limited. Therefore, in this study, we selected two algal species *Tisochrysis lutea* (previously known as the Tahitian strain of *Isochrysis*, T-Iso, Bendif *et al.* 2013) and *Nannochloropsis oculata* that

have been widely used as live food for zooplankton. The former contains high DHA and the latter has high EPA, which allow us to assess how N:P ratios affect algae on their biochemical compositions. Specifically, we aimed to assess the impact of N:P ratio on algal growth, elemental composition, fatty acid profile and the contents of protein and lipid in algal cells. The understanding of these key issues will enable us to identify the optimal nutrient regimes for algal culture to produce high nutritional algae as live food for zooplankton. This study extends our knowledge on using N:P ratios as a tool to manipulate the value of algal composition for their use as zooplankton food or in commercial industries.

## 3.3 Materials and methods

### *3.3.1 Experimental design*

We used six different N:P ratios to examine the changes in growth and chemical composition of algae including C, N, P and biochemical contents, particularly the fatty acid profile of the two algae, *N. oculata* and *T. lutea*. These two species were used to compare whether the effects of N:P ratios on algal growth and cellular contents were species-specific. The algae used in this study were obtained from South Australian Research and Development Institute Aquatic Science Centre, Adelaide. The F/2 media was used as the basal formula for nutrient ratio manipulations at N:P = 5:1 (72 µM N; 14.37 µM P), 10:1 (144 µM N; 14.37 µM P), 20:1 (287 µM N; 14.37 µM P), 30:1 (287 µM N; 9.6 µM P), 60:1 (287 µM N; 4.8 µM P), and 120:1 (287 µM N; 2.4 µM P). The N and P levels were manipulated under different proportions of N or P reduction in the F/2

medium to reach the appropriate N:P ratios. We first fixed P at 14.37 μM to explore the N impact at three N:P ratios, i.e., 5:1 (72 μM N; 14.37 μM P), 10:1 (144 μM N; 14.37 μM P), 20:1 (287 μM N; 14.37 μM P), and then we fixed N at 287 μM N to explore the P impact at three N:P ratios, ie., 30:1 (287 μM N; 9.6 μM P), 60:1 (287 μM N; 4.8 μM P), and 120:1 (287 μM N; 2.4 μM P). Therefore, the N:P ratios of 5:1,10:1, 20:1 were to explore N-limitation, while 30:1, 60:1, and 120:1 were to explore P-limitation in algal growth and other performance parameters. The amount of N and P in the seawater and the medium from algal inoculation were considered in the final nutrient manipulation at different N:P ratios. The N:P ratios were manipulated for the two algal species in triplicate, which were then compared the species at different N:P ratios. The C:N molar ratios for each treatment were 4.5 (N:P 5:1), 4.7 (N:P 10:1), 5.2 (N:P 20:1), 5.5 (N:P 30:1), 6.1 (N:P 60:1), and 6.5 (N:P 120:1). The calculations of molar ratios were based on the molar weight of each chemical element. The calculated C:N molar ratios were 4.5(324 μM C:72 μM N), 4.7 (677 μM C:144 μM N), 5.2 (1492 μM C:287 μM N), 5.5 (1579 μM C:287 μM N), 6.1 (1751 μM C: 287 μM N), 6.5 (1866 μM C: 287 μM N). Two litres of algae were harvested from the culture media of each species for biochemical analysis when the stationary growth phase was reached.

### *3.3.2 Algal growth at different N:P ratios*

The starter algal cultures of 50-100 ml in the mid exponential growth phase were used as inoculum giving initial cell densities of $2.0 \times 10^5$ cells $mL^{-1}$

for *Tisochrysis lutea* and $2.5 \times 10^5$ cells $mL^{-1}$ for *Nannochloropsis oculata*. Each experiment was carried out in 2 L aerated glass Schott bottles exposed to a light irradiance at 115 μmol photons $m^{-2}$ $s^{-1}$ with fluorescent tubes on a 12:12-h light-dark cycle. Mean temperature was 23 ± 1 °C in the light period and 20 ± 1 °C in the dark period. All cultures were aerated with 0.22-μm-filtered air enriched with 1% $CO_2$ at approximately 500 $mL^{-1}$ $min^{-1}$. Aliquots (1 ml) of each culture were collected for cell density counts. Algal cell counts (six replicates per culture) were undertaken using a Neubauer haemocytometer. The specific growth rate (μ) was calculated from the exponential phase obtained through the two points, $N_1$ and $N_2$, at the two ends of this linear phase (exponential). The specific growth rate (μ) of each culture was calculated using this equation: $\mu = \ln (N_2 - N_1)/(t_2-t_1)$ where $N_2$ = number of cells $mL^{-1}$ at time of harvest $t_2$ and $N_1$ = cells $mL^{-1}$ at time $t_1$ from day 2 until day 6 prior to the algal stationary growth phase in both species.

### *3.3.3 Algal biochemical analysis*

In order to optimize the fatty acid content, algae were harvested during the stationary phase in both algal species (Mansour *et al.* 2005). Samples for measuring protein, total lipid and fatty acid contents were centrifuged at 7000×*g* at 15 °C for 15 min to obtain concentrated algal pellets and were freeze-dried prior to analysis. Lipids were extracted with the chloroform-methanol method (Bligh & Dyer 1959; Martínez-Fernández *et al.* 2006). The fatty acid content was measured and then the samples were methylated in 5 mL of 1% $H_2SO_4$ in methanol at 70 °C for 3 h. The fatty acid methyl esters were extracted by adding 750 μL distilled

water and 2 mL of n-heptane. The heptane layer was transferred to a 2-mL vial for analysis using gas chromatography (GC, PerkinElmer Gas Chromatograph Clarus 500). Fatty acid methyl esters (FAMEs) were separated and measured on the GC equipped with a 30 m capillary column (0.32-mm internal diameter, Zebron ZB-FFAP). Helium was the carrier gas (1.5 mL $min^{-1}$). The injector temperature was set at 250 °C and the detector temperature at 300 °C. The initial oven temperature was 140 °C for 3 min, then ramped at 10.0°C $min^{-1}$ from 1 to 160 °C for 5 min followed by 10.0°C $min^{-1}$ from 1 to 230 °C for 10 min. FAMEs were identified on the GC using software of TotalChrom Navigator (version 6.3.2 0646, Perkin Elmer). The level of the internal standard 17:0 was used to calculate the FAME concentration in each sample. Protein contents were calculated from Total Kjeldahl Nitrogen (N × 6.25) by the combustion technique using an Elementar RapidN analyser where N values were obtained as a percent of dry mass for further calculations (Martínez-Fernández *et al.* 2006).

### *3.3.4 Nutrients concentrations*

Cellular N and C were analyzed using a C/H/N elemental analyzer (Carlo-Herba) (Fidalgo *et al.* 1998). Cellular P was determined using the nitric acid/hydrogen peroxide digestion method with inductively coupled plasma optical emission spectrometry (ICP-OES) (Wheal *et al.* 2011; Fong *et al.* 2004).

### *3.3.5 Data analyses*

The data in this study were expressed as mean ± SD, and the results of growth, protein, total lipid and fatty acid composition were analysed by two-way analysis of variance (ANOVA) to test the interaction between the N:P ratio and algae species. This was followed where applicable with post hoc Tukey's multiple comparison test to determine the significant differences of means between treatments for each independent factor (Ferrão-Filho *et al.* 2003). All the data were tested for normality (Shapiro-Wilk Test), homogeneity and independence (Levene's Test) to satisfy the assumptions for ANOVA. The level of significant difference was set at $P < 0.05$.

## 3.4 Results

### *3.4.1 Algal growth*

The specific growth rate between *T. lutea* and *N. oculata* was not different in all treatments of N:P ratios ($P > 0.05$, Fig. 3.1). As the N:P ratio increased from 5:1 to 20:1, the specific growth rate of *T. lutea* reached the maximum (1.42 $d^{-1}$, $P < 0.05$, Fig. 3.1) and maintained no significant changes with further increase of the N:P ratio but declined when the N:P ratio reached 120:1. Similarly, when the N:P ratio increased from 5:1 to 20:1, the specific growth rate of *N. oculata* reached the maximum (1.49 $d^{-1}$, $P < 0.05$, Fig. 3.1) but decreased significantly

with the increase of N:P ratios from 20:1 to 120:1 ($P$ = 0.004).

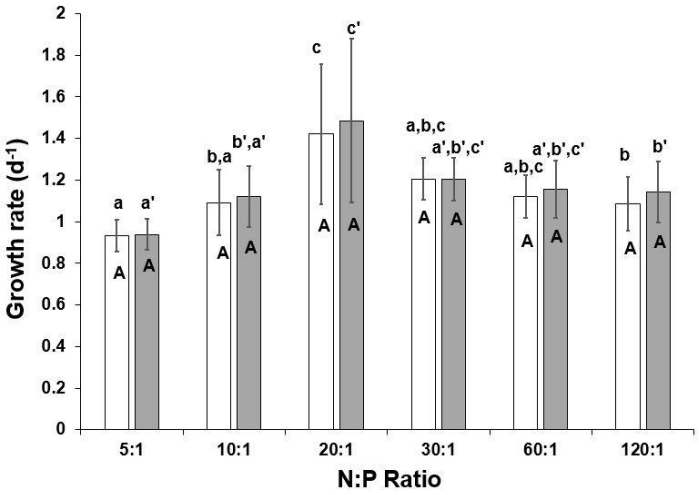



**Fig. 3.1** Growth rate of *Tisochrysis lutea* and *Nannochloropsis oculata* under different N:P ratios ($n$ = 3). Different capital letters inside the same bar type represent significant N:P ratios effect ($P$ < 0.05) between the algal species, while different small letters on the top indicate significant difference in each species ($P$ < 0.05) at different levels of N:P ratio. Error bars represent the standard deviations. Blank and grey bars represent *T. lutea* and *N. oculata*, respectively.

Growth curves of *T. lutea* and *N. oculata* under different N:P ratios are shown in Fig. 3.2. The exponential growth phase ended within the first 6 days under all test conditions in both species. At the end of the stationary phase, algal cell densities for both *T. lutea* and *N. oculata* at the N:P ratio of 20:1 were significantly higher than those at other N:P ratios ($P$ < 0.05, Fig. 3.2).

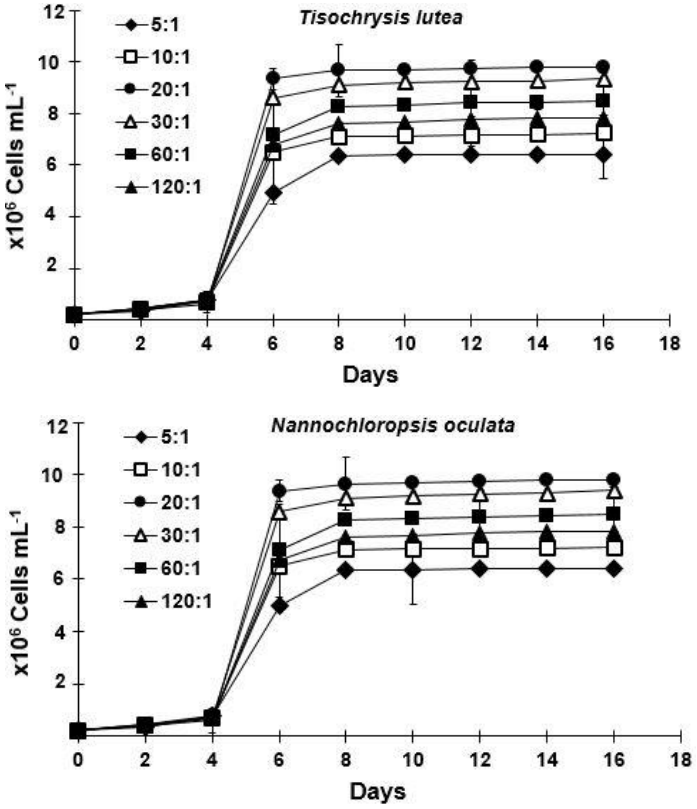
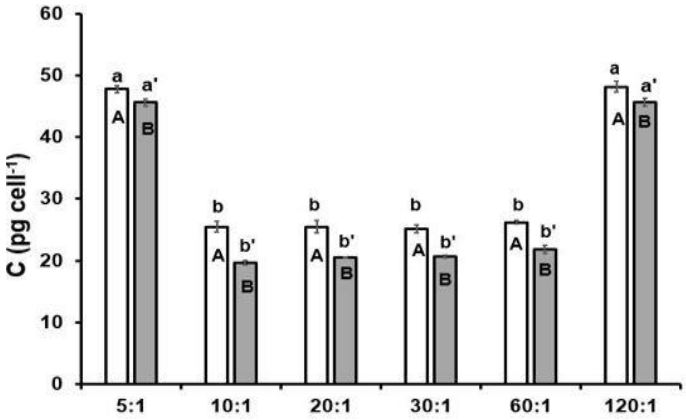



**Fig. 3.2** Growth curves of *Tisochrysis lutea* and *Nannochloropsis oculata* cultured under different N:P ratios. Data points represents mean ± standard deviations (*n* = 3).



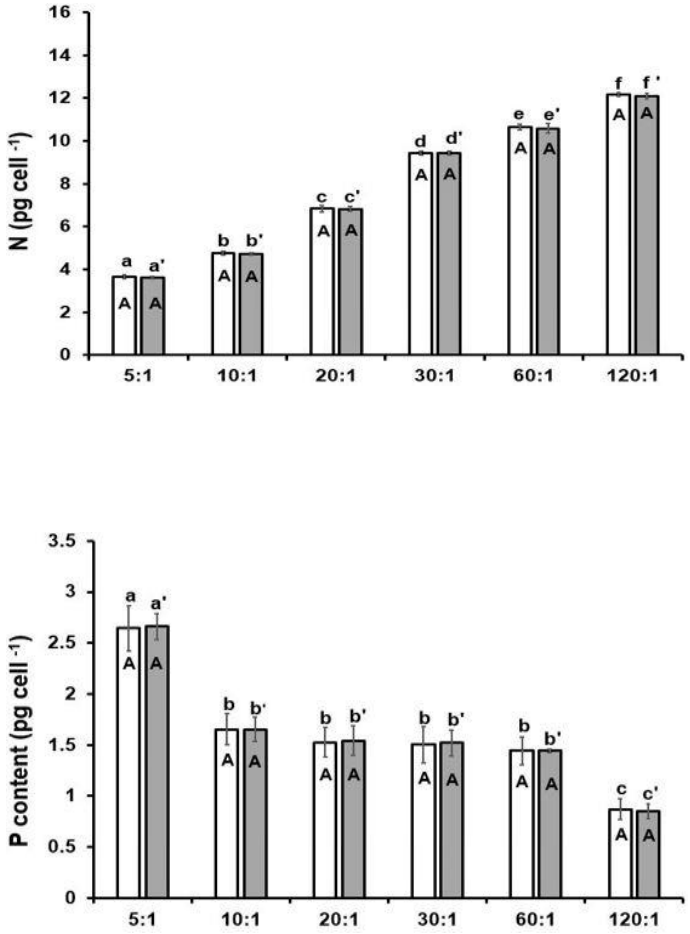



**Fig. 3.3** Changes of cellular C, N and P in *Tisochrysis lutea* and *Nannochloropsis oculata* under different N:P ratios. Different capital letters inside each bar type represent significant N:P ratio effects ($P < 0.05$) between the algal species, while different small letters on top indicate significant species effects ($P < 0.05$) at different levels of N:P ratio. Blank and grey bars represent *T. lutea* and *N. oculata*, respectively.

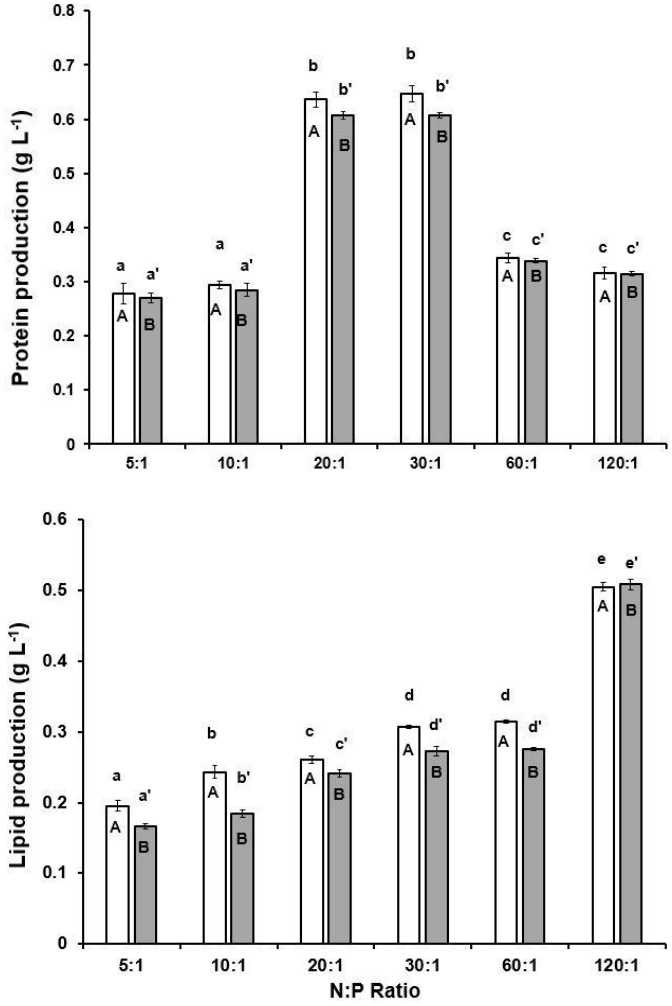



**Fig. 3.4** Protein and lipid content (% dry matter) in *Tisochrysis lutea* and *Nannochloropsis oculata* corresponding to different N:P ratios. Different capital letters inside the same bar type represent significant N:P ratio effects ($P < 0.05$) between the algal species, while different small letters on top indicate significant difference in each species ($P < 0.05$) at different levels of N:P ratio. Blank and grey bars represent *T. lutea* and *N. oculata*, respectively.

### *3.4.2 Carbon, nitrogen, and phosphorus in T. lutea and N. oculata cells*

The carbon content in *T. lutea* was significantly higher than in *N. oculata* in all N:P ratio treatments ($P = 0.001$, Fig. 3.3). The impact of N:P ratios on the

cellular C content depended on algae species ($P$ = 0.001). The highest C content was observed at the N:P ratios of 5:1 and 120:1 for both *T. lutea* and *N. oculata* ($P$ < 0.05, Fig. 3.3). The lowest C content (19.65 pg cell $^{-1}$) in *N. oculata* was found in the N:P ratio of 10:1 ($P$ = 0.001), but C contents in *T. lutea* and *N. oculata* were not significantly different as the N:P ratio increased from 10:1 to 60:1 ($P$ = 0.436). The carbon contents in both algae species depended on the level of N:P ratios ($P$ < 0.05). Cellular N contents were not different between species in all N:P ratios ($P$ > 0.05, Fig. 3.3), but as the N:P levels increased from 5:1 to 120:1, the cellular N increased in both *T. lutea* and *N. oculata* ($P$ < 0.05). Similarly, cellular P contents were not different between species in all N:P ratios ($P$ > 0.05, Fig. 3.3), but a significant declining trend of P contents was observed with the increase of N:P ratio in both *T. lutea* and *N. oculata* with highest at N:P = 5:1 and lowest at N:P =120:1 ($P$ < 0.05, Fig. 3.3).

### *3.4.3 Protein content*

The protein content increased with the increase of N:P ratio from 5:1 to 30:1 in both *T. lutea* and *N. oculata* ($P$ < 0.05, Fig. 3.4). The protein content in both species depended on N:P ratios ($P$ = 0.024). Both algal species yielded lower protein content when cultured under high (120:1) and low (5:1) N:P ratios ($P$ < 0.05). The protein contents in *T. lutea* and *N. oculata* were higher at the N:P ratios of 20:1 and 30:1 than at other N:P ratios ($P$ < 0.05), but there was no difference between these two ratios ($P$ > 0.05). The protein content in *N. oculata* was

60.67% when grown at the N:P ratio of 20:1 but it was further reduced when the ratio of N:P increased to 120:1 ($P < 0.05$, Fig. 3.4).

### *3.4.4 Lipid content*

The impact of the N:P ratio on cellular lipid depended on algal species ($P = 0.001$). The lipid content in *T. lutea* and *N. oculata* gradually increased with the increase of N:P ratio from 5:1 to 120:1 ($P = 0.001$, Fig. 3.4) and was not significantly different from the N:P ratio of 30:1 to 60:1 ($P > 0.05$) . However, the lipid content was significantly higher in the N:P ratio of 120:1 compared to that at lower N:P ratios. N:P ratios significantly affected the lipid content in both algal species ($P = 0.001$).

Fatty acid compositions in *T. lutea* and *N. oculata* under different N:P ratios are shown in Table 3.1 and Table 3.2. The fatty acid contents were significantly affected by the N:P ratio supplied to the culture ($P < 0.05$). The highest level of fatty acids in *T. lutea* was the saturated fatty acids and the content was significantly higher in the treatment of the highest N:P ratio (43.38%, N:P 120:1, Table 3.1). The amounts of myristic acid (C14:0) in *T. lutea* were higher in the N:P ratio of 120:1 (28.53 ± 0.32 %) than those at lower N:P ratios ($P < 0.05$). The increase of N:P ratios from 5:1(11.27 %) to 120:1(23.93 %) significantly augmented the C18:1 monosaturated fatty acids in *T. lutea* ($P < 0.05$, Table 3.1).

The contents of polyunsaturated fatty acids (PUFA) were significantly higher in the N:P ratio of 20:1 (37.94 %) than at other higher N:P ratios in *T. lutea* ($P <$

0.05). The highest alpha-linolenic acid (ALA) content in *T. lutea* was found in the N:P ratios of 20:1 and 30:1 (7.53 ± 0.15 %, 6.44 ± 0.10%, respectively, $P < 0.05$, Table 3.1).The amount of DHA in *T. lutea* decreased (6.73 ± 0.21 %) as N:P ratios increased from 30:1 to 120:1 ($P < 0.05$, Table 3.1). The content of C18:4 polyunsaturated fatty acids was higher in the N:P ratio of 20:1 compared to other N:P ratios ($P < 0.05$).

The saturated fatty acids were highest among other fatty acid groups in *N. oculata*, and its content was higher in algae at the N:P ratio of 120:1 than at 5:1 ($P < 0.05$, Table 3.2). The C16:0 saturated fatty acid was higher at the N:P ratio of 120:1 (38.29 ± 0.19) than that at other N:P ratios ($P < 0.05$, Table 3.2). The highest amount of monosaturated fatty acids was C16:1 under the N:P ratio of 120:1 (30.28 ± 0.12 %, $P < 0.05$). The PUFA content significantly increased when the N:P ratio increased from 5:1 to 20:1 and decreased as the N:P ratio increased to 120:1 ($P < 0.05$). Linoleic acid (C18:2) in *N. oculata* was higher (3.41± 0.09) at the N:P ratio of 20:1 than at other N:P ratios. The amount of EPA (38.67 ± 0.06 %) in *N. oculata* was significantly higher at the N:P ratio of 20:1 than at the 120:1 ratio (10.27 ± 0.06%; $P < 0.05$). The amount of EPA in *N. oculata* was significantly regulated by the N:P ratio in the culture media ($P < 0.05$). The ARA in *N. oculata* was significantly higher at the N:P ratio of 20:1 than at other ratios ($P < 0.05$).

**Table 3.1** Fatty acid composition (% total fatty acids) of *Tisochrysis lutea* grown at different N:P ratios. All values are mean ± standard error ($n$ = 3). Different letters in the same row represent significant differences at $P < 0.05$. The bold fatty acid species are described in the results.

| Species | ***Tisochrysis lutea*** | | | | | |
|---|---|---|---|---|---|---|
| | N:P ratios | | | | | |
| Fatty acids | 5:1 | 10:1 | 20:1 | 30:1 | 60:1 | 120:1 |
| Saturated | | | | | | |
| **C14:0** | **12.20 ± 0.1[a]** | **15.38 ± 0.20[b]** | **22.40 ± 0.26[c]** | **23.37 ± 0.25[d]** | **26.47 ± 0.32[e]** | **28.53 ± 0.32[f]** |
| C16:0 | 9.17 ± 0.11 | 9.48 ± 0.14 | 10.80 ± 0.36 | 10.59 ± 0.20 | 13.40 ± 0.11 | 14.85 ± 0.22 |
| Sum | 21.37 | 24.86 | 33.20 | 33.96 | 39.87 | 43.38 |
| Monosaturated | | | | | | |
| C16:1 | 3.30 ± 0.11 | 3.73 ± 0.15 | 4.74 ± 0.14 | 4.82 ± 0.14 | 4.21 ± 0.11 | 3.60 ± 0.10 |
| **C18:1[c]** | **11.27 ± 0.15[a]** | **15.87 ± 0.21[b]** | **18.31 ± 0.10[c]** | **17.26 ± 0.14[d]** | **19.27 ± 0.21[e]** | **23.93 ± 0.15[f]** |
| C22:1[c] | 0.42 ± 0.09 | 0.50 ± 0.10 | 0.90 ± 0.10 | 0.77 ± 0.15 | 0.59 ± 0.16 | 0.40 ± 0.10 |
| Sum | 14.99 | 20.10 | 23.95 | 22.85 | 24.07 | 27.93 |
| Polyunsaturated | | | | | | |
| C18:2[b] | 4.30 ± 0.10 | 4.31 ± 0.18 | 5.63 ± 0.15 | 4.02 ± 0.06 | 3.71 ± 0.24 | 3.43 ± 0.25 |
| **C18:3[a](ALA)** | **6.27 ± 0.21[a]** | **6.37 ± 0.15[a]** | **7.53 ± 0.15[b]** | **6.44 ± 0.10[b]** | **4.13 ± 0.13[c]** | **3.56 ± 0.14[c]** |
| **C18:4[a]** | **15.20 ± 0.17 [a]** | **15.50 ± 0.17[a]** | **16.40 ± 0.11[b]** | **15.50 ± 0.15[a]** | **11.50 ± 0.15[c]** | **8.80 ± 0.15[d]** |
| C20:4[b](ARA) | 0.37 ± 0.07 | 0.38 ± 0.06 | 0.51 ± 0.10 | 0.42 ± 0.10 | 0.33 ± 0.10 | 0.30 ± 0.10 |
| C20:5[a](EPA) | 0.34 ± 0.02 | 0.35 ± 0.03 | 0.57 ± 0.06 | 0.43 ± 0.06 | 0.31 ± 0.03 | 0.29 ± 0.04 |
| **C22:6[a](DHA)** | **6.80 ± 0.30[a]** | **7.20 ± 0.10[a]** | **7.30 ± 0.26[a]** | **8.20 ± 0.10[b]** | **7.02 ± 0.26[a]** | **6.73 ± 0.21[a]** |
| Sum | 33.28 | 34.11 | 37.94 | 35.01 | 27.00 | 23.11 |

[a]ω-3 fatty acids, [b]ω-6 fatty acids, and [c]ω-9 fatty acids.
ALA: Alpha-linolenic acid; ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

**Table 3.2** Fatty acid composition (% total fatty acids) of *Nannochloropsis oculata* grown at different N:P ratios. All values are mean ± standard error ($n$ = 3). Different letters in the same row represent significant differences at $P < 0.05$. The bold fatty acid species are described in the results.

| **Species** | ***Nannochloropsis oculata*** | | | | | |
|---|---|---|---|---|---|---|
| **Treatments** | | | | | | |
| **Fatty acids** | **5:1** | **10:1** | **20:1** | **30:1** | **60:1** | **120:1** |
| ***Saturated*** | | | | | | |
| **C14:0** | 1.10 ± 0.06 | 1.20 ± 0.02 | 1.27 ± 0.01 | 1.53 ± 0.01 | 1.41 ± 0.02 | 1.33 ± 0.02 |
| **C16:0** | **16.50 ± 0.34$^{a}$** | **17.66 ± 0.30$^{b}$** | **19.71 ± 0.37$^{c}$** | **33.55 ± 0.40$^{d}$** | **36.26 ± 0.22$^{e}$** | **38.29 ± 0.19$^{f}$** |
| **Sum** | 17.60 | 18.86 | 20.98 | 35.08 | 37.67 | 39.62 |
| ***Monosaturated*** | | | | | | |
| **C16:1** | **21.00 ± 0.17$^{a}$** | **21.80 ± 0.13$^{b}$** | **26.62 ± 0.14$^{c}$** | **28.54 ± 0.39$^{d}$** | **29.44 ± 0.23$^{d}$** | **30.28 ± 0.12$^{d}$** |
| **C18:1$^{c}$** | 0.36 ± 0.01 | 1.29 ± 0.01 | 2.04 ± 0.06 | 2.56 ± 0.04 | 2.66 ± 0.03 | 3.32 ± 0.07 |
| **C22:1$^{c}$** | 0.13 ± 0.01 | 0.17 ± 0.15 | 0.19 ± 0.10 | 0.20 ± 0.10 | 0.13 ± 0.06 | 0.15 ± 0.03 |
| **Sum** | 21.49 | 23.26 | 28.85 | 31.30 | 32.23 | 33.75 |
| ***Polyunsaturated*** | | | | | | |
| **C18:2$^{b}$** | **2.02 ± 0.08$^{a}$** | **2.55 ± 0.07$^{b}$** | **3.41 ± 0.09$^{c}$** | **2.03 ± 0.11$^{a}$** | **1.46 ± 0.12$^{d}$** | **1.00 ± 0.18$^{e}$** |
| **C18:3$^{a}$(ALA)** | 0.35 ± 0.22 | 0.57 ± 0.21 | 0.79 ± 0.13 | 0.59 ± 0.10 | 0.37 ± 0.15 | 0.27 ± 0.15 |
| **C18:4$^{a}$** | 0.50 ± 0.15 | 0.50 ± 0.10 | 0.35 ± 0.01 | 0.65 ± 0.12 | 0.45 ± 0.11 | 0.39 ± 0.09 |
| **C20:4$^{b}$(ARA)** | **5.20 ± 0.10$^{a}$** | **5.20 ± 0.10$^{a}$** | **6.31 ± 0.11$^{b}$** | **4.19 ± 0.10$^{c}$** | **3.60 ± 0.10$^{d}$** | **3.32 ± 0.10$^{d}$** |
| **C20:5$^{a}$(EPA)** | **21.53 ± 0.06$^{a}$** | **21.57 ± 0.06$^{a}$** | **38.67 ± 0.06$^{b}$** | **25.40 ± 0.08$^{c}$** | **14.33 ± 0.09$^{d}$** | **10.27 ± 0.06$^{e}$** |
| **C22:6$^{a}$(DHA)** | 0.15 ± 0.02 | 0.23 ± 0.03 | 0.34 ± 0.02 | 0.47 ± 0.02 | 0.33 ± 0.03 | 0.21 ± 0.03 |
| **Sum** | 29.75 | 30.62 | 49.87 | 33.33 | 20.54 | 15.46 |

$^{a}$ω-3 fatty acids, $^{b}$ω-6 fatty acids, and $^{c}$ω-9 fatty acids.

ALA: Alpha-linolenic acid; ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

The interactive effects of algal species and N:P ratios were detected on major species of polyunsaturated fatty acids ($P < 0.05$). The EPA content was not affected by N:P ratios in *T. lutea*, but it was highest at the N:P ratio of 20:1 and lowest at the N:P ratio of 120:1 ($P < 0.05$). The DHA content was significantly higher in *T. lutea* compared to *N. oculata* regardless of N:P ratios ($P < 0.05$). The ALA content was not affected by N:P ratios in *N. oculata*, but it was highest at the 20:1 or 30:1 N:P ratio and lowest at the 60:1 or 120:1 N:P ratio ($P < 0.05$) in *T. lutea*. In contrast, the ARA content was not affected by N:P ratios in *T. lutea*, but it was highest at the N:P ratio of 20:1 and lowest at 60:1 or 120:1 N:P ratio in *N. oculata* ($P < 0.05$).

## 3.5 Discussion

In this study, algal growth was enhanced when the N:P ratio increased from 5:1 to 20:1, and then there was a gradual decline from 20:1 to 120:1 N:P ratios. This finding is similar to a previous study by Vanucci *et al.* (2012) where final cell yields of a dinoflagellate *Ostreopsis ovata* was increased from a low N:P ratio of 5:1 to a high N:P ratio of 16:1, which is consistent with another study using similar N:P ratios on the same dinoflagellate *O. ovata* (Vidyarathna & Granéli 2013). Similarly, Mayers *et al.* (2014) have found that the maximum cell density was obtained when the *Nannochloropsis* sp. was cultured under the N:P ratios of 16:1 and 32:1 compared to 64:1 and 80:1. Our results demonstrated that the N:P ratio of 20:1 increased the final cell densities in *T. lutea* and *N. oculata* compared to those at the N:P ratio of 5:1. Furthermore, the current results showed

that within each species algal growth was affected by the range of N:P ratios. Geider & La Roche (2002) stated that phytoplankton is N-limited at N:P <16, and that it is P-limited at N:P >16 based on the Redfield ratios (Redfield 1934). However, in the present study, the highest growth rate was obtained for both algal species under the N:P ratio of 20:1 compared to the rest of the N:P ratios. This finding is consistent with a previous study that the N:P ratio of 20:1 favoured the growth of *Thalassiosira* sp. and *Chroomonas salina* (Zhang & Hu 2011). In contrast, another study on other algal species such as *Cylindrotheca closterium* and *Platymonas helgolandica* var. *tsingtaoensis* (Sun *et al.* 2004) showed a different pattern where the algal growth rate is higher at the N:P ratios of 160:1 and 80:1 respectively than at 16:1 and this suggests that algal preference for optimal N:P ratios varies among algal species as reported in previous studies (Clark *et al.* 2002; Flynn *et al.* 2002). The response of algal growth to N:P ratios depends on the physiological requirement for nutrients and varies among species (Lagus *et al.* 2004). Our results demonstrate that the higher growth rates of *T. lutea* and *N. oculata* occurred close to the N:P ratio of 20:1, which is slightly higher than the Redfield ratio of N:P = 16:1.

The increase of N:P ratio from 5:1 to 120:1 increased the cellular N content of *T. lutea* and *N. oculata* in the current study. Similarly, in a previous study, the increase of N:P ratio from 22:1 to 3000:1 also increased the cellular N content in *Calcidiscus leptoporus* (Langer *et al.* 2012). In the treatments of N:P ratios at 20:1 and 30:1 where phosphorus was limited, the excess N may be easily incorporated into protein as shown in a previous study on *Scenedesmus* sp. when

N was in excessive supply relative to P in the medium (Rhee 1978). Our results demonstrated that the increase of the N:P ratios from 5:1 to 120:1 (ascending N supply) increased the cellular N contents, but phosphorus limitation might lead to the accumulation of excess nitrogen stored as protein in the cells of *T. lutea* and *N. oculata*. The increase of N:P ratio from 20:1 to 120:1 leads to P limitation and a tendency of PUFA reduction in both algal species, which is consistent with the report on *Dunaliella parva* where the N:P ratio of 118:1 decreased the cellular P content compared with the N:P ratios of 30:1 and 6:1, though the PUFA contents were not reported by Suzuki *et al.* (1997).

Cellular C contents of algae at the N:P ratios of 5:1 and 120:1 were high in *T. lutea* and *N. oculata* in the present study, in line with a previous result on *C. leptoporus* where lower N:P ratios of 0.17:1 and higher N:P ratios of 3000:1 increased C content compared with the N:P ratio of 22:1 (Langer *et al.* 2012). Similarly, N:P ratios of 0.57:1 and 115:1 also increased the carbon contents in *T. tutea* (Marchetti *et al.* 2012). Under the optimum range of N:P ratios (20:1 - 30:1), carbon contents were lower than the high (120:1) and low N:P ratios (5:1) in *T. lutea* and *N. oculata*. Langer *et al.* (2012) also found that in *C. leptoporus* where the N:P ratio of 22:1 resulted in low carbon contents compared to the N:P ratios at 0.17:1 and 3000:1. At a high or low N:P ratio, algae can produce excess C that may be stored internally as starch and lipids for metabolism and reproduction (Sterner & Hessen 1994; Claquin *et al.* 2002). This suggests that carbohydrates may be the preferential storage at low N:P ratios, while lipids might be the preferential storage at high N:P ratios. However, in this study we did not measure

the conversion of cellular C to carbon compounds and further investigation is needed to identify whether a high C in the low N:P ratio can be converted to lipids or starch for physiological functions in algae.

In this study, protein contents in *T. lutea* and *N. oculata* were affected by N:P ratios. The protein contents of algae at the N:P ratios of 20:1 to 30:1 were higher than other N:P ratios. In another study, the high protein yield in *Prorocentrum donghaiense* also happened to fall into the range of the N:P ratios of 8:1 to 64:1 (Lai *et al.* 2011). Besides, Berdalet *et al.* (1994) and Kilham *et al*. (1997) reported that algae at high N:P ratios of 200:1 to 486:1 resulted in a lower protein concentration than at a medium N:P ratio of 20:1 to 24:1 due to phosphorus deficiency, which coincides with the low protein content at the high N:P ratio in this study. The amount of N in the N:P ratios from 20:1 to 30:1 was 287 µM which coincides with high protein contents in both algae species. This finding is consistent with a previous study that the protein level is high in *Microcystis aeruginosa* at medium N:P ratios from 18:1 to 50:1 and the reason is that these N:P ratios with high ambient N promote protein synthesis (Downing *et al.* 2005). Similarly, the N content was higher in the N:P ratios of 20:1 to 30:1 (287 µM) than that in 5:1 (72 µM) and 10:1 (144 µM) in both algal species, suggesting that a relatively low N:P ratio with absolutely high N concentration favours a high constituent of protein in algae (Flores & Herrero 2004). Thus, a lack of N would result in a decrease in the rate of protein synthesis (Berdalet *et al.* 1994), as shown in the low protein content under the N:P ratios of 5:1 and 10:1 in the current study. Protein is the major macromolecular pool of intracellular N, and

thus N availability in the medium can influence protein synthesis (Zhao *et al.* 2009). Hence, the N:P ratios from 20:1 to 30:1 are recommended to increase the protein content of algae as long as the N concentration in the medium is sufficient for protein synthesis.

Nitrogen and phosphorus are essential elements for algal metabolism, since their deficiency can affect biochemical synthesis in algae (Kilham *et al.* 1997). A high N:P ratio above 22:1 indicates a P limiting condition (Healey & Hendzel 1979; Nalewajko *et al.* 1981). For *T. lutea* and *N. oculata*, at the N:P ratio of 120:1, the lipid accumulation was higher than at low N:P ratios, indicating that *T. lutea* and *N. oculata* can accumulate lipids at a high N:P ratio. In accordance with our results, *Chlorella* sp. can accumulate a high content of lipids under high N:P ratios of 55:1 and 110:1 (P limitations) compared to the lower N:P ratios of 11:1 and 7:1 (N limitations) (Liang *et al.* 2013). A few algal species, including *Chlorella* spp. (Reitan *et al.* 1994; Liang *et al.* 2013), *Dunaliella* spp. (Gordillo *et al.* 1998; Takagi *et al.* 2006), *Parietochloris incisa* (Bigogno *et al.* 2002), *Neochloris oleoabundans* (Tornabene *et al.* 1983), and *Botryococcus braunii* (Li & Qin 2005), have also been reported to have the capacity of accumulating large quantities of lipids in cells at high N:P ratios. A high N:P ratio usually indicates low P availability and may cause P deficiency for algal growth and alter their lipid biosynthetic pathways towards the formation and accumulation of lipids (Hu *et al.* 2008). When there is a lack of P source in the medium, cell division will be reduced and C source is absorbed continuously by algal cells, leading to high lipid synthesis in the Krebs cycle (Ratledge & Wynn

2002), suggesting that the N:P ratio of 120:1 in this study has a lower P content and thus may have increased the lipid synthesis by stimulating the lipid metabolic pathways. Unfortunately, we did not measure the mechanism of lipid metabolic pathways in algae under a low P concentration. Further research is required to examine the lipid metabolic pathways at high N:P ratios to gain more understanding on the process of lipid synthesis at low P concentrations. The N:P ratios can be manipulated based on the need of cultivation. For instance, if the aim of cultivation is for lipid production, then higher N:P ratios should be used and vice versa (Mayers *et al.* 2014).

In this study, the impact of N:P ratio on the contents of ARA, ALA, EPA and DHA depended on algal species. To our best knowledge, this is the first study reporting the impact of N:P ratios on biochemical properties between algae species that possess different EPA and DHA profiles. The increase of N:P ratio from 20:1 to 120:1 decreased the amount of EPA and DHA in *N. oculata* and *T. lutea*, which is consistent with a previous study where a higher N:P ratio of 555:1 (P limitation) decreased the amounts of EPA and DHA in *Thalassiosira pseudonana* and *Chaetoceros calcitrans* compared to a lower N:P ratio of 14:1 (Harrison *et al.* 1990). Similarly, the EPA and ARA contents in *Phaeodactylum tricornutum* and *Porphyridium cruentum* were higher at the 20:1 N:P ratio than at the 7:1 N:P ratio, while DHA in *T. tutea* and ALA in *Chlorella vulgaris* and *Dunaliella tertiolecta* were increased at the N:P ratio of 20:1 compared with at the N:P ratio of 7:1 (Breuer *et al.* 2012). In comparison, we found that cellular DHA and ALA in *T. lutea* and cellular ARA and EPA in *N. oculata* were low at low

N:P ratios (5:1 - 10:1) and high at N:P ratios of 20:1 to 30:1. Under an unfavourable condition such as low N and low P that inhibits biochemical synthesis, algae are unable to increase the PUFA content by de novo synthesis, but algae in a medium with sufficient nitrogen and phosphorus can more efficiently produce energy-rich PUFA (Solovchenko *et al.* 2008).

This chapter focused on the relative concentrations of N and P needed for algal growth. Nutrient ratios are an important parameter in determining the potential nutrient requirement for algal growth. In addition, the N:P ratio can also affect the biochemical composition of algae. Once the appropriate N:P ratio is set, we can proportionally increase the N and P concentration so that the nutrients in the culture can sustain longer. The N:P ratios in the culture medium were to explore which nutrient was limiting algal growth and influencing the chemical composition. Therefore, when considering the range of N:P ratios, we first fixed P and varied N, then we fixed N and varied P. The reason we used a broad range of N:P ratio was to explore the role of N and P limitation in algal culture on a laboratory scale basis. Furthermore, the results obtained through this study are expected to be referred as the core for basal N:P ratio formula for aquaculturists to use during algal medium preparations.

In conclusion, the N:P ratio significantly affected the growth, cellular elements, and biochemical composition in both *T. lutea* and *N. oculata*. The N:P ratios of 20:1 and 30:1 favoured algal growth and protein content, while the high N:P ratio (120:1) reduced algal growth and protein content but increased lipid in

both algae. The N:P ratio of 20:1 increased the EPA content in *N. oculata* while the N:P ratio of 30:1 increased the DHA content in *T. lutea*. This study indicates that N:P ratio manipulation is an effective strategy to change the nutritional content in algae. Further research is needed to understand the physiological mechanisms of the biochemical changes in *T. lutea* and *N. oculata* under different N:P ratios and to enhance the production of valuable biochemical properties such as lipid and PUFA in algae.

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# Chapter 4

## Effects of dietary microalgae on fatty acids and digestive enzymes in copepod *Cyclopina kasignete*, a potential live food for fish larvae

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## 4.1 Abstract

The copepod *Cyclopina kasignete* is a potential live food in aquaculture and its fatty acid components and digestive enzymes were investigated. Three dry algal products (mixed algae, *Melosira* sp. and *Nannochloropsis oculata*) and two fresh microalgae (*Tisochrysis lutea* and *N. oculata*) were fed to the copepod for 30 days. The essential fatty acids (EFA) in copepods were altered by feeding different types of dry algae. The copepod fed dry *Melosira* sp. or fresh *T. lutea* contained higher eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA), protease and trypsin than fed on other algae. The copepod contained a similar fatty acid profile and digestive enzymes by feeding either dry or fresh *N. oculata*. Between fresh algal species, the copepod fed *T. lutea* contained higher EPA, DHA and ARA than that fed fresh *N. oculata*. The amounts of EPA and DHA in copepods were more concentrated than those in the dietary algae, suggesting that the copepod has the ability to accumulate some essential fatty acids (EFA). This study indicates that dietary algae can modify the nutritional composition and digestive enzymes in copepods, which in turn may be able to transfer suitable nutrients and digestive enzymes to fish larvae in aquaculture.



**Keywords:** algae, digestive enzymes; fatty acids; copepods, live food.

## 4.2 Introduction

Copepods are the main food source for most planktivores at higher trophic levels, such as fish and shrimp (Penchenik 2004; Frederiksen *et al.* 2006). In marine hatchery, copepods can complement the nutrient supply from rotifers and *Artemia* nauplii as they contain higher levels of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and other polyunsaturated fatty acids (PUFA) that are essential for the growth and health of marine fish larvae (Støttrup 2000; McKinnon *et al.* 2003). Copepods are an important live food for fish larvae because of their high PUFA content that is less available in rotifers and *Artemia* (Shields *et al.* 1999; McKinnon *et al.* 2003). It has been proven that the addition of copepod as a live feed can enhance the growth and survival of fish larvae compared to live food with sole rotifers and *Artemia* nauplii (Watanabe *et al.* 1983; Stottrup *et al.* 1999; Shields *et al.* 1999; Payne *et al.* 2001). Therefore, there is an increasing interest on the use of copepods to improve larval fish nutrition (Evjemo *et al.* 2003). However, there is little information on the nutritional manipulation of copepods via their diet, in particular of the algae-copepod nutritional relationship as live food in aquaculture.

Currently, live feeds such as *Artemia* and rotifers have been used widely in the culture of fish larvae. In addition to their inherent nutrition, these live feeds also can be enriched using the oil emulsion containing various amounts of DHA ethyl ester and EPA ethyl ester (Satoh & Takeuchi 2009; Satoh *et al.* 2009b; Satoh *et al.* 2009a). However, some fish larvae require specific nutrients such as a high content of PUFA that rotifers or *Artemia* nauplii do not have even after enrichment. Therefore,

copepods can be used to fill the nutrition gap and serve as an important food for fish larvae.

In larval fish culture, an important approach in fish nutrition is the use of a co-feeding regime where live feed and formulated feed are offered to the same larval fish tank. At first feeding, the digestive system of fish larvae is not ready to process the formulated feed (Kolkovski 2001). In such a feeding scheme, fish can use digestive enzymes from live feed to help digestion of formulated feed (Kolkovski *et al.* 1997; Kolkovski 2001; Zhao *et al.* 2013). Fish larvae utilize enzymes from live prey to facilitate the process of digestion until the larval alimentary system is fully functional and can independently produce digestive enzymes (Jancarik 1964; Kurokawa *et al.* 1998; Lazo *et al.* 2000). In addition to the abundant polyunsaturated fatty acids (PUFAs), copepods contain exogenous digestive enzymes that can directly facilitate the digestion in fish larvae.

In the studies on turbot *Scophtalmus maximus* and Atlantic herring *Clupea harengus* larvae, copepods not only donate their digestive enzymes (protease and trypsin) but also activate zymogens in the larval gut (Pedersen & Hjelmeland 1988; Munilla-Moran *et al.* 1990; Sun *et al.* 2013). It is notable that the enzyme activities in copepods are depending on the types of algae in the food and copepod species (Freese *et al.* 2012). To our best knowledge, only one species of copepod (*Calanus helgolandicus*) has been tested on its digestive enzyme activities when fed different algae, and trypsin and amylase can be accumulated in the digestive track of copepod after feeding on algae (Harris *et al.* 1986). Since the growth and reproduction of

copepods depend on food quality (Freese *et al.* 2012), it is essential to investigate the effects of nutritional compositions of microalgae on fatty acids and digestive enzymes in copepods.

Unlike rotifers and *Artemia*, the nutrient content in copepods cannot be manipulated through existing enrichment techniques (Rasdi & Qin 2014). Due to the sensory chemoreceptors on the mouthpart setae, copepods show rejection, avoidance and morbidity when exposed to some excess chemical substances in the nutritional enrichment product (Huntley *et al.* 1986; Kleppel & Burkart 1995). Therefore, the change of copepod nutrition has to be done through feeding copepods with different types of diet. Moreover, the ability of copepods to synthesize some PUFAs is limited (Kainz *et al.* 2004; Burns *et al.* 2011). Some copepods are able to convert unsaturated fatty acids between fatty acid families, but most calanoid copepods cannot elongate and desaturate α-linolenic acid (ALA) (18:3n-3) to produce significant amounts of longer chain PUFA (Støttrup & Jensen 1990; Bell *et al.* 2007; Jonasdottir *et al.* 2009). Therefore, some essential fatty acids have to be supplied from microalgae (Von Elert 2002) to support copepods for the need of reproduction and development.

There is a motivation to identify which kind of algae that can be used to enrich or change the content of copepod nutrition. Most studies have used fresh algae, but there is little information for using dry microalgae to feed zooplankton (Lubzens *et al.* 1995; Mostary *et al.* 2010). Although non-living microalgae are unlikely to provide adequate nutrition for zooplankton growth and reproduction (Baer & Goulden 1998; Mostary *et al.* 2007), dry microalgae is still a good option to feed

copepods when the supply of fresh algae is insufficient (Mostary *et al.* 2010). In a recent study, the 20:1 N:P ratio in the culture medium favoured EPA production in *Nannochloropsis oculata* and the 30:1 N:P ratio favours DHA production in *Tisochrysis lutea* (Rasdi & Qin 2015). However, the algae cultivated from the optimized nutrition regime have not been tested as live food to feed copepods.

The objective of this study was to understand the impact of different microalgae diets on nutritional and enzymatical responses of copepods. Specifically, we aimed to understand (1) if the nutritional composition and digestive enzymes in copepods are affected by dietary algae; and (2) whether copepods are able to accumulate certain fatty acids from the microalgae diets. The understanding of these key issues will help improve copepod nutrition condition and its digestive enzymes as live feed for fish larvae in aquaculture.

## 4.3 Materials and method

### *4.3.1 Experimental organisms*

The copepod *Cyclopina kasignete* was obtained from a continuous culture that has been maintained over 100 generations in the Aquaculture Laboratory at Flinders University since October 2012. Berried females were originally collected from the Arno Bay Jetty at Eyre Peninsula, South Australia. Since then a laboratory population was maintained at 20–22 °C and 22–23 practical salinity units (PSU) at an irradiance level of 115 μE $m^{-2}$ $s^{-1}$ with a 12⁄12 h photoperiod. The culture medium was prepared from filtered seawater and the desired salinity was obtained by the addition of demineralized water. The *C. kasignete* were fed with the same amount of fresh *N.*

*oculata* and *T. lutea* cultures (1:1 by number) at least for 2 weeks prior to the feeding experiment. The microalgae were cultured using the recommended N:P ratio of 20:1 for *N. oculata* and 30:1 for *T. lutea* (Rasdi & Qin 2014).

### *4.3.2 Experimental designs*

Five types of microalgae (two fresh and three dry algae) with different composition of fatty acids (Table 4.1) were separately provided to *C. kasignete* in the feeding trial with three replicates each. All the dry microalgae were contributed by James Cook University/MBD Microalgae R&D Facility. Gravid females of *C. kasignete* were isolated from the stock culture and placed in a beaker filled with seawater medium before copepod hatch. Nauplii were used only after 24 - 48 h of hatch. The nauplii were separately fed each of the five different algae treatments in 1 litre jars with three replicates until the adult stage reached. Three dry algae types included a mixed green algae product (*Schroederiella apiculata, Scenedesmus pectinatus, Tetraedrom minimum, Mesotaenium* sp. and *Desmodesmus* sp.), dry *Nannochloropsis oculata* and dry *Melosira* sp. Each type of dry algae was fed to the copepod at a daily ration of 4.18 µg dry weight $mL^{-1}$ which is equivalent to 3.16 µg C $mL^{-1}$ and $1.67 \times 10^5$ algal cells $mL^{-1}$. The fresh *Tisochrysis lutea* and *Nannochloropsis oculata* were cultured in the laboratory following a previous protocol in Rasdi & Qin (2015) and were separately fed to the copepod in the equivalent daily ration of the dry algae at 3.16 µg C $mL^{-1}$ (Espinosa-Rodríguez *et al.* 2012). The experimental jars were exposed to an irradiance of 115 µE $m^{-2}$ $s^{-1}$ provided with fluorescent tubes on a 12:12 hour light-dark cycle. Mean temperature was 23 ±

1°C in the light period and 20 ± 1°C in the dark period. Continuous aeration was also provided to the experimental jars to ensure homogeneous distribution of food and oxygen supply.

*4.3.3 Fatty acid analysis in algae and copepods*

Samples for quantifying fatty acids in algae were centrifuged at 7000×*g* at 15 °C for 15 min to obtain concentrated algal pellets and were freeze-dried prior to analysis after the experiment lasted 30 days. Lipids were extracted with the chloroform-methanol method (Bligh & Dyer 1959; Martínez-Fernández *et al.* 2006). The fatty acid content was measured and then the samples were methylated in 5 mL of 1% $H_2SO_4$ in methanol at 70 °C for 3 h. The fatty acid methyl esters were extracted by adding 750 µL distilled water and 2 mL of n-heptane. The heptane layer was transferred to a 2-mL vial for analysis using gas chromatography (GC, PerkinElmer Gas Chromatograph Clarus 500). Fatty acid methyl esters (FAMEs) were separated and measured on the GC equipped with a 30-m capillary column (0.32-mm internal diameter, Zebron ZB-FFAP). Helium was the carrier gas (1.5 mL $min^{-1}$). The injector temperature was set at 250 °C and the detector temperature at 300 °C. The initial oven temperature was 140 °C for 3 min, then ramped at 1:10.0 $min^{-1}$ from 1 – 160 °C for 5 min followed by 10.0 $min^{-1}$ from 1 – 230 °C for 10 min. FAMEs were identified on the GC using software of TotalChrom Navigator (version 6.3.2 0646, Perkin Elmer Inc., Shelton, CT, USA). Heptadecanoic acid (17:0, Sigma-Aldrich, St. Louis, MO, USA) was used as the internal standard to calculate the

FAME concentration in each sample. The results were expressed as a percentage of total fatty acids (FAME) in 1 g dry weight of sample.

Adult copepods (50-200) were collected on a 120-µm mesh net and transferred to new jars (Nanton & Castell 1999). Copepods were starved for 24 h to allow for the clearance of dietary algae present in the gut and rescreened to remove any accumulated waste products. Over 200 animals per replicated jar were isolated in this manner for fatty acids analysis. Subsequently, the copepods were captured on Whatman filter paper and rinsed with distilled water prior to freeze drying for fatty acids analysis. The rest procedures for fatty acid analysis were the same to those used in algae.

*4.3.4 Enzyme assays in C. kasignete*

In this study, total protease and trypsin were analysed as these enzymes have been detected as exogenous digestive enzymes in copepods (Kolkovski 2001) that aid in fish larval digestion (Zaleha & Busra 2012). Copepods were homogenized in five volumes (v/w) of ice-cold distilled water. The mixtures including extracts of copepods and substrate were incubated and then the reaction was stopped by adding 500 µl trichloroaceticacid (TCA) at 120 g $L^{-1}$ (Kumar *et al.* 2005; Mitra *et al.* 2007). Trypsin and protease were assayed according to Naz (2008) and Kumar *et al.* (2005) respectively. Samples for protease and trypsin activities were expressed as micromoles of substrate hydrolysed per minute per milligram protein (i.e., Units per mg of protein; U $mg^{-1}$ protein). The absorbance was read at 410 nm for 3 min and

366 nm for 5 min for trypsin and protease assays respectively (Kumar *et al*., 2005).

All measurements were carried out in triplicate.

**Table 4.1** Fatty acid compositions of five different dietary algae. All values are mean ± standard deviation ($n$ = 3). Fatty acid species in bold letters are fully described in the text. Values are in per g fatty acids ($100g^{-1}$ total fatty acids).

| **Fatty acids** | **Dry *Melosira* sp.** | **Mixed dry algae** | **Dry *Nannochlopsis oculata*** | **Fresh *Nannochloropsis oculata*** | **Fresh *Tisochrysis lutea*** |
|---|---|---|---|---|---|
| ***Saturated*** | | | | | |
| **C12:0** | 0 | 0 | 0 | 0 | 0 |
| **C14:0** | 9.38 ± 0.04 | 0.58 ± 0.01 | 4.89 ± 0.02 | 1.27 ± 0.01 | 23.37 ± 0.25 |
| **C16:0** | 15.72 ± 0.07 | 17.19 ± 0.09 | 24.00 ± 0.29 | 19.71 ± 0.37 | 10.59 ± 0.20 |
| **C18:0** | 0.66 ± 0.01 | 1.05 ± 0.01 | 0.61 ± 0.01 | 0 | 0 |
| **C20:0** | 0 | 0 | 0 | 0 | 0 |
| **C24:0** | 0 | 1.03 ± 0.02 | 0 | 0 | 0 |
| **Sum** | 25.76 | 19.85 | 29.50 | 20.98 | 33.96 |
| ***Monosaturated*** | | | | | |
| **C16:1** | 30.21 ± 0.10 | 0.46 ± 0.03 | 26.85 ± 0.10 | 26.62 ± 0.14 | 4.82 ± 0.14 |
| **C18:1[c]** | 0.39 ± 0.16 | 7.22 ± 0.01 | 5.40 ± 0.08 | 2.04 ± 0.06 | 17.26 ± 0.14 |
| **C20:1[c]** | 0 | 0 | 0 | 0 | 0 |
| **C22:1[c]** | 0 | 0 | 0 | 0.19 ± 0.10 | 0.77 ± 0.15 |
| **Sum** | 30.60 | 7.68 | 32.25 | 28.85 | 22.85 |
| ***Polyunsaturated*** | | | | | |
| **C18:2[b]** | 0.43 ± 0.01 | 5.82 ± 0.10 | 1.94 ± 0.02 | 3.41 ± 0.09 | 4.02 ± 0.06 |
| **C18:3[a](ALA)** | **2.39 ± 0.02** | **38.02 ± 0.02** | **0.16 ± 0.01** | **0.79 ± 0.13** | **6.44 ± 0.10** |
| **C18:4[a]** | 0 | 0 | 0 | 0.35 ± 0.01 | 15.50 ± 0.15 |
| **C20:2[b]** | 0 | 0 | 0 | 0 | 0 |
| **C20:4[b](ARA)** | **1.95 ± 0.05** | **0.88 ± 0.03** | **6.90 ± 0.04** | **6.31 ± 0.11** | **0.42 ± 0.10** |
| **C20:5[a] (EPA)** | **23.09 ± 0.04** | **1.01 ± 0.06** | **25.93 ± 0.50** | **38.67 ± 0.06** | **0.43 ± 0.06** |
| **C22:5[b]** | 0 | 0 | 0 | 0 | 0 |
| **C22:6[a] (DHA)** | **2.42 ± 0.06** | **0** | **0** | **0.34 ± 0.25** | **6.02±0.15** |
| **Sum (PUFA)** | 30.28 | 45.73 | 34.93 | 49.87 | 32.83 |

Value of 0 indicates that FA was not detected.
[a]ω-3 fatty acids, [b]ω-6 fatty acids, and [c]ω-9 fatty acids.
ALA: alpha-linolenic acid; ARA: Arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

Enzyme activity was calculated by the following equation (Kumar *et al.* 2005) :

$$\text{Activity} = \frac{\Delta \text{ Abs (410 nm) /min} \times 1000 \times \text{volume of reaction mixture}}{8800 \times \text{mg protein in the reaction mixture}}$$

## 4.4 Data analysis

Results are given as mean ± standard deviation (SD). All statistical analyses were conducted using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Comparisons of fatty acids and enzymatic activities in different algal diets were made using one-way analysis of variance (ANOVA), and *t*-test was used to compare the essential fatty acids (EFA) contents between dietary algae and copepods. Differences were considered significant at the $P < 0.05$ level. When the main treatment effect was significant, *post-hoc* comparisons were made using Tukey's test. All the data were tested for normality, homogeneity and independence to satisfy the assumptions for ANOVA.

## 4.5 Results

### *4.5.1 Fatty acid composition of copepods*

Five types of saturated fatty acids were found in copepods fed different algae (Table 4.2). The amount of palmitic acid (C16:0) was higher in the copepod fed the mixed dry microalgae (30.42 ± 0.09) than dry *Melosira* sp. (20.33 ± 0.07) or dry *N. oculata* (29.84 ± 0.06, $P < 0.05$). Copepods fed fresh or dry *N. oculata* had similar contents of C16:0 fatty acids ($P > 0.05$). Copepods fed fresh *N. oculata* contained more C16:0 fatty acids than those fed fresh *T. lutea* ($P < 0.05$).

Three types of monosaturated fatty acids were detected in copepods (Table 4.2). The amounts of C16:1 fatty acid in the copepod fed the mixed dry algae (30.00 ± 0.02) or dry *N.oculata* (30.20 ± 0.09) were higher than those fed *Melosira* sp. (20.38 ± 0.03; $P < 0.05$, Table 4.2), but the C18:1 fatty acid in the copepods fed *Melosira* sp. was higher (3.43 ± 0.01) than those fed other two types of dry algae ($P < 0.05$). When fed dry *N. oculata*, copepods had higher C16:1 fatty acid, but lower C18:1 and C20:1 fatty acids than those fed fresh *N. oculata* ($P < 0.05$). Copepods fed *T. lutea* contained higher C16:1 fatty acid, but lower C18:1 and C20:1 fatty acids than those fed fresh *N. oculata* ($P < 0.05$).

Among seven types of polyunsaturated fatty acids in copepods, α-linolenic acid (ALA), α- arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were the major essential fatty acids for fish larvae. Copepods fed dry *Melosira* sp. contained higher ARA, EPA and DHA than those fed either mixed dry algae or dry *N. oculata* ($P < 0.05$, Table 4.2). The ALA content was not significantly different in copepods fed different dry algal diets ($P > 0.05$). There was no significant difference in EPA and DHA between copepods fed dry *N. oculata* and fresh *N. oculata* ($P > 0.05$; Table 4.2). Copepods fed fresh *T. lutea* contained higher ARA, EPA and DHA than those fed fresh *N. oculata* ($P > 0.05$, Table 4.2).

**Table 4.2** Fatty acid compositions of *Cyclopina kasignete* fed different dietary algae. All values are mean ± standard deviation ($n$ = 3). Major fatty acid components are shown in bold letters. Fatty acid species in bold letters are fully discussed in the text. Values are in per g fatty acids (100 $g^{-1}$ total fatty acids).

| Fatty acids | Dry *Melosira* | Mixed dry algae | Dry *N. oculata* | Fresh *N. oculata* | Fresh *T. lutea* |
|---|---|---|---|---|---|
| ***Saturated*** | | | | | |
| **C12:0** | 0.45 ± 0.01 | 0.14 ± 0.03 | 0.18 ± 0.10 | 0.16 ± 0.07 | 0.44 ± 0.01 |
| **C14:0** | 2.02 ± 0.01 | 9.97 ± 0.52 | 8.83 ± 0.11 | 8.72 ± 0.05 | 5.01 ± 0.01 |
| **C16:0** | **20.33 ± 0.07** [a] | **30.42 ± 0.09** [b] | **29.84 ± 0.06** [c] | **29.89 ± 0.17** [d,c] | **20.10 ± 0.06** [e, a] |
| **C18:0** | 0.64 ± 0.07 | 0.83 ± 0.05 | 0.88 ± 0.03 | 0.86 ± 0.01 | 0.62 ± 0.06 |
| **C24:0** | 0.20 ± 0.01 | 0 | 0.13 ± 0.01 | 0.47 ± 0.16 | 0.21 ± 0.01 |
| **Sum** | 23.64 | 41.36 | 39.86 | 40.10 | 26.38 |
| ***Monosaturated*** | | | | | |
| **C16:1** | **20.38 ± 0.03**[a] | **30.00 ± 0.02**[b] | **30.20 ± 0.09** [c] | **22.72 ± 0.09** [d] | **24.10 ± 0.06** [e] |
| **C18:1**[c] | 3.43 ± 0.04 | 0.49 ± 0.04 | 0.38 ± 0.11 | 4.92 ± 0.12 | 3.54 ± 0.09 |
| **C20:1**[c] | 0.17 ± 0.01 | 0.38 ± 0.04 | 0.35 ± 0.03 | 0.34 ± 0.11 | 0.17 ± 0.01 |
| **C22:1**[c] | 0 | 0 | 0 | 0 | 0 |
| **Sum** | 23.98 | 30.87 | 30.93 | 27.98 | 27.81 |
| ***Polyunsaturated*** | | | | | |
| **C18:2**[b] | 2.49 ± 0.17 | 0.60 ± 0.11 | 1.34 ± 0.10 | 1.54 ± 0.11 | 2.68 ± 0.15 |
| **C18:3**[a]**(ALA)** | **0.11 ± 0.08** [a] | **0.12 ± 0.07** [a] | **0.17 ± 0.06** [a] | **0.33 ± 0.06** [b, a] | **0.17 ± 0.06** [c, a] |
| **C18:4**[a] | 0.76 ± 0.01 | 0.31 ± 0.07 | 0.23 ± 0.02 | 0.58 ± 0.04 | 0.76 ± 0.01 |
| **C20:4**[b]**(ARA)** | **5.78 ± 0.08** [a] | **1.36 ± 0.05** [b] | **1.56 ± 0.02** [c] | **3.05 ± 0.64** [d] | **6.02 ± 0.15** [e,a] |
| **C20:5**[a]**(EPA)** | **27.50 ± 0.84**[a] | **18.54 ± 0.28** [b] | **25.28 ± 0.52** [c] | **25.52 ± 0.18** [d,c] | **27.71 ± 0.30** [e,a] |
| **C22:5**[b] | 0 | 0 | 0 | 0 | 0.0 |
| **C22:6**[a]**(DHA)** | **5.78 ± 0.08** [a] | **2.52 ± 0.15** [b] | **0.52 ± 0.07** [c] | **0.54 ± 0.09** [d, c] | **8.20 ± 0.10** [e] |
| **Sum (PUFA)** | 42.42 | 23.45 | 29.10 | 31.56 | 45.54 |

Value of 0 indicates that FA was not detected. Different letters indicate significant difference between treatments (a,b,c and d).
[a]ω-3 fatty acids, [b]ω-6 fatty acids, and [c]ω-9 fatty acids.
ALA: alpha-linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

*4.5.2 EPA, DHA, ARA and ALA contents in algae and copepods*

There was a significant difference in the contents of essential fatty acids between algae and copepods in all feeding treatments except in ALA and EPA contents of the copepod fed dry *N. oculata* ($P < 0.05$, Fig. 4.1).The EPA contents in copepods were higher than in algal diets when copepods fed the mixed dry algae, dry

*Melosira* sp. or fresh *T.lutea* ($P < 0.05$). The content of DHA in copepods fed dry *Melosira* sp., mixed dry algae, fresh *T. lutea*, dry *N. oculata* or fresh *N. oculata* was significantly higher than that in the diets ($P < 0.05$). The ALA contents in copepods were low in all feeding treatments ($P < 0.05$, Fig. 4.1). The ARA contents in copepods were higher than in algal diets when copepods fed dry *Melosira* sp., mixed dry algae or fresh *T. lutea* ($P < 0.05$).

### *4.5.3 Protease and trypsin activities in copepods*

There was a significant difference in the protease and trypsin activities of copepods in all feeding treatments ($P < 0.05$, Table 4.3) except in those fed fresh *N. oculata* or dry *N. oculata* ($P > 0.05$). The production of digestive enzymes in copepods depended on the types of dietary algae ($P < 0.05$, Table 4.3). For instance, copepods fed dry *Melosira* sp. showed significantly higher protease and trypsin activities ($55.52 \pm 0.07$ U mg $^{-1}$ protein and $36.92 \pm 0.03$ U mg $^{-1}$ protein, respectively) than copepods fed the mixed dry algae or dry *N. oculata* ($P < 0.05$). Copepods fed fresh *N. oculata* or dry *N. oculata* produced a similar amount of trypsin ($25.48 \pm 0.36$ U mg $^{-1}$ protein, $25.30 \pm 0.46$ U mg $^{-1}$ protein, respectively) and protease ($36.12 \pm 0.16$ U mg $^{-1}$ protein and $35.97 \pm 0.12$ U mg $^{-1}$ protein, respectively, $P > 0.05$). Furthermore, protease and trypsin activities in copepods were significantly different between copepods fed fresh *T. lutea* and fresh *N. oculata* ($P > 0.05$, Table 4.3) and both digestive enzymes in copepods were higher when copepods were fed with fresh *T. lutea* than those fed with fresh *N. oculata.*

**Table 4.3** Protease and trypsin activities of *Cyclopina kasignete* fed different dietary algae. All values are mean ± standard deviation ($n$ = 3). Different letters indicate significant differences between feeding groups at $P < 0.05$ level. Values are in U $mg^{-1}$ protein.

| Enzyme activities | Dry *Melosira* | Mixed dry algae | Dry *N. oculata* | Fresh *N. oculata* | Fresh *T. lutea* |
|---|---|---|---|---|---|
| **Protease** | 55.52 ± 0.07 [a] | 6.56 ± 0.03 [b] | 35.97 ± 0.10 [c] | 36.12 ± 0.16 [d,c] | 39.25 ± 0.05 [e] |
| **Trypsin** | 36.92 ± 0.03 [a] | 3.99 ± 0.02 [b] | 25.30 ± 0.46 [c] | 25.48 ± 0.36 [d,c] | 32.79 ± 0.22 [e] |

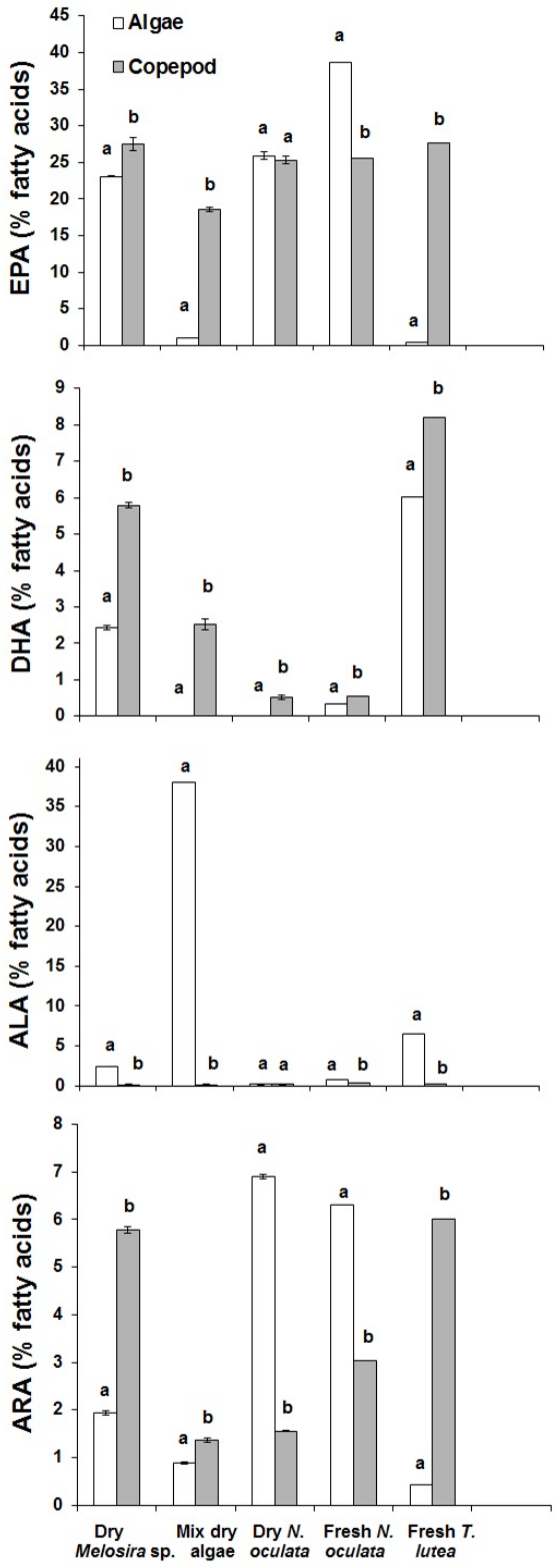
Algae
Copepod
EPA (% fatty acids)
DHA (% fatty acids)
ALA (% fatty acids)
ARA (% fatty acids)
Dry Melosira sp.
Mix dry algae
Dry N. oculata
Fresh N. oculata
Fresh T. lutea

**Fig. 4.1**. Production of EPA, DHA, ARA and ALA by *Cyclopina kasignete* corresponding to different dietary algae. Different letters represent significant differences ($P < 0.05$) between each microalgae diets and copepods on EPA, DHA, ARA and ALA production. Blank and grey bars represent the FA in algae and copepod, respectively.

## 4.6 Discussion

In this study, the food types have significant impacts on the profiles of saturated, monosaturated and PUFAs in copepods. The contents of PUFAs including EPA, DHA and ARA in copepods depended on the type of dry algae. Copepods fed the dry diatom (*Melosira* sp.) produced higher contents of EPA, DHA and ARA than those fed other dry microalgae, which is consistent with a previous study that copepods fed the diatom *Thalassiossira weisflogii* (Veloza *et al.* 2006) contain high PUFA in their body.

There was no difference on the essential fatty acid (EFA) contents in copepods fed either dry *N. oculata* or fresh *N. oculata* in this study. Likewise, rotifers fed dry or frozen *Nannochloropsis* sp. produced similar EFA as those fed live microalgae (Lubzens *et al.* 1995; Mostary *et al.* 2010). Moreover, the larval seabream *Sparus aurata* achieved a similar growth rate by feeding rotifers enriched either by live or dry *N. gaditana* (Cañavate & Fernández-Díaz 2001). As reported by Lubián & Yúfera (1989), the buoyancy of live or dry algae cells determines the success of rotifer culture. Due to the small and spherical shape, *Nannochloropsis* sp. may provide

similar accessibility to rotifers regardless of a fresh or a frozen form (Lubián 1981). In the present study, the fatty acid composition was different between fresh and dry *N. oculata*. Fresh *N. oculata* contained higher proportions of PUFA than dry *N. oculata*. In general, dried microalgae are subject to reduction in food quality due to the change in biochemical composition in algal cells, but the nutritional value of dried algae for copepods is still controversial (Albentosa *et al.* 1997; Dobberfuhl & Elser, 1999). In previous studies, the use of dried diatom resulted in lower growth rates (Cnudde *et al.* 2011) and nutritional value (Albentosa *et al.,* 1997) in harpacticoid copepods. In hatchery, the unexpected crash of algal cultures will impact the live feed supply to zooplankton and negatively influence the fish production in hatcheries (Mostary *et al.,* 2010). We found that the nutritional quality of copepods fed on dried algae was comparable to those fed on fresh algae especially in the EPA and DHA contents in copepods. Thus it is possible to use dried algae to maintain copepod cultures in short supply of fresh algae (Martinez & Chavez, 1994). Despite the low contents of EPA, DHA and ARA in dry *Melosira* sp. and mixed dry algae, copepods showed higher EPA, DHA and ARA when fed on these corresponding algae than other diets. This is similar to the study on the harpacticoid copepod *Attheyella trispinosa* where EPA and DHA were detected in the copepods despite the negligible amounts of these PUFA in the dietary algae *Leptolyngbya foveolarum* (Norsker & Stottrup 1994; Nanton & Castell 1998; Nanton & Castell 1999; Caramujo *et al.* 2008). In present study, the absence or a low of DHA and EPA in dry *Melosira* sp. resulted in high EPA and DHA in copepods, suggesting that copepods can accumulate these PUFAs.

It has been reported that EPA and DHA can be synthesized from 18:3n-3 (ALA) as reported in other copepods species such as *Tachidius discipes* and *Tisbe* sp. (Norsker & Stottrup 1994; Nanton & Castell 1998; Nanton & Castell 1999; Caramujo *et al.* 2008). Moreover, Desvilettes *et al.* (1997) have also suggested that the cyclopoid copepod *Paracyclopina nana* was able to convert ALA to EPA and DHA when fed on *Tetraselmis suecica*, an ALA rich green microalga, which coincides with the result of our study on *C. kasignete.* Therefore, cyclopoid copepods may incorporate dietary ALA from microalgae into their own EPA and DHA.

This study also showed that the content of digestive enzymes in copepods was associated with the types of dietary microalgae. The highest protease and trypsin activities were achieved when the copepod was fed with dry *Melosira* sp. Our result is consistent with a study on copepod *Tisbe biminiensis* that some diatoms species (*Thalassiosira* sp. and *Chaetoceros muelerii*) can increase the amount of trypsin and proteases in copepods (França *et al.* 2010). This study suggests that copepods fed dry *Melosira* sp. can produce higher amounts of protease and trypsin compared with those fed other dry algae, and copepods contain similar fatty acids and digestive enzymes by feeding fresh or dry *N. oculata.* Furthermore, fatty acids and digestive enzymes in copepods are dependent on the types of fresh algae as food. The digestive enzymes found in the copepod from our study are very likely to be donated to the first feeding fish larvae before the fish enzymatic system is established.

This study attempts to link the digestive enzymes of trypsin and protease in the copepod and its food. The levels of digestive enzymes in copepods were not different

between copepods fed dry *N. oculata* and fresh *N. oculata*. However, there was a significant difference between copepods fed fresh *N. oculata* and fresh *T. lutea*. A previous study reported that the difference of enzymatic responses in copepods depends on the nutritional quality of algae (Kreibich *et al.* 2011), which is consistent with our results. For instance, in this study the dry *Melosira* sp. and fresh *T. lutea* with high EFA enabled copepods to contain a higher level of digestive enzymes than other microalgae diets. Therefore, it is likely that the protease and trypsin in copepods are closely associated with the EFA content in the diets. Although the content of EFA can be used as an indicator of the food quality of microalgae (Brett & Muller-Navarra 1997), further study is still required to verify the amount of PUFA for the minimum requirement in copepods.

Our results showed that the protease activities in copepods ranged from 6.56 ± 0.03 to 55.52 ± 0.07 U mg $^{-1}$ proteins. The highest content in copepods was obtained when the animals were fed dry *Melosira* sp. and the lowest was from the animal fed mixed dry microalgae. The level of protease detected in this study was higher than in other zooplankton species such as *Daphnia carnata* (0.28 - 0.55 U mg $^{-1}$ protein) (Kumar *et al.* 2005) , *Calanus sinicus* (5 - 16.5 U mg $^{-1}$ protein) (Han *et al.* 2002), and *Daphnia magna* (7.5 - 40.3 U mg $^{-1}$ protein) (Agrawal *et al.* 2005). The trypsin levels in the copepod was also dependent on microalgae diets, but the level of trypsin (between 3.99 ± 0.02 and 36.92 ± 0.03 U mg $^{-1}$ protein) in the copepod was similar to other zooplankton species, such as 0.21 - 47.3 U mg $^{-1}$ protein in *Tisbe biminiensis* (Franca *et al.* 2010), and 9.3 U mg $^{-1}$ protein in *Artemia salina* and 47.3 U mg $^{-1}$

protein in *Moina* sp. (Lauff 1983). Since the overall amount of digestive enzymes (protease and trypsin) in the copepod was higher than other zooplankton as above, it is possible that the copepod can contribute more digestive enzymes to fish larvae, promote the digestive activity, and thus improve the growth and survival of fish larvae at the early stage.

In conclusion, the amounts of fatty acids and digestive enzymes in copepods can be modified by feeding copepods with different dietary algae. The copepod fed dry *Melosira* sp. and fresh *T. lutea* had higher PUFA (EPA, DHA and ARA) and digestive enzymes (protease and trypsin) than those fed other microalgae. The EFA profile and content in copepods were similar between the copepods feeding dry *N. oculata* and fresh *N. oculata.* The contents of EPA and DHA in copepods are higher regardless of the low contents of these PUFAs in the algal diets. As shown from the results, the copepods have higher EPA and DHA after ingesting algae with high ALA content, suggesting that copepods might have the ability to accumulate and bio-convert ALA in the diets to EPA and DHA. The results are adequate to show the differential quantities of EPA and DHA between the diets and copepods. Furthermore, copepods had higher levels of EPA and DHA than those in microalgae diets, suggesting that the copepod is able to accumulate essential fatty acids. This study indicates the potential of using microalgae to improve the nutritional composition and digestive enzyme contents in copepods. As a result, it may provide suitable nutrition and essential digestive enzymes to fish larvae.

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# Chapter 5

## Impact of food type on growth, survival and reproduction of the cyclopoid copepod *Cyclopina kasignete* as a potential live food in aquaculture

This chapter has been submitted as:

Rasdi N.W., Qin J.G., Li Y. (2015) Impact of food type on growth, survival and reproduction of the cyclopoid copepod *Cyclopina kasignete* as a potential live food in aquaculture. Submitted to *Aquaculture Research* (under review).

## 5.1 Abstract

This study compared the efficacy of different dietary algae on the growth and reproduction of the cyclopoid copepod *Cyclopina kasignete*, a potential live food species for fish larvae in aquaculture. The experimental diets for the copepod consisted of three monoalgal diets (*Nannochloropsis oculata*, *Tisochrysis lutea* and dry *Melosira* sp.) and two mixed algae diets (*T. lutea* + *N. oculata*, *T. lutea* + dry *Melosira* sp.). The experiment was carried out for 30 days and the population growth, survival and reproductive performance (generation time, hatching rate, life spawning times, daily offspring production, eggs per sac, lifespan and sex ratio) were used to assess the responses of *C. kasignete* to different food types. Population growth, survival and reproductive capacities of *C. kasignete* were significantly affected by the mono and binary species of algal diets. The results showed that copepods exhibited superior growth, survival and productivity when fed on fresh *T. lutea*, dry *Melosira* sp., and a mixture of both species compared to other dietary treatments. Copepods produced comparable growth, survival and productivity when fed on diatoms (dry *Melosira* sp.) as a single or in combination with other algae. This study indicates that cyclopoid copepod *C. kasignete* grow fast and have the potential to serve as a live food for aquaculture. The algae *T. lutea*, dry *Melosira* sp. and their combination are appropriate food to sustain the growth and reproduction of this copepods in mass culture as a potential live food in fish hatchery.



**Keywords:** algae; zooplankton, live food, copepod.

## 5.2 Introduction

Copepods are a common live feed for most fish larvae in the wild (Støttrup 2000). The nutritional values of copepods to fish larvae is known to be superior to rotifer *Branchionus* spp. and brine shrimp *Artemia* spp, though the latter two are often used in aquaculture hatcheries (Milione & Zeng 2008). However, the currently available live feed organisms may not be suitable for the size and nutrition required by some fish species such as bluefin tuna (De Lima *et al.* 2013; Solgaard *et al.* 2007) as these fish larvae primarily feed on planktonic copepods in the ocean that are not usually available as live feed in most hatcheries (VanderLugt & Lenz 2008). The use of copepods as supplemental live feed can improve the survival for a variety of fish larvae (Sipaúba-Tavares & Pereira 2008; Drillet *et al.* 2006; Rajkumar & Kumaraguruvasagam 2006; Camus & Zeng 2009). Copepods, therefore, have been considered a promising candidate as a live feed for the culture of highly valued and delicate marine fish species in hatchery (Qin 2013).

Calanoids and harpacticoid copepods have been the major targets for mass culture. Most calanoid copepods are pelagic and can only be cultured at a low density, while harpacticoid copepods can be cultured at a higher density but their benthic habitat limits the access to the pelagic fish larvae (Lee *et al.* 2013). Cyclopoid copepod cultures have also been tried on several species such as *Oithona* sp. and *Paracyclopina nana* (Lee *et al.* 2006), and the results have been encouraging since cyclopoids are able to be produced at high densities, and they are planktonic and reachable by pelagic fish larvae.

Food quality is a regulating factor for the growth and reproduction of copepods (Peterson 2001). Development of nauplius copepods at early stage appears to be less sensitive to food quantity, whereas the quality of food is important for juvenile and adult copepods (Daase *et al.* 2011). The biochemical composition of algae affects the development of copepods nauplii at late stages due to either the presence of inhibitory compounds (Carotenuto *et al.* 2002; Ianora *et al.* 2004) or the lack of essential nutritional components for copepod development (Breteler *et al.* 2005). Polyunsaturated fatty acids, in particular, the long-chained omega-3 fatty acids EPA and DHA, are produced exclusively in marine algae, and play a vital role in reproduction, growth and metabolism of marine zooplankton (Daase *et al.* 2011). The usefulness of omega-3 fatty acids for copepod offspring production, egg hatching success and growth has been reported in the field (Dutz *et al.* 2005) and small-scaled experiments (Dutz *et al.* 2005; Jónasdóttir *et al.* 2009). However, the response of copepod growth and productivity has been rarely tested across a variety of mono or binary algal diets in dry or fresh forms.

As diatoms comprise the major portion of phytoplankton in the ocean, diatoms have been used as food for most copepod species in culture conditions (Koski *et al.* 2008; Wichard *et al.* 2008; Jeyaraj & Santhanam 2013). For instance, several diatoms such as *Nitzschia closterium* and *Chaetoceros muelleri* have been successfully used to feed some species of copepods such as *Tisbe bimiensis* (Pinto *et al.* 2001) and *Euterpina acutifrons* (Camus & Zeng 2012). Diatoms can provide sufficient nutrients to support copepod growth especially during the early stages of nauplii (Carotenuto *et*

*al.* 2012), but there are still some contradictory results such as low egg hatching rates in copepods after feeding on diatoms (Ianora *et al.* 2003). Traditionally, the use of dry diatom in copepod culture is not common due to its little supply for zooplankton mass culture. With the recent development on algal biotechnology, however, there is no doubt that microalgae can be cultured at a large scale for producing either algal paste or dry algal powder for marketing as functional aquafeed (Li *et al.* 2015) .

In a previous study, we found that a cyclopoid copepod (*Cyclopina kasignete*) contained 27% EPA and possessed high quantity of digestive enzymes after feeding on dry *Melosira* sp., fresh *N. oculata*, and fresh *T. lutea* (Rasdi *et al.* 2015). However, the growth and reproductive responses of this copepod to food quality have not been studied. The objective of this study was to evaluate the effects of different mono and binary algal diets on the growth, survival and reproduction of this copepod. Specifically, we aimed to understand (1) if the growth and population density of this copepod are affected by the mono and binary algal diets; and (2) whether the algal diets affect the survival of copepods from hatching to the adult stage; (3) if the algal diets affect the key reproductive parameters related to the productivity of the copepod *C. kasignete*.

## 5.3 Materials and method

### *5.3.1 Copepod stock culture*

*Cyclopina kasignete was* originally collected using a plankton net from the Arno Bay Jetty at Eyre Peninsula, South Australia. The *C. kasignete* were continuously cultured and sustained in our laboratory since October 2012 at 20–22 °C

and 22–23 practical salinity units (PSU) at an irradiance level of 115 μE $m^{-2}$ $s^{-1}$ with a 12⁄12 h photoperiod. The culture medium was prepared from filtered seawater and the desired salinity was obtained by the addition of demineralised water. The *C. kasignete* were fed with mixed microalgae including *N. oculata* and *T. lutea* (1:1 in ratio by number) for at least 2 weeks prior to the experiment. During stock maintenance, each culture was examined daily; all exuvia and any dead individuals were removed; and up to 30% of water was also changed daily.

### *5.3.2 Experimental design*

A total of five diet treatments including three single algal diets (*N. oculata*, *T. lutea* and dry *Melosira* sp.) and two mixed algae treatments composed of two binary algae diets (dry *Melosira* sp. + *T. lutea*, and *T. lutea* + *N. oculata*) were set up (Table 5.1). The *C. kasignete* were transferred from the stock culture to 1 L beakers with respective diets for at least one generation to remove any effects of previous diets on the copepods. The preconditioning stocks were placed under the same condition as the stock culture above, and then were also assigned to each feeding treatment. The copepods were fed the same ration of 3.16 μg C $mL^{-1}$ in all treatments and for the binary algae diets a ratio of 1:1 by number was given to the copepods during the preconditioning period and throughout the experiments.

**Table 5.1** Microalgal diets used for feeding *Cyclopina kasignete*.

| Diets | Algal species |
|---|---|
| Monoalgal | Fresh *T. lutea*<br>Fresh *N. oculata*<br>Dry *Melosira* sp. |
| Binary | Mixed algae (Dry *Melosira* sp. + Fresh *T. lutea*)<br>Mixed algae (Fresh *T. lutea*+ Fresh *N. oculata*) |

### *5.3.3 Offspring production, spawning and lifespan*

One pair of mature male and female copepods with five replicates for each diet treatment was placed into a 50 ml beaker from the preconditioning stocks and covered with aluminium foil with holes for ventilation. The copepods were fed with the mono and binary algal diets with five replicates each and the beakers were monitored daily for occurrence of new egg sacs. After spawning, each male-female pair was transferred to a new beaker to facilitate observation of the subsequent spawning. The individuals produced from the previous egg sacs were counted in a Sedgewick-Rafter chamber (Pyser – SGI Limited, Kent, UK) on a dissecting stereo-microscope (Model Nikon: SMZ, 1500, Tokyo, Japan) by collecting through a 40 µm mesh. Daily offspring production and offspring per egg sacs were obtained from the mean of five replicate pairs of *C. kasignete* in each diet treatment. Total spawns in lifetime were recorded and the lifespan of females was obtained by averaging all individual life duration in 30 days.

### *5.3.4 Hatching rate, survival and generation time*

In each diet treatment, fifty eggs were randomly collected from each beaker and placed in a 30 ml petri dish with five replicates for each treatment. The eggs were checked every 6 h for counting newly hatched nauplii over a 24- h period. The nauplii were then transferred to a new petri dish with the same algal diet as the broodstock to evaluate the subsequent copepod survival from hatching to adulthood. The number of unhatched eggs was counted after 48 h, and the hatching rate was calculated as: Hatching rate = 1 - (number of unhatched eggs / number of total eggs) (Pan *et al.* 2012). The hatched nauplii in each treatment were subsequently monitored daily for mortality to determine overall survival from hatching to the adult stage. Throughout the experiment, food was added daily to each beaker according to each algal treatment.

One single egg was placed in a petri dish with five replicates for each dietary treatment to allow for determination of developmental period from nauplii to the copepodite stage. The development time of *C. kasignete* was monitored every 6 h until the copepod reached mature copepodite VI stage and the development time to each post-embryonic stages was obtained from the mean of five replicates for each treatment. The developmental stages were observed under a microscope to calculate the development time (i.e., from a nauplius to copepodite; from a copepodite to an adult and from a nauplius to a gravid female).

### *5.3.5 Population density, specific population growth rate and sex ratio*

A culture of *C. kasignete* population consisting of nauplii (10 ind. $mL^{-1}$), copepodite (6 ind. $mL^{-1}$) and adult (4 ind. $mL^{-1}$) was separately fed each of the five algae treatments in 1000 mL beakers with five replicates and cultured for 30 days. The cultures were conducted at 28 ±1°C under natural illumination with gentle aeration. Each feeding treatment consisted of five replicates and the copepods were fed the same ration of 3.16 μg C $mL^{-1}$ in all dietary treatments and the binary algae diets (1:1 ratio by algal number) were given to the copepods until the end of the experiment.

A 3-mL subsample was taken daily from the culture medium and the number of copepods at each development stage (nauplii, copepodite, and adult) was recorded. Triplicate counting was made from the subsamples. Specific population growth rate ($r$) was calculated from the population density data using the following equation (Lee *et al.* 2013):

$$r = (\ln N_e - \ln N_i) / t$$

Where, $t$ is culture days, $N_i$ and $N_e$ are the initial and end density of copepods, respectively. All male and female adults were separately counted to obtain the sex ratio.

## 5.4 Data analysis

Data are presented as mean ± standard deviation (SD). All statistical analyses were conducted using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Comparisons of reproductive and population parameters of copepods in different algal diets were made using one-way analysis of variance (ANOVA). Differences were considered significant at the $P < 0.05$ level. When the main treatment effect was significant, post-hoc comparisons were made using Tukey's test. All the data were tested for normality, homogeneity and independence to satisfy the assumptions for ANOVA.

## 5.5 Results

### *5.5.1 Population density and growth*

Fig. 5.1 shows the overall population density of copepods in each dietary treatment after culture for 30 days. The highest population densities of copepods occurred when copepods were fed with monoalgal diets of fresh *T. lutea* (204.8 ± 29.64 ind $mL^{-1}$), dry *Melosira* sp. (201.6 ± 29.88 ind $mL^{-1}$), and binary diets of dry *Melosira* sp. and fresh *T. lutea* (208.6 ± 26.53 ind $mL^{-1}$), compared with other feeding treatments ($P < 0.05$, Fig. 5.1). Copepods fed fresh *N. oculata* produced lower population density (84 ± 20.84 ind $mL^{-1}$ ) than those fed mixed fresh algae (*T. lutea* and fresh *N. oculata*) (120 ± 27.86 ind $mL^{-1}$ ) but these were not statistically different ($P > 0.05$, Fig. 5.1). The nauplii and copepodite densities were higher in copepods fed a single diet of fresh *T. lutea*, dry *Melosira* sp., or mixed algae (fresh *T. lutea* + dry *Melosira sp.*) than other dietary treatments ($P < 0.05$, Fig. 5.1). The specific

population growth rate of copepods depended on food type ($P < 0.05$; Table 5.2). Copepods fed the binary diets of dry *Melosira* sp. and fresh *T. lutea* and single diets of fresh *T. lutea* or dry *Melosira* sp. achieved higher growth rates than in other feeding treatments ($P < 0.05$).

**Table 5.2** The specific population growth rate of copepods fed different mono and binary algal diets. All values are mean ± standard deviation ($n = 5$). The different small letters indicate significant difference between different treaments ($P < 0.05$).

| **Diets** | **Specific population growth rate (mean ± SE)** |
|---|---|
| Fresh *T. lutea* | 0.154 ± 0.014 **a** |
| Fresh *N. oculata* | 0.094 ± 0.017 **b** |
| Dry *Melosira* sp. | 0.153 ± 0.010 **a** |
| Mixed algae (Dry *Melosira* sp. + Fresh *T. lutea*) | 0.156 ± 0.008 **a** |
| Mixed algae (Fresh *T. lutea*+ Fresh *N. oculata*) | 0.111 ± 0.028 **b** |

*5.5.2 Offspring production*

The total number of offspring per egg sacs in 30 days depended on food type ($P = 0.008$, Fig. 5.2) and was higher in treatments using a single diet of fresh *T. lutea* or dry *Melosira* sp., or the mixture of both than in other treatments ($P < 0.05$, Fig. 5.2). The daily production of offspring copepods was also significantly associated with the type of mono and binary algal diets ($P = 0.001$). Daily offspring production was lowest in the treatment using fresh *N. oculata* compared to other dietary treatments ($P < 0.05$, Fig. 5.2) but there are no significant difference between treatments of fresh *T. lutea*, dry *Melosira* sp. and the mixture of both ($P > 0.05$).

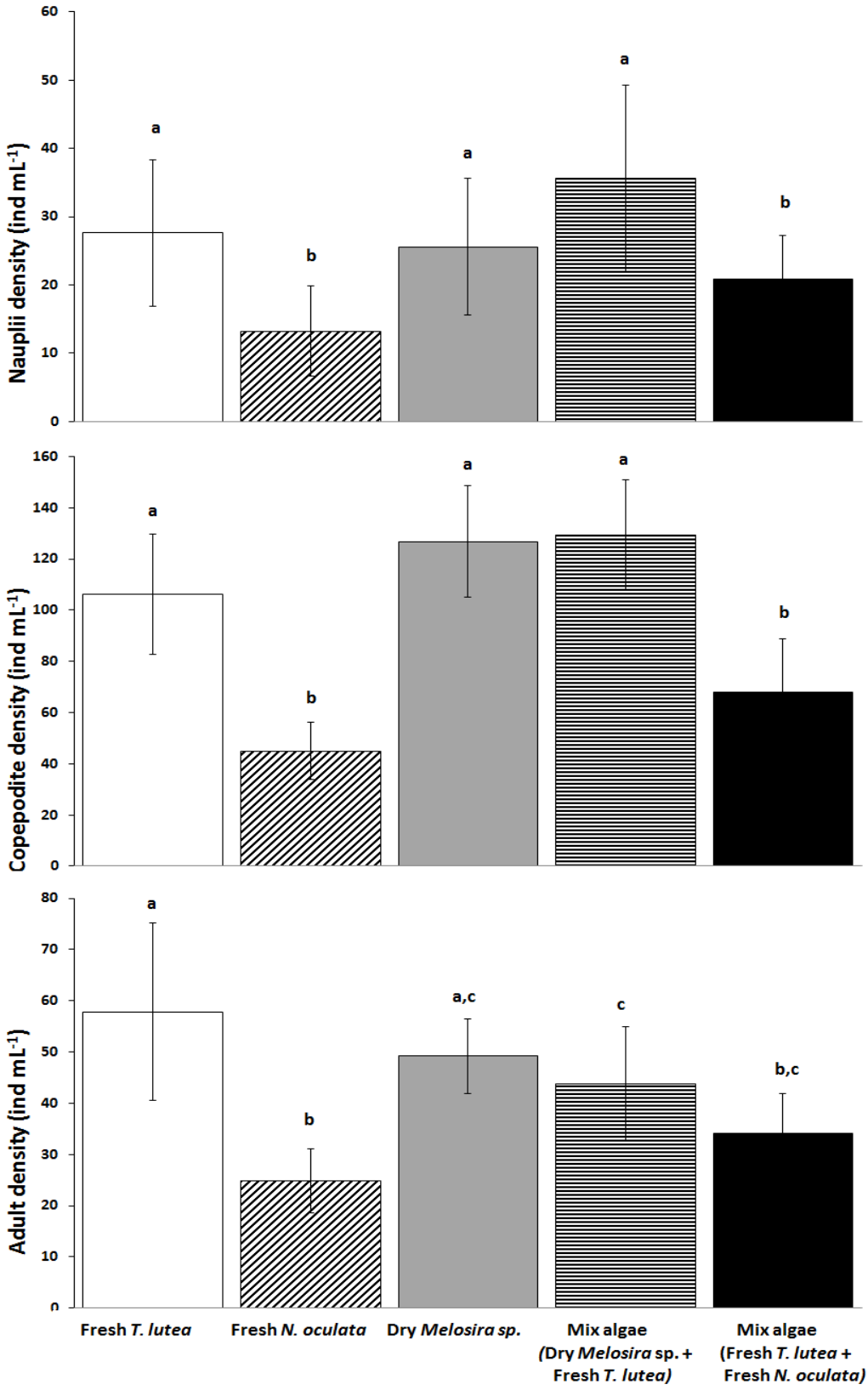
Nauplii density (ind mL-1)
60
50
40
30
20
10
0
a
b
a
a
b
Copepodite density (ind mL-1)
160
140
120
100
80
60
40
20
0
a
b
a
a
b
Adult density (ind mL-1)
80
70
60
50
40
30
20
10
0
a
b
a,c
c
b,c
Fresh T. lutea
Fresh N. oculata
Dry Melosira sp.
Mix algae (Dry Melosira sp. + Fresh T. lutea)
Mix algae (Fresh T. lutea + Fresh N. oculata)

**Fig. 5.1**. Population density of *C. kasignete* fed different mono and binary algal diets. Different letters represent significant differences ($P < 0.05$) between each algal diets. Bars indicate standard deviations ($n = 5$).

### *5.5.3 Lifespan and hatching rate*

The lifespans of female *C. kasignete* under different dietary treatments are shown in Fig. 5.3. The longer lifespan was observed in treatments with monoalgal diets of fresh *T. lutea* (26 ± 1.58 days), dry *Melosira* sp. (25.8 ± 1.48 days), or in the mixture of both algae (26 ± 1.87 days). The shortest lifespan occurred in copepods fed single fresh *N. oculata* (22 ± 1.58 days; $P < 0.05$, Fig. 5.3). Algal diets had significant impact on the lifespan of female *C. kasignete* ($P = 0.005$).

The type of algal food significantly affected the female copepods' hatching rate ($P < 0.05$, Fig. 5.3). Lower hatching rate occurred when copepods fed monoalgal diets of fresh *N. oculata* (50.8 ± 8.73 %) compared to other dietary treatments ($P = 0.001$; Fig. 5.3). However, there was no significant difference in hatching rate between treatments of fresh *T. lutea*, dry *Melosira* sp. and the mixture of both algae ($P > 0.05$, Fig. 5.3).

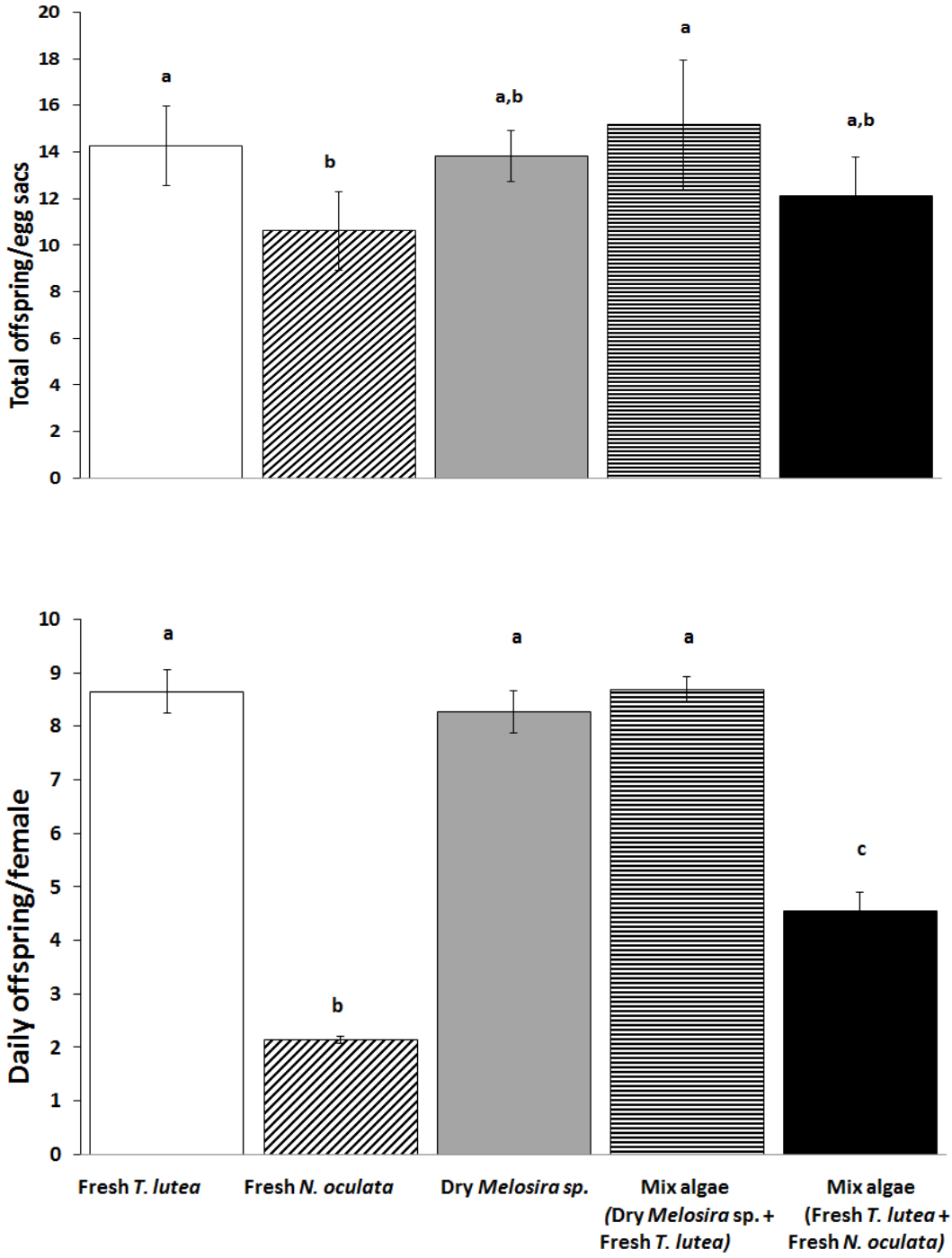


**Fig. 5.2**. Offspring production in copepods fed different mono and binary algal diets. Different letters represent significant differences ($P < 0.05$) between each dietary algae. Bars indicate standard deviations.

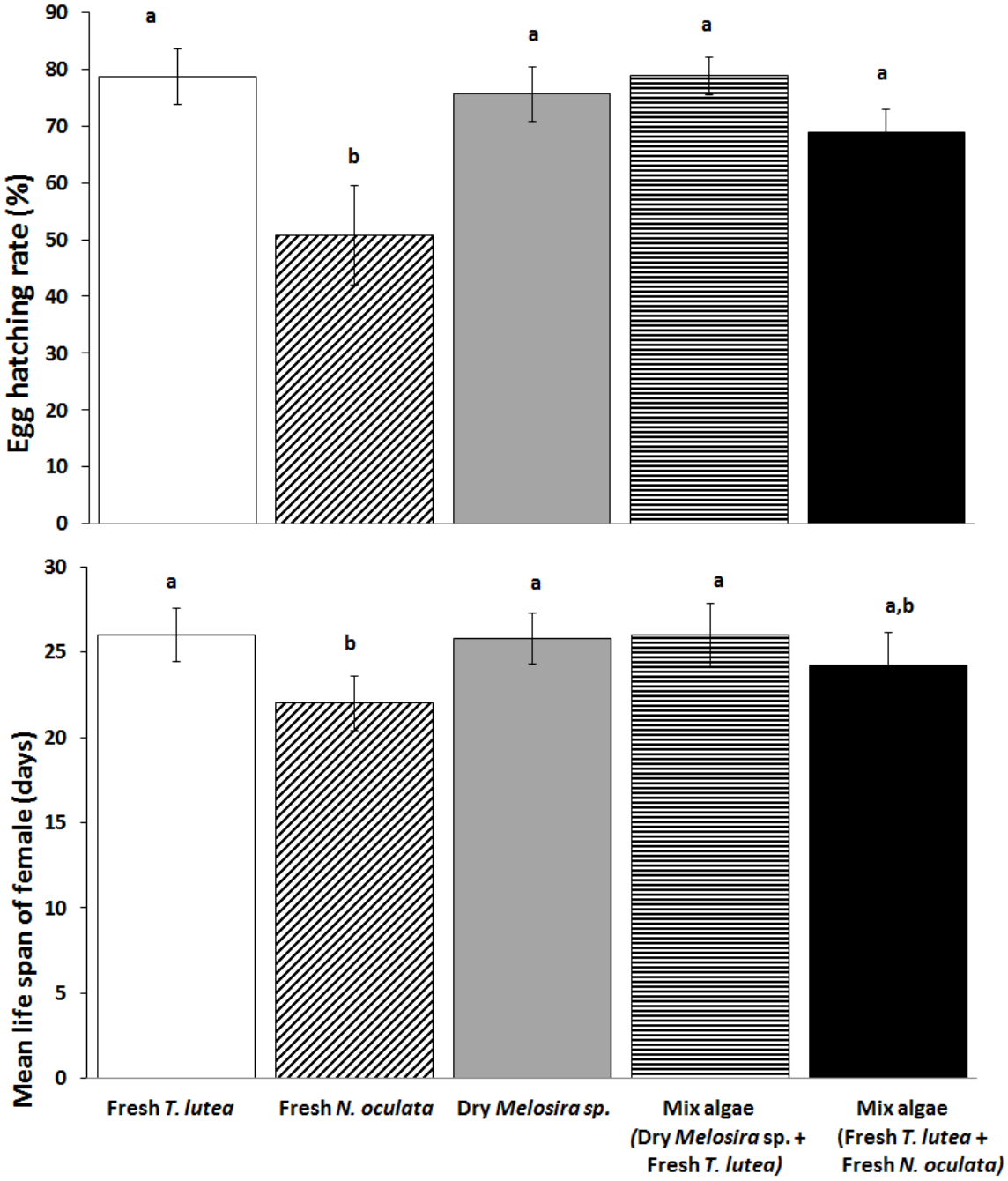


**Fig. 5.3**. Hatching rate and lifespan of *C. kasignete* fed different mono and binary algal diets. Bars indicate standard deviations. Different letters indicate significant differences at $P < 0.05$ level. Bars indicate standard deviations.

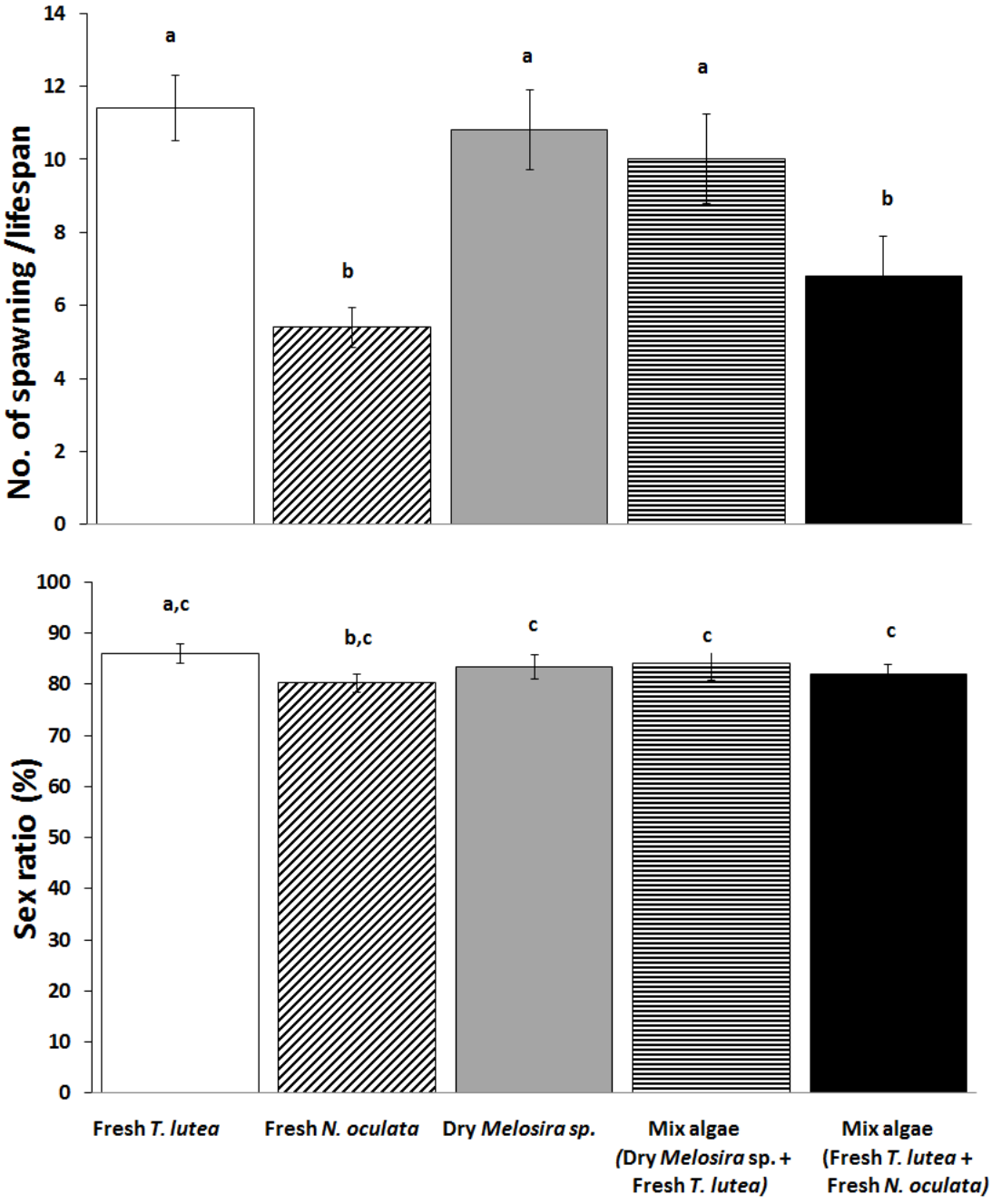


**Fig. 5.4**. Number spawns per lifespan and sex ratio of *C. kasignete* fed different mono and binary algal diets. Different letters indicate significant difference ($P < 0.05$). Bars indicate standard deviations.

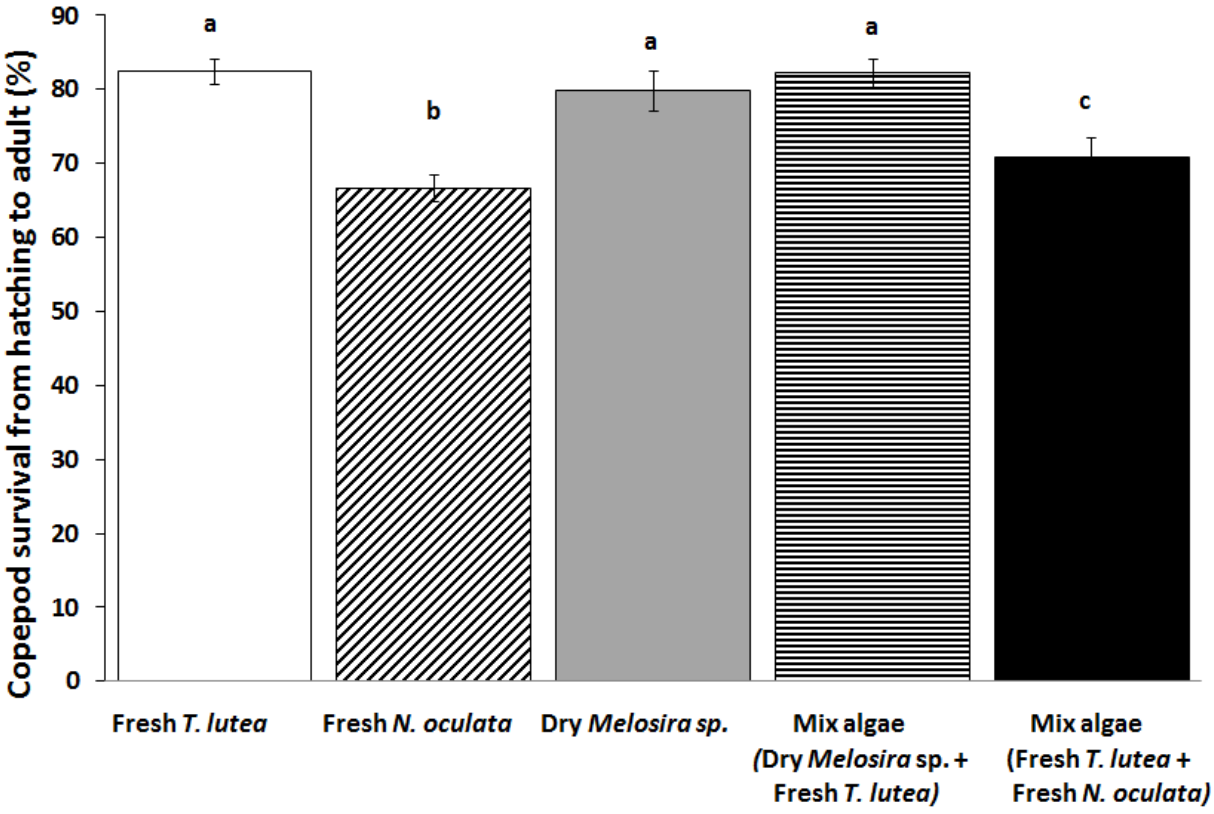


**Fig. 5.5**. Survival of copepods from hatching to adult stage after feeding on different mono and binary algal diets. Different letters indicate significant difference ($P < 0.05$). Bars indicate standard deviations.

*5.5.4 Spawning, sex ratio, survival and generation time*

Copepods had most spawns in lifetime when fed on fresh *T. lutea* (11.4 ± 0.89), dry *Melosira* sp. (10.8 ± 1.10) or the mixture of fresh *T. lutea* and dry *Melosira* sp. (10 ± 1.22) but lowest spawns in copepods fed fresh *N. oculata* (5.4 ± 0.55) or the mixture of fresh *T. lutea* and *N. oculata* (6.8 ± 1.10; $P < 0.05$; Fig. 5.4). The number of spawns in lifetime was impacted by the type of algal diets ($P = 0.001$, Fig 5.4).

There are no difference on the sex ratio of the copepods ($P > 0.05$. Fig. 5.4) except in treatments between fresh *T. lutea* and fresh *N. oculata* ($P = 0.005$, Fig. 5.4) where females were highly dominant, ranging from 80.2 ± 1.48 (monoalgal diets of fresh *N. oculata*) to 86.0 ± 1.87 % (monoalgal diets of *T. lutea*) in the adult population.

The survival of copepods from hatching to adult stage depended on food type ($P = 0.001$; Fig. 5.5). Higher survival rate of copepods (from hatching to adult stage) was achieved on single diets of fresh *T. lutea* (82.4 ± 1.67%), dry *Melosira* sp. (79.8 ± 2.77%) or binary diets of dry *Melosira* sp. and fresh *T. lutea* (82.2 ± 1.92 %) ($P < 0.05$, Fig. 5.5). It is worth noting that the survival of copepods ranged from 66.6 ± 1.82% when fed fresh *N. oculata* to 82.4 ± 1.67% when fed fresh *T. lutea*.

Development time from newly hatched nauplii stage 1 to stage 6 was significantly different among food types ($P = 0.001$, Table 5.3). Copepods fed fresh *N. oculata* had the longest development time (5.8 ± 0.8 days) compared to other dietary treatments ($P < 0.05$; Table 5.3). Maturation period of copepodite was not

significantly different among food types ($P = 0.091$, Table 5.3). The generation time from nauplii to a gravid female was longer in copepods fed fresh *N. oculata* (12.14 ± 1.30 days) or the mixture of fresh *N. oculata* + fresh *T. lutea* (13.48 ± 1.32 days) than the other diets ($P < 0.05$, Table 5.3).

**Table 5.3** Generation time of nauplii and copepodite in *C. kasignete*. All values are mean ± standard deviation ($n$ = 5). The different small letters indicate significant difference between different treatments ($P < 0.05$).

| **Diets** | **Hatching time (day)** | **Generation time (Nauplii I-VI) (day)** | **Generation time (Copepodite I- adult) (days)** | **Generation time (Nauplii I- gravid) (days)** |
|---|---|---|---|---|
| Fresh *T. lutea* | 2.01 ± 0.08$^{a}$ | 4.00 ± 0.68$^{a}$ | 3.00 ± 0.54$^{a}$ | 9.16 ± 1.19$^{a}$ |
| Fresh *N. oculata* | 3.00 ± 0.14$^{b}$ | 5.80 ± 0.84$^{b}$ | 4.20 ± 0.82$^{a}$ | 13.48 ± 1.32$^{b}$ |
| Dry *Melosira* sp. | 1.98 ± 0.05$^{a}$ | 4.20 ± 0.51$^{a}$ | 3.40 ± 0.66$^{a}$ | 9.92 ± 0.92$^{a}$ |
| Mixed algae (Dry *Melosira* sp. + Fresh *T. lutea*) | 1.93 ± 0.12$^{a}$ | 4.00 ± 0.60$^{a}$ | 3.20 ± 0.46$^{a}$ | 9.66 ± 0.92$^{a}$ |
| Mix algae (Fresh *T. lutea*+ Fresh *N. oculata*) | 2.25 ± 0.13$^{c}$ | 4.80 ± 0.49$^{a,b}$ | 3.60 ± 0.81$^{a}$ | 12.14 ± 1.30$^{b}$ |

## 5.6 Discussion

The results of the present study clearly demonstrated that the food was crucial to the culture success of *C. kasignete*, significantly affecting its population growth and reproductive parameters. The population growth, offspring production, survival and development of nauplii and copepodites clearly depended on the mono and binary algae diets used for feeding. In most cases, the monoalgal diets of fresh *T. lutea*, dry *Melosira* sp. and the binary diet dry *Melosira* sp. + fresh *T. lutea* favoured growth, survival and productivity of *C. kasignete*. In this study, the growth rate of *C. kasignete* (0.156 ± 0.008) was higher than some other cyclopoid species reported previously such as *Apocyclops royi* (0.096 ± 0.0138) and *Tigriopus japonicas* (0.086 ± 0.0143) (Lee *et al.* 2013; Lee *et al.* 2006). This indicates that *C. kasignete* also has a mass growth potential as a live food for fish larval rearing.

Several studies have indicated that diatom *Thalassiosira weissflogii* can reduce the population growth and offspring production in copepods (Ianora *et al.* 2003; Dutz *et al.* 2008) . However, our study demonstrated that copepods fed a dry diatom *Melosira* sp. produced high population density, growth rate and offspring production regardless of using as mono *Melosira* diet or binary diet with fresh *T. lutea*. Similarly, diatom *Nitzschia closterium* was also reported to promote population growth and offspring production of the copepod *Tisbe biminiensis*, compared to a single green alga diet of *Tetraselmis gracilis* (Pinto *et al.* 2001). This may be associated with the high contents of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in dried *Melosira* sp. (Rasdi *et al.* 2015) since they are essential dietary nutrients for copepods (Pinto *et al.* 2001).The similar result was also found on copepod *Euterpina acutiferons* feeding on diatom *Chaetoceros*

*muelleri* (Camus & Zeng 2012). Moreover, the offspring production of copepods with dry *Melosira* sp. diet is consistent with previous studies that diatoms as food are able to produce high offspring production of copepods *Acartia* sp., and *Calanus* sp. (Ismar *et al.* 2008; Irigoien *et al.* 2002). In current study, *C. kasignete* produced maximum growth and offspring production when fed the monoalgal diet of dry *Melosira* sp. or the dinary diets of dry *Melosira* sp. and fresh *T. lutea*. It seems that the diatom *Melosira* sp. can be used as either single or mixed form with other algae such as *T. lutea* to sustain the growth and reproduction of *C. kasignete*.

Our results also showed that fresh *T. lutea* sustained high population growth and density, and increased reproductive capacities in *C. kasignete*. Similarly, a cyclopoid copepod *Paracyclopina nana* fed *Isochrysis* sp. produced maximum population densities and increased reproduction capacities compared to feeding on *Tetraselmis suecica* (Lee *et al.* 2006). Given a high EPA content in *N. oculata* diet (Rasdi *et al.* 2015), the copepod obtained relatively low population growth rate and offspring production, which was mainly due to derived low DHA and other HUFAs content of copepods (Morehead *et al.* 2005; Rasdi *et al.* 2015). As highlighted by Payne and Rippingale (2001), the toughness and indigestibility of its cell wall would be the main reason for the poor performance of *Nannochloropsis* sp., even feeding to the *Artemia* culture. With this regards, *Isochrysis* sp. could positively promote the productivity of copepods *Acartia bilobata* (Lee *et al.* 2006; Pan *et al.* 2012). Moreover, the present study showed that *T. lutea* was also a superior food to increase *C. kasignete* offspring production.

In the present study, algal quality significantly impacted the offspring production and generation time of *C. kasignete*. The binary diet (dry *Melosira* sp. +

fresh *T. lutea*) produced more offspring, fast hatching rate, the shortest developmental period and longer female lifespan. These results were comparable to the levels in the treatments of single fresh *T. lutea* or dry *Melosira* sp. Microalga *T. lutea* was also recommended as a suitable diet to produce higher number of offspring and shorter developmental period of copepod *Parvocalanus crassirostris* (Alajmi & Zeng 2013). Improvement in offspring production of copepods fed fresh *T. lutea* + dry *Melosira* sp. may be related to their complementary essential fatty acids profiles such as EPA and DHA (Camus *et al.* 2009). Since dry *Melosira* sp. and fresh *T. lutea* contains a high amount of essential fatty acids such as EPA and DHA (Rasdi *et al.* 2015), this shows that diet quality clearly impacts the offspring production of this copepod.

Hatching rate is a vital parameter to assess copepod production since this parameter is directly associated with maternal nutrition as egg quality determines hatching success (Evjemo *et al.* 2003; Jónasdóttir & Kiørboe 1996). Our results showed that successful egg hatching and number of spawns during the lifetime in copepods depended on the algal diets. The similar results were also reported on the copepods *Acartia sinjiensis* and *Bestiolina similis* (Milione & Zeng 2007) but contrary on the copepod *P. crassirostris* where there are no difference on egg hatching success under different algal treatments (Alajmi & Zeng 2015). It seems that the relationship of reproductive success of copepods with food quality is species-specific. Therefore, it also highlights the necessary of studying the diet requirement for each copepod species, in particular of those ones having potential for aquaculture as live food.

There are no much different in sex ratio among all food types except the one between *T. lutea* and *N. oculata* treatments where females were more than males. In the studies on the sex ratios of other copepods *Bestiolina similis* and *Tigriopus californicus,* the results have indicated that the sex ratio of copepods is more likely inherited, rather than being affected by environmental factors (Camus & Zeng 2010; Voordouw *et al.* 2005). This study also suggests that the sex ratio of copepods does not depend on food quality.

In conclusion, the population growth, survival and reproductive capacities of *C. kasignete* are affected by mono and binary algal diets. Copepods produced better growth, survival and productivity when fed on fresh *T. lutea*, dry *Melosira* sp., and binary diets of fresh *T. lutea* and dry *Melosira* sp. To a large extent, copepod productivity increased with the high essential fatty acids profiles such as EPA and DHA in the algal diets. Our results demonstrated that the fresh *T. lutea*, dry *Melosira* sp. and the combination of both algae can contribute to a proper feeding regime for the cyclopoid copepod *Cyclopina kasignete* as live feed in aquaculture.

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# Chapter 6

## Copepod supplementation as live food improved growth and survival of Asian seabass *Lates calcarifer* larvae

This chapter will be submitted as:

Rasdi N.W. and Qin J.G. (2015) Copepod supplementation as live food improved growth and survival of Asian seabass *Lates calcarifer* larvae. *Aquaculture Research.*

## 6.1 Abstract

The copepod *Cyclopina kasignete* was supplemented to the Asian seabass *Lates calcarifer* larvae to evaluate its potential use as a live food supplement during larval rearing. Initial larval fish feeding began with sole rotifers as live food from 3 to 7 days post hatch (DPH) followed by the addition of copepod, *Artemia* nauplii and their combination from 8 to 21 DPH. Fish were sampled every 5 days to measure growth and food selectivity on 11, 15, 19, and 21 DPH. The larvae fed the combination diets involving copepods resulted in better growth, survival, and increased the essential fatty acid contents of EPA and DHA. In terms of food selectivity, seabass larvae selected for copepods over rotifers and *Artemia*. This study indicates that the potential use of copepod as a supplementary live food can improve larval fish performance and thus overcome low survival and slow growth in fish larval aquaculture. The present study demonstrates that the nutritional value of the cyclopoid copepod *C. kasignete* is suitable for fish larvae and this copepod can be used as a sole or supplementary live food for rearing fish larvae.

**Keywords:** copepods, fatty acids, food selectivity, live food, growth, survival

## 6.2 Introduction

Copepods have become increasingly important as a live feed for larval fish due to the similarity of their nutritional profile to the need of fish larvae (Rajkumar & Kumaraguruvasagam 2006), but the sole use of copepods in larval fish rearing has been problematic due to low mass production of most copepod species in captivity. Current practices on larval rearing have used rotifers and *Artemia* to feed fish larvae though these live foods do not contain adequate nutrition for fish development even after nutrient enrichment (Rasdi & Qin, 2014). The motivation driving this research was to test if supplementation of copepods to the current live food regime would improve larval fish performance. Larval fish are planktivorous and select for appropriate live food along the course of their ontogenetic development to obtain proper nutrient and energy after yolk depletion, therefore live food composition and quality are critical for larval fish development and growth (Ma *et al.* 2013).

Asian seabass *Lates calcarifer* also known as barramundi is distributed through the northern Indian and tropical western Indian Ocean, from Iran to northern Australia, including Taiwan and Papua New Guinea (Tucker, 2002). This species has been cultured in Australia since 1980s due to its high demand from consumers, fast growth, and ability to adapt to either a marine or freshwater environment in aquaculture (Schipp *et al.* 2007). The *L. calcarifer* culture is very important in Australia (Battaglene & Cobcroft 2007) but one of the major constraints in hatchery production of this species is slow growth during larval rearing from first feeding and low survival during the early developmental period (Curnow *et al.* 2006). Furthermore, the seabass larvae often underwent sporadic high mortalities which is caused by nutritional deficiency in the type of live food

(Rimmer *et al.* 1994). By choosing suitable feed during the larval phase is very important to ensure fish survival and growth in a hatchery. In intensive-rearing systems, first-feeding marine fish larvae are most commonly fed on rotifers *Brachionus* spp. with a range in lorica size from 100 to 340 μm (Theilacker & McMaster 1971; Støttrup 2000), followed by larger sized *Artemia* nauplii in a later developmental stage. The brine shrimp cysts from a large variety of strains vary in size, energy content and nutritional quality and the newly hatched *Artemia* nauplii measure 422 - 517 μm in length (Sorgeloos *et al.* 2001). As copepods provide a wide size range of nauplii (80-160 μm) and copepodites (200-450 μm) and contain essential nutrients that rotifers and *Artemia* do not have, but it is not clear if fish larvae would show better growth and survival with copepod supplementation.

To date, the production of high quality fish larvae is constrained by poor fish survival and growth in many commercial marine hatcheries when fish are only fed traditional live feed species such as *Artemia* and rotifers (Zaleha *et al.* 2012). More specifically, inadequate nutritional supply from live feed species has caused low survival and high deformity in fish larvae (Rajkumar & Kumaraguruvasagam 2006). Rotifers and *Artemia* do not fulfil all the nutritional requirements in some marine fish larvae even after nutrient enrichment on live food (Rasdi & Qin, 2014).

The cyclopoid copepod *Cyclopina kasignete* has been successfully mass cultured and reported as a potential live food for fish larvae (Rasdi *et al.* 2015). Since this copepod contains the essential nutrition needed by fish larvae, it is necessary to test if this copepod species can be effectively ingested by fish larvae where *Lates calcalifer* larvae can be used as a model fish to examine food selectivity on copepods when other traditional live foods exist. The *C. kasignete* is

proven to contain high EPA, DHA, protease and trypsin when fed on *T. lutea*, which is essential for growth and digestion in fish larvae (Rasdi *et al.* 2015). Thus, the growth, survival and nutrition content of *L. calcarifer* larvae were evaluated when the *C. kasignete* as a live food was introduced to fish tanks.

The objective of this study was to test the efficacy of copepods in fish larvae emphasizing on food selectivity, growth, survival and PUFA contents of *L. calcarifer*. This study tested three hypotheses: (1) growth and survival of fish larvae depends on the type of live food; (2) fatty acid contents in *L. calcarifer* depends on live food types and (3) fish larvae select for copepods over *Artemia* and rotifers during the transition of co-feeding period. The understanding of these key issues would help improve the success and efficiency of larval fish rearing in hatcheries and the efficacy of live food use for fish larvae at different developmental stages.

## 6.3 Materials and method

### *6.3.1 Fish larvae and live food culture*

One day old *L. calcarifer* larvae were obtained from the hatchery at West Beach Aquaculture, Adelaide and transported to the Aquaculture Laboratory at Flinders University. The larvae were transported in plastic bags filled with pure oxygen and were acclimatized in 250 L tanks for 2 days prior to the experiment. During acclimatization, fish were fed rotifers and the environmental condition was maintained at 28 ± 1°C, 30-35 ppt salinity and >5.5 mg dissolved oxygen $L^{-1}$. Water exchange rate was at 50% of the tank volume per day.

*Cyclopina kasignete* were originally collected from the Arno Bay Jetty at Eyre Peninsula, South Australia. The *C. kasignete* were continuously cultured and

sustained in the laboratory since October 2012 at 20–22 °C, 22–23 practical salinity units (PSU) and 115 μE $m^{-2}$ $s^{-1}$ irradiance with a 12⁄12 h photoperiod. The *C. kasignete* were fed with mixed microalgae including *N. oculata* and *T. lutea* (1:1 in ratio by cell number).

The rotifers *Brachionus plicatilis* were cultivated in the Animal House, Flinders University at 28 $^{o}$C in 250 L tanks with 30 ppt salinity and continuous aeration. The rotifers were fed with algae *Tisochrysis lutea* and *Nannochloropsis oculata* daily. The *Artemia* cysts (Inve Technologies, Belgium) were decapsulated and hatched according to the standard procedures (Rajkumar & Kumaraguruvasagam 2006). The hatched *Artemia* nauplii and rotifers were harvested, washed and prepared for the feeding experiments according to the feeding protocols in Table 6.1. The compositions of fatty acids in *Artemia* nauplii, rotifers and copepods relevant to this study are shown in Table 6.2.

**Table 6.1**. Feeding protocols of *Lates calcarifer* larvae from 3 to 21 DPH.

| Treatments | Days Post Hatch | Diets (ind/mL) |
|---|---|---|
| A | 3 - 7 | Rotifer (10) |
| | 8 - 21 | Rotifer (7) + *Artemia* (7) + Copepod (7) |
| B | 3 - 7 | Rotifer (10) |
| | 8 - 21 | Rotifer (7) + Copepod (7) |
| C | 3 - 7 | Rotifer (10) |
| | 8 - 21 | Rotifer (7) + *Artemia* (7) |

*6.3.2 Experimental design and sampling procedures*

After acclimatization, 300 larval fish were transferred to each of the twelve 30 L experimental tanks at 10 larvae $L^{-1}$ with a rate of water exchange at 50% of the tank volume per day. Three feeding regimes are illustrated in Table 1 with four replicates each. The experiment was divided into two feeding phases. Phase 1 (3-7 DPH) started with rotifers as a sole live food and then moved on to a period of co-feeding with additional two other live feeds: *Artemia* nauplii, copepods and a mixture of *Artemia* and copepods in phase II (8-21 DPH).

Fish were sampled on 7, 11, 15, 19 and 21 DPH for growth measurements and gut content analysis. Growth was determined by specific growth rate (SGR) in % $d^{-1}$ using this equation (Hopkins 1992): $SGR = 100(lnSL_f - lnSL_i)/\Delta t$, where $SL_f$ and $SL_i$ were the final and initial larval fish standard length (mm), respectively, and $\Delta t$ was the time between sampling days. Five larval fish from each replicate were anaesthetized in 0.5% AQUI-S (AQUI-S New Zealand Ltd., Lower Hutt, New Zealand) before preservation in 10% neutrally buffered formalin. Each fish larvae were dissected and the entire gut was extracted under a microscope at 100× magnification. The number and type of live food items in each fish gut were determined. Copepods in the fish gut were identified by the presence of antennae, eyes and head. The number of rotifers ingested by fish larvae was determined by the number of mastax and trophi found in the gut while for *Artemia* nauplii, the eye and whole body in the gut were used as evidence of fish larvae ingestion, and the number of partially digested exoskeleton was counted for prey ingestion. Prey selectivity was calculated based on the method by Ivlev (1961).

Food selectivity = (r – p) / (r + p)

Where r is the percentage of prey type in each larval gut, and p is the percentage of prey type in the rearing medium. This index ranged from -1.00 to + 1.00, where –1 indicates absence of prey in the stomach, negative values suggest avoidance of those prey types, and positive values suggest active selection on those prey types. The number of dead larvae was counted daily to estimate fish survival.

### *6.3.3 Fatty acid and data analyses*

At the end of the feeding experiments, fish were euthanized with 40 mL $L^{-1}$ Aqui-STM until medullary collapse and then fish were measured, weighed and the number of fish was recorded. The larvae were chill-killed and stored frozen at −80 °C for subsequent determination of fatty acid compositions. The fatty acid content was measured and then the samples were methylated in 5 mL of 1% $H_2SO_4$ in methanol at 70 °C for 3 h. The fatty acid methyl esters were extracted by adding 750 µL distilled water and 2 mL of n-heptane. The heptane layer was transferred to a 2-mL vial for analysis using gas chromatography (GC, PerkinElmer Gas Chromatograph Clarus 500). Fatty acid methyl esters (FAMEs) were separated and measured on the GC equipped with a 30-m capillary column (0.32-mm internal diameter, Zebron ZB-FFAP). Helium was the carrier gas (1.5 mL/min). The injector temperature was set at 250 °C and the detector temperature at 300 °C. The initial oven temperature was 140 °C for 3 min, then ramped at 1:10.0 $min^{-1}$ at 1 – 160 °C for 5 min followed by 10.0 $min^{-1}$ at 1 – 230 °C for 10 min. FAMEs were identified on the GC using software of TotalChrom Navigator (version 6.3.2 0646, Perkin Elmer). Heptadecanoic acid (17:0, Sigma-Aldrich, Australia) was used as the internal standard to calculate the FAME concentration in each sample. The results

were expressed as a percentage of total fatty acids (FAME) in 1 g dry weight of sample.

Dietary treatments were compared using one way ANOVA. Duncan's multiple range test was applied to determine any significant differences between feeding treatments. All the above mentioned statistical analyses were performed using SPSS statistical software (ver 17.0 for Windows, SPSS, Chicago, IL, USA). The level of significance was set at $P < 0.05$.

## 6.4 Results

### *6.4.1 Fatty acid composition of fish*

Four species of saturated fatty acids were found in fish fed a combination of different types of live food (Table 6.3). The amounts of C18:0 (4.86 ± 0.06%) and palmitic acid C16:0 (10.74 ± 0.26%) were higher in the fish fed rotifer and *Artemia* than fed a mixture of rotifer, *Artemia* and copepod, or a mixture of rotifer and copepod ($P < 0.05$), but the C12:0 and C14:0 fatty acids in fish were not affected by the feeding treatments ($P > 0.05$, Table 6.3). Three monosaturated fatty acids were detected in larval fish (Table 6.3). The amount of C16:1 (10.42 ± 0.57%) and C20:1 (0.64 ± 0.03 %) fatty acids in fish fed a combination of rotifer and *Artemia* were lower than those fed the mixture of rotifer, *Artemia* and copepod, or rotifer and copepod ($P < 0.05$, Table 6.3), but the C18:1 and C22:1 fatty acids in fish were not affected by the feeding regimes ($P > 0.05$).

Among seven polyunsaturated fatty acids in copepods, α-linolenic acid (ALA), α- arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were the major essential fatty acids for fish larvae.

Fish fed the mixture of rotifer, *Artemia* and copepod, or the mixture of rotifer and copepod contained higher EPA (11.28 ± 1.44%, 11.40 ± 1.36 % respectively), DHA (12.30 ± 0.14%, 12.00 ± 0.11% respectively) and ARA (2.00 ± 0.08%, 2.03 ± 0.05% respectively) than those fed rotifers or *Artemia* (EPA 8.63 ± 0.48%, DHA 11.09 ± 0.28% and ARA 1.82 ± 0.05%, $P < 0.05$, Table 6.3). The ALA content in larval fish was higher when fed the mixture of rotifer and copepod compared to other feeding treatments (6.10 ± 0.04%, $P < 0.05$, Table 6.3). The fatty acids in larval fish depended on the type of live food used for feeding ($P < 0.05$).

**Table 6.2**. Fatty acid composition in live feeds (mean ± SD), expressed as percentage of total lipids. The fatty acid contents in *Artemia* nauplii and rotifer were adapted from Santhanam (2012) and the copepod, *Cyclopina kasignete* from Rasdi *et al.* (2015).

| **Fatty acids** | ***Artemia* nauplii** | **Rotifer** | **Copepod** |
|---|---|---|---|
| **C12:0** | 0 | 0 | 0.44 ± 0.01 |
| **C14:0** | 1.53 ± 0.32 | 4.26 ± 0.20 | 5.01 ± 0.01 |
| **C16:0** | 12.70 ± 0.38 | 20.01 ± 0.38 | 20.10 ± 0.06 |
| **C18:0** | 4.76 ± 0.17 | 5.20 ± 0.81 | 0.62 ± 0.06 |
| **C24:0** | 0.13 ± 0.01 | 0.26 ± 0.060 | 0.21 ± 0.01 |
| **Sum(Saturated)** | 19.12 | 29.73 | 26.38 |
| **C16:1** | 5.80 ± 2.07 | 12.68 ± 0.15 | 24.10 ± 0.06 |
| **C18:1[c]** | 8.60 ± 0.41 | - | 3.54 ± 0.09 |
| **C20:1[c]** | 0.82 ± 0.15 | 3.07 ± 0.22 | 0.17 ± 0.01 |
| **C22:1[c]** | 0 | 0.58 ± 0.05 | 0 |
| **Sum(Monosaturated)** | 15.22 | 16.33 | 27.81 |
| **C18:2[b]** | 0 | 6.51 ± 0.12 | 2.68 ± 0.15 |
| **C18:3[a](ALA)** | 20.20 ± 0.22 | 0.16 ± 0.28 | 0.17 ± 0.06 |
| **C18:4[a]** | 2.20 ± 1.14 | 0.16 ± 0.05 | 0.76 ± 0.01 |
| **C20:4[b](ARA)** | 1.10 ± 0.27 | 3.72 ± 0.06 | 6.02 ± 0.15 |
| **C20:5[a] (EPA)** | 4.00 ± 0.17 | 6.31 ± 0.13 | 27.71 ± 0.30 |
| **C22:5[b]** | 0 | 0 | 0.0 |
| **C22:6[a] DHA)** | 2.90 ± 0.35 | 0.20 ± 0.49 | 8.20 ± 0.10 |
| **Sum (PUFA)** | 30.40 | 17.06 | 45.54 |

Value of 0 indicates that FA was not detected. [a]ω-3 fatty acids, [b]ω-6 fatty acids, and [c]ω-9 fatty acids.
ALA: alpha-linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

**Table 6.3**. Fatty acid compositions of *Lates calcarifer* fed different feeding treatments. All values are mean ± standard error ($n$ = 3). Fatty acid species in bold letters are fully described in the text.

| **Fish species** | ***Lates calcarifer*** | | |
|---|---|---|---|
| | **Feeding treatment** | | |
| **Fatty acids** | **Rotifer + *Artemia* + Copepod** | **Rotifer + Copepod** | **Rotifer + *Artemia*** |
| ***Saturated*** | | | |
| **C12:0** | 0.29 ± 0.03 | 0.30 ± 0.03 | 0.35 ± 0.09 |
| **C14:0** | 1.14 ± 0.05 | 1.20 ± 0.12 | 1.23 ± 0.10 |
| **C16:0** | 7.66 ± 1.01 | 7.85 ± 1.13 | 10.74 ± 0.26 |
| **C18:0** | 6.38 ± 0.17 | 6.03 ± 0.31 | 4.86 ± 0.06 |
| **C24:0** | 0 | 0 | 0 |
| **Sum** | 15.47 | 15.38 | 17.18 |
| ***Monosaturated*** | | | |
| **C16:1** | 11.26 ± 0.11 | 11.05 ± 0.33 | 10.42 ± 0.57 |
| **C18:1[c]** | 12.27 ± 2.59 | 12.03 ± 2.53 | 10.84 ± 0.07 |
| **C20:1[c]** | 0.28 ± 0.10 | 0.37 ± 0.08 | 0.64 ± 0.03 |
| **C22:1[c]** | 3.54 ± 0.27 | 3.31 ± 0.08 | 3.14 ± 0.08 |
| **Sum** | 27.35 | 26.76 | 25.04 |
| ***Polyunsaturated*** | | | |
| **C18:2[b]** | 9.10 ± 0.48 | 8.95 ± 0.40 | 8.39 ± 0.12 |
| **C18:3[a](ALA)** | **5.78 ± 0.20 [a]** | **6.10 ± 0.04 [b]** | **3.38 ± 0.13 [c]** |
| **C18:4 [a]** | 2.30 ± 0.39 | 2.32 ± 0.33 | 1.91 ± 0.17 |
| **C20:2 [b]** | 0.21 ± 0.05 | 0.20 ± 0.04 | 0.27 ± 0.01 |
| **C20:4[b](ARA)** | **2.00 ± 0.08 [a]** | **2.03 ± 0.05 [a]** | **1.82 ± 0.05 [b]** |
| **C20:5[a] (EPA)** | **11.28 ± 1.44 [a]** | **11.40 ± 1.36 [a]** | **8.63 ± 0.48 [b]** |
| **C22:5[b]** | 0.65 ± 0.04 | 0.66 ± 0.04 | 0.59 ± 0.01 |
| **C22:6[a] (DHA)** | **12.30 ± 0.14 [a]** | **12.00 ± 0.11 [a]** | **11.09 ± 0.28 [b]** |
| **Sum (PUFA)** | 43.62 | 43.66 | 36.08 |

Value of 0 indicates that FA was not detected. [a]ω-3 fatty acids, [b]ω-6 fatty acids, and [c]ω-9 fatty acids.

ALA: alpha-linolenic acid; ARA: Arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

### *6.4.2 Larval fish growth and survival*

The specific growth rate of *L. calcarifer* was not affected by the feeding treatments from 1 to 7 DPH ($P > 0.05$, Fig. 6.1). However, the SGR of larval fish significantly depended on the type of live food used from 8 to 21 DPH ($P < 0.05$, Fig. 6.1). The larval fish resulted in a better growth rate when fed on a mixture of rotifer, *Artemia* and copepod (6.35 ± 0.45 % $day^{-1}$), or a mixture of rotifer and copepod (6.33 ± 0.25% $day^{-1}$) than feeding on a mixture of rotifer and *Artemia* (4.93 ± 0.43% $day^{-1}$; $P < 0.05$).

Larval fish survival was not significantly different between treatments from 1 to 7 DPH ($P < 0.05$, Fig. 6.2). In contrast, fish survival was also significantly affected by the feeding treatments from 8 to 21 DPH ($P < 0.05$, Fig. 6.2). Fish had the highest survival rate (64.20 ± 3.56 %) when fed on a mixture of rotifer, *Artemia* and copepods while lowest survival was observed when fish were fed with a mixture of rotifer and *Artemia* from 8 to 21 DPH (42.60 ± 4.51%). The survival rate in larval fish depended on the type of live food ($P < 0.05$, Fig. 6.2).

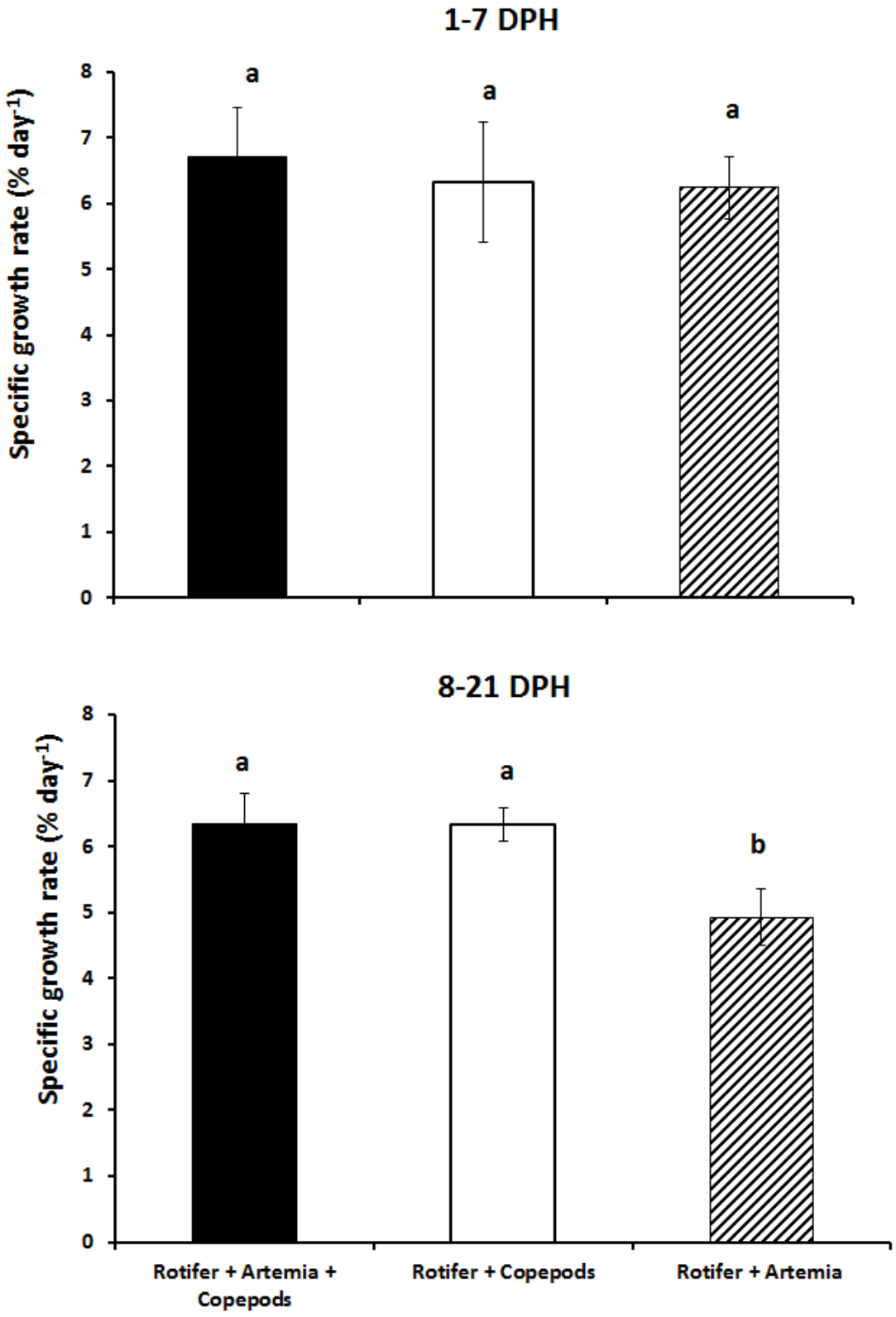


**Fig. 6.1** The specific growth rate of *Lates calcarifer* larvae in different feeding treatments. Different letters represent significant difference ($P < 0.05$).

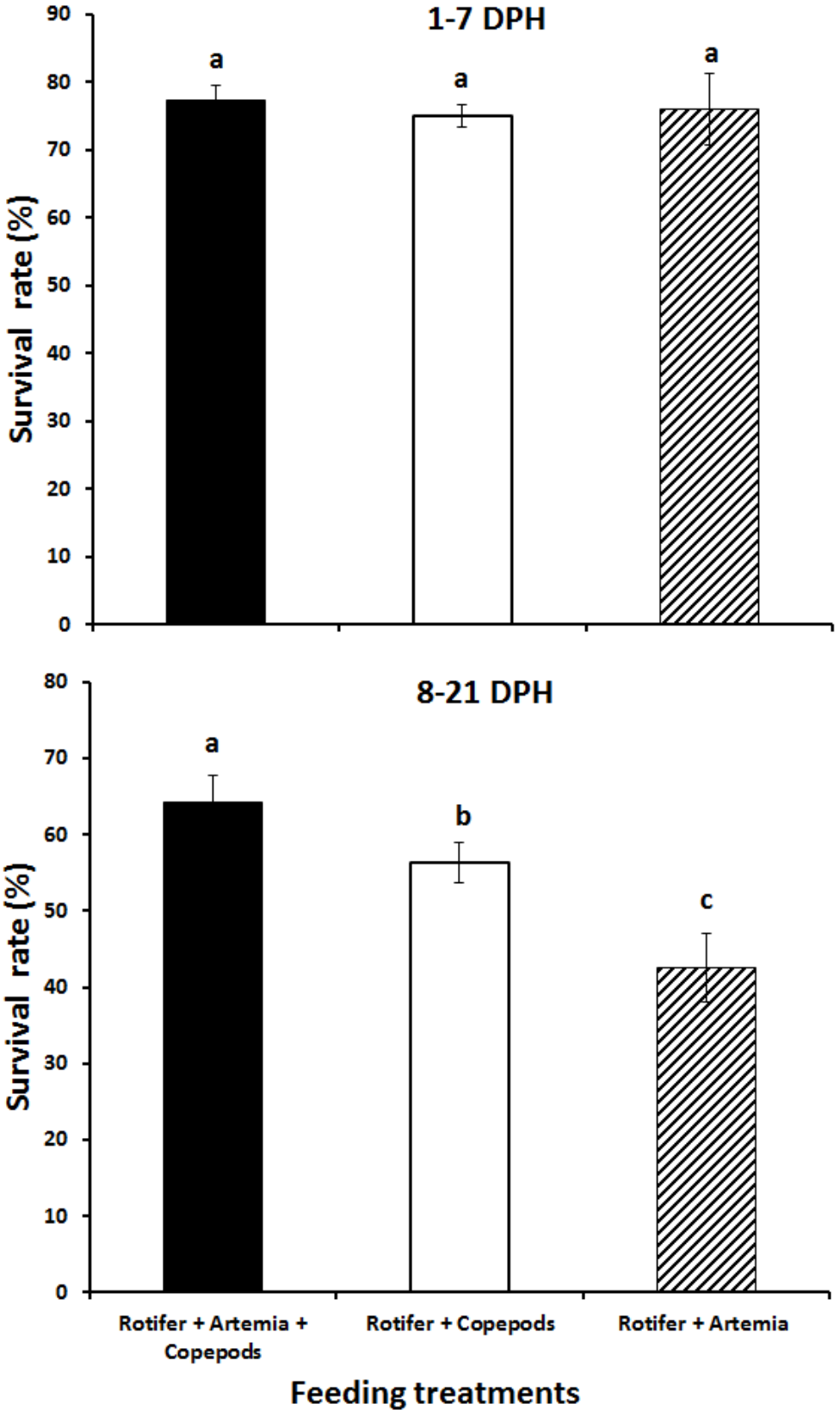


**Fig. 6.2** Survival rate in *Lates calcarifer* larvae in different dietary treatments. Different letters represent significant difference ($P < 0.05$).

### *6.4.3 Food selectivity*

Fish in each feeding groups showed different prey selectivity ($P < 0.05$, Fig. 6.3). In group A during the period of co-feeding of rotifer, *Artemia* and copepod, larval fish preferred copepod and *Artemia* and selected against rotifer on 11 DPH (Fig. 6.3). While in group B, copepods were selected as a preferred prey, but in group C, rotifers were selected for compared to *Artemia* on 11 DPH (Fig. 6.3). On 15 DPH, copepods became the preferred prey for fish when fed a mixture of rotifer, *Artemia* and copepod (group A) and similar results were also observed in fish fed a mixture of copepod and rotifer (group B). Similar trends were also observed on 19 and 21 DPH where in both feeding groups (group A and B), copepods were selected for and rotifers selected against by fish. However, in group C, fish selected for *Artemia* and against rotifer from 15 to 21 DPH. Prey selectivity by larval fish depended on the live food used for feeding ($P < 0.05$, Fig. 6.3).

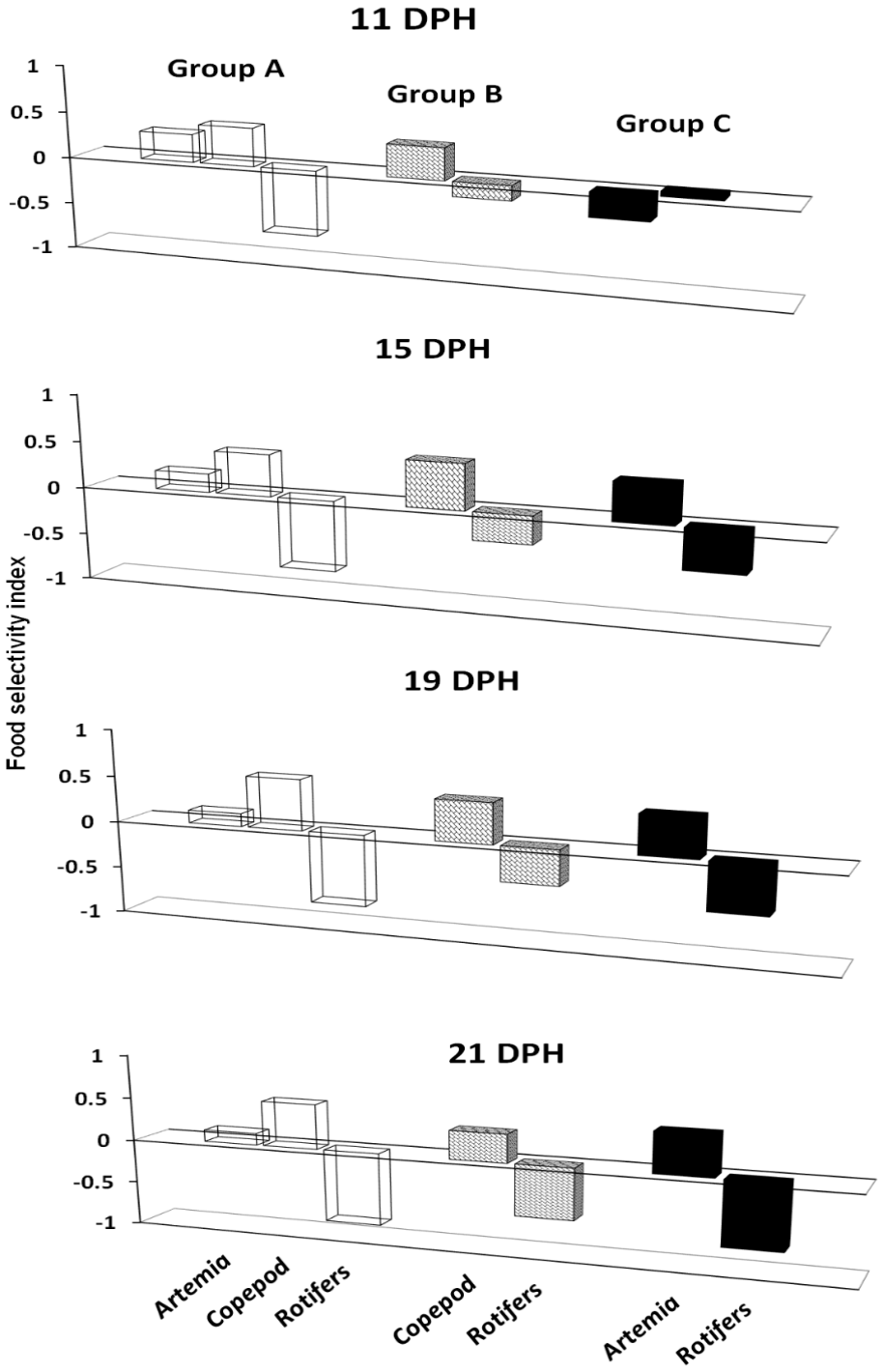


**Fig. 6.3**. Food selectivity by *Lates calcafier* larvae fed different live foods.

## 6.5 Discussion

In this study, the type of live food significantly impacted the profiles of saturated, monosaturated and polyunsaturated fatty acids in fish larvae. The contents of essential fatty acids such as EPA, DHA and ARA in larval fish depended on the composition of live food combination in the rearing tank. The high content of essential fatty acids in fish fed copepod is possibly due to the content of n-3 highly unsaturated fatty acids (n-3 HUFA) in *Cyclopina kasignete* as reported by Rasdi *et al.* (2015). In the present study, the seabass larvae contained a lower amount of essential fatty acids when fed on the mixture of rotifer and *Artemia* due to nutritional deficiencies in these live foods (Payne *et al.* 2001; Watanabe *et al.* 1978). In another study, Bravo *et al.* (1996) reported that the contents of n-3 highly unsaturated fatty acids (n-3 HUFA) in two species of copepods (*Pseudodiaptomus* sp. and *Acartia* sp.) are 2-3 times higher than in rotifer and *Artemia*. The nutritional value of live feeds, particularly the quantity and quality of n-3 highly unsaturated fatty acids (n-3 HUFA) are directly related to the EPA and DHA contents in fish larvae (Payne *et al.* 2001). In another study, the high percent of EPA and DHA in a copepod, *Temora longicornis* resulted in a high percent of EPA and DHA in the halibut larvae *Hippoglossus hippoglossus* compared to fish fed *Artemia franciscana* (Evjemo *et al.* 2003). The Dover sole *Solea solea* larvae fed copepod rich in EPA and DHA showed darker pigmentation and rapid development of the nervous system such as brain and retina (Heath & Moore 1997). However, in the present study no observations were made on the variation in pigmentation and development of nervous system in the larval fish among dietary treatments, which warrants further investigation in future.

Growth and survival rate were improved in larval fish fed a mixture diet containing *C. kasignete* in the present study, suggesting that the nutritional profile of live food is related to growth and survival in fish larvae, which is supported by the study of Santhanam (2012) that the inclusion of copepod *Oithona rigida* results in better growth and survival of the seabass *Lates calcarifer* larvae. The poor growth and survival in seabass larvae fed live food only involving rotifer and *Artemia* nauplii could be a reflection of the inadequate nutritional value in food supply. Similar result was also observed on larval clownfish *Amphiprion clarkii* where highest growth and survival were attained when larvae were fed mixtures of copepod *Tisbe* sp. with either rotifer or *Artemia* nauplii compared to combined diets of *Artemia* and rotifer (Olivotto *et al.* 2008). In contrast, there was no growth difference between rotifer- and copepod-fed larvae of striped patio *Eugerres brasilianus,* but the survival of the larvae was higher when fed with copepods than rotifers (Hernández Molejón & Alvarez-Lajonchère 2003). Similarly, the use of copepod *Acartia clausi* as live food improves the growth and survival of *Lates calcarifer* larvae compared to those fed rotifers and *Artemia* nauplii (Rajkumar & Kumaraguruvasagam 2006). A possible explanation for the low growth and survival in seabass larvae fed rotifers and *Artemia* nauplii is that these live foods contain lower HUFA which are essential for the growth and survival in marine fish larvae (Kanazawa 2003). In the present study, seabass larvae fed a combined diet involving the copepod *C. kasignete* resulted in better growth and survival performance when compared to other feeding treatments. As a result, supplementation of copepod to fish larvae is thus highly recommended in fish hatchery.

In another study, the nauplii and copepodite of the copepod *Oithona oculata* (65-500 µm long) could successfully replace *Artemia* nauplii and rotifers in rearing *Eugerres brasilianus* larvae (Hernández Molejón & Alvarez-Lajonchère 2003). In the present study, the nauplii and copepodite of *C. kasignete* (80-450µm) were successfully replace the traditional live food species in fish larviculture. Moreover, the slow growth in fish fed rotifer and *Artemia* could also be attributed to the fact that the larvae spend more energy searching for *Artemia* and rotifers which have tendency to swim along the tank walls (Laurence 1977) compared to copepods which have jerking (i.e. zigzag) swimming motion with subsequent pauses for floating, providing a visual stimulus for fish larvae, which can be easily detected by visual feeding fish larvae (Barroso *et al.* 2013).

A strong positive selection for copepods was observed in the seabass larvae from day 11 to day 21 DPH. The two feeding groups in this study showed that seabass larvae preferred copepod over rotifer, which is consistent with the result of a previous study where turbot larvae selected for copepod over rotifer when these feed organisms were offered together and the growth and survival were better in the treatments with a mixture of rotifer and copepod compared to the mixture of copepod and cladoceran *Padon* sp. (Kuhlmann *et al.* 1981). Similarly, our study showed that the growth and survival of *L. calcarifer* were better when fed on the combination diet involving copepods compared to other feeding treatments. In another study, the larval red snapper *Lutjanus argentimaculatus* were offered with the copepod *Acartia sinjiensis* and rotifers in the same tank for evaluation of prey selectivity and the larvae showed strong preference on copepods over rotifers with fast growth and high survivorship at the end (Ohno *et al.* 1997). Information on prey

selectivity is vital for optimization of live food supply in larval fish rearing because during the course of fish ontogeny, larval fish gradually change food preference due to the change of size and morphology of feeding apparatus in fish larvae (Shaw *et al.* 2006). Such changes in food selectivity are the way how larval fish adapt to food availability in the environment and to optimize energy intake (van der Meeren 1991). In the ocean, copepods are the principal prey of most fish larvae (McKinnon *et al.* 2003) and our study proves that seabass larvae opt for copepods more than the conventional live feeds of rotifer and *Artemia*.

In conclusion, seabass larvae contain high amounts of essential fatty acid such as EPA and DHA when fed live food involving copepods. The growth and survival rates were low in seabass larvae fed live food only containing rotifers and *Artemia*. In terms of food selectivity, seabass larvae selected copepod over rotifer and *Artemia* from 11 to 21 DPH. This study indicates that the supplementation of copepods improved the performance of larval fish rearing and increased production efficiency and profit in fish larval aquaculture. Our finding highlights the importance of incorporating copepods into the feeding regimes for seabass larvae, which may be applicable to the culture of fish larvae for other species.

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# Chapter 7

# General Discussion and Conclusion

Understanding food, nutrition and feeding behaviour of fish larvae is pivotal to the success of sustainable production of fish larvae in hatcheries (Mahjoub *et al.* 2013). The availability and supply of highly nutritious live food are a major concern in fish larviculture. To date, many aquaculturists face difficulties in finding appropriate live food with sufficient nutrients for larval fish and still rely on few available live food species such as rotifers and *Artemia* nauplii to feed fish and crustacean during their early life stages (Marcus & Murray 2001). Unfortunately, rotifers and *Artemia* nauplii are insufficient in providing necessary nutrition for some marine fish larvae due to incomplete nutrient content (Stottrup 2006) and erratic swimming behaviour (von Herbing & Gallager 2000) of these live food organisms. Even after artificial enrichment using emulsified oils and other common enrichment products, rotifers and *Artemia* are still nutritionally incomplete and cannot provide adequate nutrition for some fish larvae. On the other hand, copepods are deemed more suitable and substantial in nutrition for most marine fish larvae as they are the common live food of marine fish larvae in the ocean (Rasdi & Qin 2014). This thesis addressed the issues of nutrition improvement in algae through nutrient manipulation in the culture medium, nutritional and enzymatic responses of copepods to their food, copepod growth and productivity, and the use of copepods to feed fish larvae. The major findings of this thesis research are summarized as follows:

**Major findings**

1. The range of optimal N:P ratios for the cultivation of *Tisochrysis lutea* and *Nannochloropsis oculata* were identified as 30:1 to 20:1. Culturing algae at the N:P

ratio of 120:1 led to reduction in growth performance and low protein content in both species of algae. The 20:1 N:P ratio favoured eicosapentaenoic acid (EPA) production in *N. oculata* and the 30:1 N:P ratio favoured docosahexaenoic acid (DHA) production in *T. lutea.*

2. The copepods fed with dried *Melosira* sp. or fresh *T. lutea* contained higher levels of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (ARA), protease and trypsin than those fed with other algal diets. The amounts of EPA and DHA in the copepod were higher than those in the dietary algae, suggesting that the copepod has the ability to accumulate some essential fatty acids (EFA).

3. Both food quality and algal types are the key controlling factors for the growth and reproduction of the copepod *Cyclopina kasignete*. Copepods showed better growth, survival and productivity when fed the single or combined diets of dried *Melosira* sp. and fresh *T. lutea.*

4. The inclusion of live copepods in the diet improved fish survival and growth, and increased the contents of essential fatty acids (EPA and DHA) in seabass larvae. In relation to food selectivity, seabass larvae preferred feeding copepods over rotifers and *Artemia* from 8 to 21 DPH (days post-hatch).

## 7.1 Knowledge advance and significance of the thesis research

### 7.1.1 Significance of N:P ratio manipulation for improvement of algal nutrition

Algal quality plays an important role as a tool to ensure successful cultivation of copepods (Rasdi & Qin 2014). The algae *T. lutea* and *N. oculata* with the former containing the essential fatty acids of DHA and the latter being rich in EPA are common algal species used as live food in aquaculture (Rasdi & Qin 2015). It is well known that there are huge variations in the profile of fatty acids between different species of microalgae. The fatty acid profile is algal species-specific but is also influenced by the culture condition, especially nutrient supply (Roncarati *et al.* 2004; Dunstan *et al.* 1993). In the present study, the manipulation of N:P ratios is proven to be an effective strategy in changing the biochemical composition of algae. In Chapter 3, the culture medium with N:P ratios of 20:1 and 30:1 favoured algal growth and increased the protein content, while the higher N:P ratio (120:1) reduced algal growth and protein content but increased lipid in both species of algae. The N:P ratio of 20:1 increased the EPA content in *N. oculata* while the N:P ratio of 30:1 increased the DHA content in *T. lutea*. Similarly, Harrison *et al.* (1990) reported that the higher N:P ratio of 555:1 (P limitation) reduced the amount of EPA and DHA in *Thalassiosira pseudonana* and *Chaetoceros calcitrans* compared to a lower N:P ratio of 14:1. To my best knowledge, this thesis was the first study reporting the impact of N:P ratios on biochemical properties between algae species with contrasting EPA and DHA profiles. Therefore, the knowledge of appropriate N:P ratios for algal

cultivation obtained through this study is useful to produce algae with better nutrition composition which could enhance the nutrition of copepods. In Chapter 4, I tested these nutritionally modified algae including fresh *T. lutea*, *N. oculata* and other types of algae as food for copepods to evaluate the efficacy of these diets for improvement of copepod nutrition.

### 7.1.2 Modification of fatty acids and digestive enzymes in copepods

The nutrient content of copepods cannot be manipulated through the existing enrichment techniques used for rotifers and *Artemia* nauplii as copepods show rejection, avoidance and morbidity when they are subject to some excess chemical substances in the nutritional enrichment formula (Huntley *et al.* 1986; Kleppel & Burkart 1995). Therefore, the change of copepod nutrition has to be done by feeding copepods with suitable algae to improve the value of copepod nutrition. Most studies have used fresh algae, but there is little information using dried microalgae to feed zooplankton especially when fresh algae are in short supply for copepod culture. Findings in Chapter 4 indicate that dried algae can be used alone or with fresh algae to feed copepods, which is similar to a previous study on another zooplankton species where dried *Chlorella* were used as a supplementary feed for rotifers in the absence of fresh algae (Mostary *et al.* 2007). Chapter 4 also found that the amounts of fatty acids and digestive enzymes in copepods can be modified to feed copepods with different dietary algae. Even when the contents of EPA, DHA and ARA in dried *Melosira* sp. were low, *C. kasignete* still contained higher EPA, DHA and ARA when

fed on these corresponding algae than other diets. This finding is consistent with a previous study on the harpacticoid copepod *Attheyella trispinosa* where EPA and DHA were accumulated in the copepods despite the negligible amount of PUFA in the dietary algae *Leptolyngbya foveolarum* (Caramujo *et al.* 2008). Chapter 4 specifically reported that the absence or low DHA and EPA in dried *Melosira* sp. resulted in high EPA and DHA in *C. kasignete*, suggesting that this copepod species can accumulate these PUFAs in its body tissues. Furthermore, Chapter 4 documented that the highest protease and trypsin enzymes in copepods were detected when fed on dried *Melosira* sp., which is consistent with a study on the copepod *Tisbe biminiensis* that dietary diatoms *Thalassiosir*a sp. and *Chaetoceros muelerii* can increase the amounts of trypsin and proteases in copepods (França *et al.* 2010). The result in Chapter 4 provides important evidence that copepods are able to bioconvert some essential fatty acids, and dietary algae can change the amount of fatty acids and digestive enzymes in copepods. By having this option, aquaculturists can select the suitable species of algae to improve nutritional composition of copepods. The three best algae identified to increase PUFA and digestive enzymes in copepods are dried *Melosira* sp., fresh *N. oculata*, and fresh *T. lutea*, which were further used in Chapter 5 to test the effects of single or combination algal species in accelerating the growth and reproduction of the copepods *C. kasignete.*

### 7.1.3 Population growth, survival and reproduction of *C. kasignete*

In Chapter 5, the *C. kasignete* showed maximum growth and offspring production when the copepod fed the monoalgal diet of dried *Melosira* sp. or the

binary diet of dried *Melosira* sp. and fresh *T. lutea*. The result demonstrates that the diatom *Melosira* sp. can be used as either a single or mixed form with other algae such as *T. lutea* to sustain the growth and reproduction of *C. kasignete*. Similar results were also reported on the copepod *Euterpina acutiferons* feeding on the diatom *Chaetoceros muelleri* (Camus & Zeng 2012). Moreover, the offspring production of copepods fed dried *Melosira* sp. is consistent with previous studies where diatoms as food are able to enhance offspring production of copepods *Acartia* sp., and *Calanus* sp. (Ismar *et al.* 2008; Irigoien *et al.* 2002). In a similar study, the cyclopoid copepod *Paracyclopina nana* fed on *Isochrysis* sp. produced maximum population densities and increased reproduction capacities compared to when it fed on *Tetraselmis suecica* (Lee *et al.* 2006). Given the high EPA content of *N. oculata* as the copepod diet, the copepod gained relatively low population growth and offspring production, suggesting that a high EPA in the diet does not sustain the nutritional requirement of copepods (Morehead *et al.* 2005; Rasdi *et al.* 2015). With regards to this, the *Isochrysis* sp. could positively promote the productivity of copepods *Acartia bilobata* (Lee *et al.* 2006; Pan *et al.* 2012). The present study also showed that *T. lutea* (formerly known as *Isochrysis galbana*) is a superior food for the growth and offspring production of *C. kasignete*. Chapters 4 and 5 demonstrate that dried *Melosira* sp. and fresh *T. lutea* could increase fatty acids, digestive enzymes, growth and reproduction of *C. kasignete* either when used as a single or in a combination with other algae. Lastly in Chapter 6, the copepods with superior nutritional quality after feeding on modified nutritious algae produced in the trial described in Chapter 5

were fed to seabass larvae *Lates calcarifer* to examine the potential as a prominent live food for larval fish.

### 7.1.4 Growth, survival and food selection of seabass larvae on live foods

Chapters 4 and 5 show that *C. kasignete* is proven to contain high EPA, DHA, protease and trypsin after feeding on enriched algae which support fast growth and high reproduction rates of copepods (Rasdi *et al.* 2015). This finding led to the comparative study in Chapter 6 where the seabass *L. calcarifer* larvae were fed with *C. kasignete* and other conventional live food species (rotifers and *Artemia* nauplii). Chapter 6 clearly indicates the merit of using this copepod for seabass larvae evaluated by growth, survival, food selectivity and fatty acid contents of the larval fish.

Larval seabass showed better growth and survival when fed on a mixture of diets involving copepods, suggesting that the nutrition provided by copepods could enhance the growth and survival of fish larvae, which is consistent with the results by Santhanam (2012) that the inclusion of copepod *Oithona rigida* results in better growth and survival of the seabass *Lates calcarifer* larvae. The slow growth and low survival in seabass larvae in Chapter 6 by feeding fish with a live diet with sole rotifers or *Artemia* nauplii may be a consequence of inadequate nutrition in the diet. A similar result was also observed in a previous study on *Amphiprion clarkii* where the highest growth and survival rates were obtained when larvae were fed the mixture of copepod *Tisbe* sp. with either rotifer or *Artemia* nauplii compared to a combined

diet of *Artemia* and rotifers (Olivotto *et al.* 2008). As a live food source, copepods could meet the nutritional requirements of fish larvae compared to the conventional diets of rotifers and *Artemia* nauplii (van der Meeren 1991). The growth and survival of *L. calcarifer* larvae were comparatively higher when copepods were supplemented into the diet mixed with rotifers and *Artemia* since copepods contain higher HUFAs which are essential for the growth and survival of most marine fish larvae (Kanazawa 2003).

Chapter 6 indicates a strong selection for copepods by the seabass larvae compared to rotifers and *Artemia* nauplii. The feeding trial in this study showed that seabass larvae preferred copepods over rotifers, which is consistent with a previous study on turbot larvae, where copepods were preferably selected over rotifers when these feeds were offered together to the fish (Kuhlmann *et al.* 1981). Information on prey selectivity is vital for optimization of live food supply in larval fish rearing because during the course of fish ontogeny, larval fish gradually change food preference (Shaw *et al.* 2006). Such changes in food selectivity is an adaptation for larval fish to optimize their energy intake (van der Meeren 1991). In the ocean, copepods are the principal prey of virtually all fish larvae (McKinnon *et al.* 2003) and the present study proves that seabass larvae opt for copepods more than the conventional live feeds (rotifer and *Artemia*). Furthermore, seabass larvae contain high amounts of essential fatty acid such as EPA and DHA when fed the combination diets involving copepods compared to a diet mixture with only rotifers and *Artemia* nauplii.

## 7.2 Conclusions

To improve the production of a potential copepod species in hatcheries, this thesis explored four key issues that influence the production efficiency of copepods and its use as live food for fish larvae, including (1) modification of algal nutritional composition, (2) utilization of nutrient-enriched algae as food for copepods, (3) food type and feeding requirement for improving copepod growth and productivity, and (4) prospective use of copepod as live food for fish larvae. The results from this thesis provide significant contribution in providing the essential knowledge of copepods nutrition and important information to guide management, practice and protocols for successful production of copepods. The major conclusions are summarized as below:

1. Alteration of medium N:P ratios to improve algal nutritional composition

The N:P ratio of 20:1 favoured the growth and protein contents in algae, while the N:P ratio of 120:1 reduced algal growth and protein synthesis but increased algal lipid contents. The 20:1 N:P ratio favoured EPA production in *N. oculata* and the 30:1 N:P ratio favoured DHA production in *T. lutea.* The outcomes from this research indicate that N:P ratio manipulation could change the biochemical composition in algae and the N or P limitation tends to lower PUFA contents in algae.

2. Fatty acids and digestive enzymes in copepods can be altered through dietary algae

The amounts of fatty acids and digestive enzymes in *C. kasignete* can be modified by feeding copepods with different dietary algae. The copepods fed with

dried *Melosira* sp. and fresh *T. lutea* contained more PUFAs (EPA, DHA and ARA) and digestive enzymes (protease and trypsin) than those fed other species of microalgae. Furthermore, copepods fed dried *Melosira* sp. and fresh *T. lutea* had higher levels of EPA and DHA than those fed other microalgae diets, suggesting that copepods are able to enrich the content of body essential fatty acids through feeding proper algae. This study indicates the potential use of microalgae to improve the nutritional composition and digestive enzyme contents in copepods. As a result, dietary copepods may provide suitable nutrition and essential digestive enzymes to fish larvae.

3. Copepod growth, survival and reproduction can be improved through food manipulation

The use of the dried diatom *Melosira* sp. to feed copepods could improve copepod growth, survival and productivity as a single diet or in combination with other algae. The cyclopoid copepod *C. kasignete* has shown fast population growth and can be a potential live food for fish larvae. The fresh *T. lutea* and dry *Melosira* sp. or their combination are ideal food for copepods.

4. The copepod *Cyclopina kasignete* as live food improved the performance of seabass larvae

The inclusion of *Cyclopina kasignete* as live food alone or in combination with rotifers and *Artemia* nauplii enhanced fish growth, survival and the EPA and DHA contents in seabass larvae compared to the combination of only rotifers or

*Artemia* nauplii alone, or their combination. Seabass larvae showed preference by selecting for copepods as food when other live foods such as rotifers and *Artemia* nauplii were present in the environment. The improved performance of seabass larvae could be due to the suitable size or nutrition of *Cyclopina kasignete* as a live food.

## 7.3 Recommendations

The thesis outcomes could contribute not only to the understanding on the interactions among nutrients, algae, zooplankton and fish larvae, but also to the application for managing nutrients in algal culture, algae supply in copepod culture, and copepod supplementation as live food in larval fish culture. The specific recommendations of the research outcomes are summarised as follows:

1. The biochemical composition of algae is a crucial measure of algal quality in commercial application. The growth rate and fatty acid contents in *N. oculata* and *T. lutea* could be manipulated by N:P ratios in the algal medium. Therefore, it is recommended that the efficient production of fatty acids and optimization of algal growth in *N. oculata* and *T. lutea* could be improved by careful monitoring the N:P ratios in the algal culture medium. The optimal N:P ratio is recommended from 20:1 to 30:1 to produce algae as food for copepods.

2. The quality and type of algae can concurrently impact the amount of fatty acids and digestive enzymes in copepods. The selection for ideal dietary algae is important to improve copepod nutrition. Dried *Melosira* sp. and fresh *T. lutea* are

recommended as the basal diet for copepods to maximize the fatty acid content and digestive enzymes in copepods.

3. The use of mono and binary species of algae can enhance the growth and reproduction of copepods. The recommended diet to facilitate the growth and reproduction in copepods is either a single or a combined diet of dried and fresh microalgae such as *N. oculata* and *T. lutea*. The dried *Melosira* sp. as a substitution of fresh algae can maintain copepod growth and reproduction to overcome the shortage of fresh algal supply in copepod culture.

4. The continuous availability of live food of high nutrition plays a vital role in successful growth and survival of marine fish larvae. It is recommended that the sole use or supplementation of copepods as live food be considered in the culture of marine fish larvae to improve fish growth and survival during the early and critical development stages.

## 7.4 Future Research

The outcomes of this research provide fundamental understanding of food requirement, population growth and reproduction of *C. kasignete*, and the potential use of this copepod as live food for fish larvae. Nevertheless, some questions are still prevalent and future research is needed to tackle the following unsolved issues:

1. Although the nutritionally-modified algae in this study stimulated the copepod growth and reproduction, achieving sustainable mass production of copepods is still a challenge. Exploitation on the use of other algal species or algal supplementation in copepod culture is still needed. In copepod mass culture, the optimal supply of algae (density, biomass or energy) should be further determined in future research. In addition, it is also pivotal to determine the optimal harvest frequency and sustainable harvest quantity of copepods in mass culture. Due to facility limitation and workload overflow, the current study did not test the impact of these factors in copepod culture. However, these questions are important to develop copepod as a sustainable live food for fish larvae.

2. It is important to find ways to store subitaneous or diapause eggs of *C. kasignete* to make copepods more reliable to access like *Artemia* cysts. The life history of *C. kasignete* needs further research to understand the conditions for the production of resting or diapause eggs. Due to time and facility limitation, the current study did not test the possibility of producing resting or diapause eggs of *C. kasignete* for storage. This is an important issue because there is a need to increase the availability of copepod seeds to start copepod culture in a fish hatchery.

3. Further research should also consider other aspects of copepod nutrition such as the factors regulating specific amino acids in the algal diets. In addition, it is important to find suitable methods to enrich the micronutrient contents such as

selenium, copper, vitamin C and E in copepods to meet the nutritional requirement of fish larvae. Further research in these areas will improve the understanding of copepod nutrition and its use as live food for fish larvae.

4. It should be pointed out that the efficient production of any newly exploited copepod species such as *C. kasignete* can only be measured by the success of larval fish culture in a commercial setting. Therefore, it is important to further test this copepod species on a commercial scale to prove its practicality as live food in fish larviculture.

## 7.5 References


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## Appendix

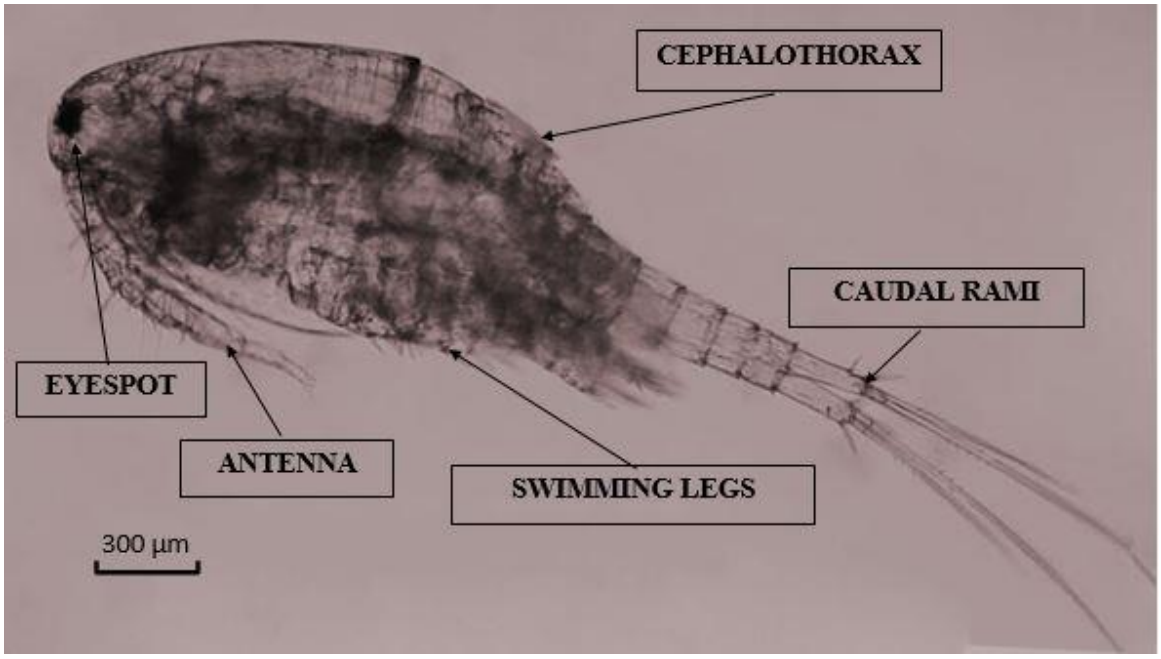


Fig. 8.1 Morphology of adult male copepods

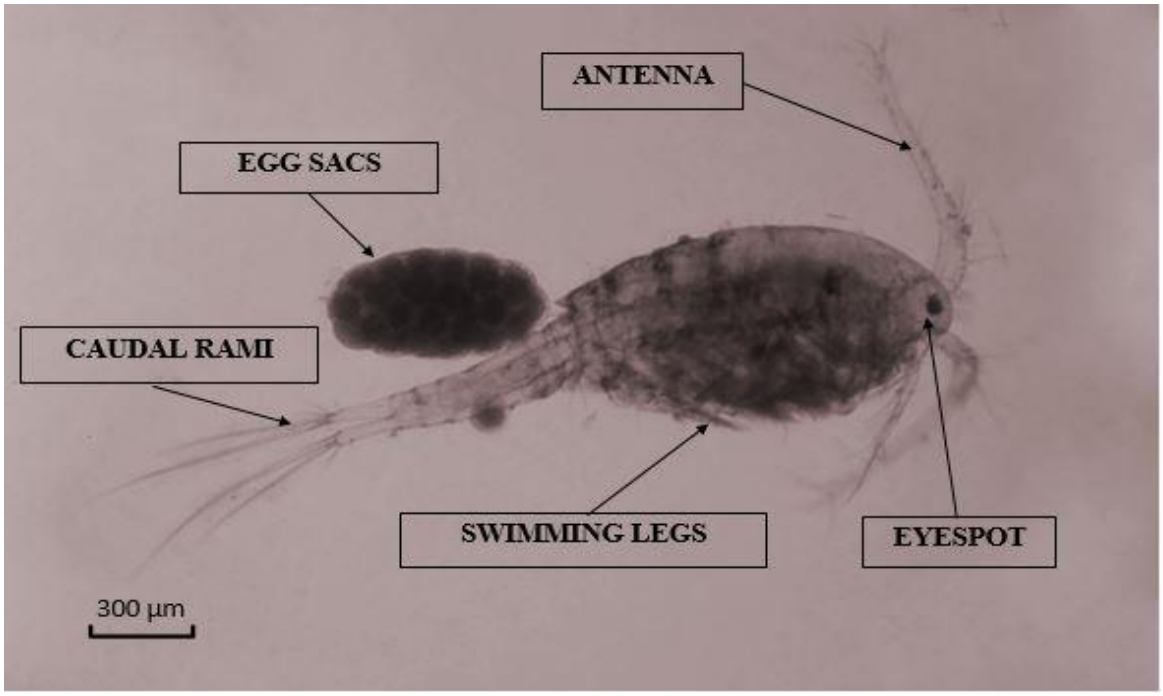


Fig. 8.2 Morphology of female copepods carrying eggs

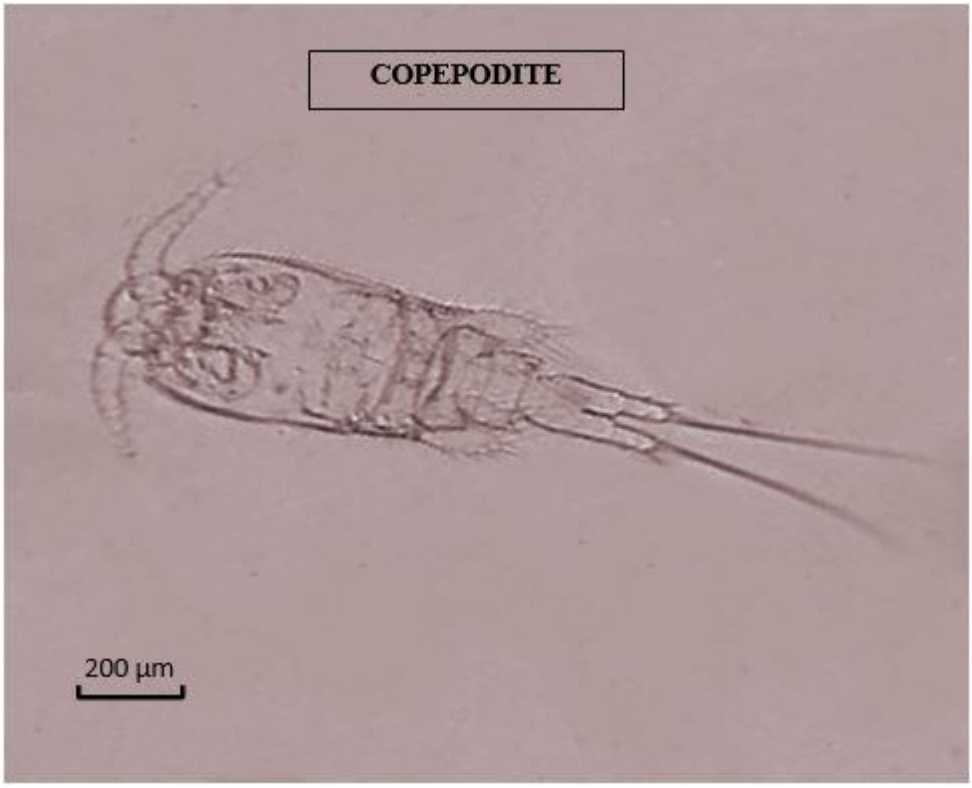


Fig. 8.3 Copepodite stage

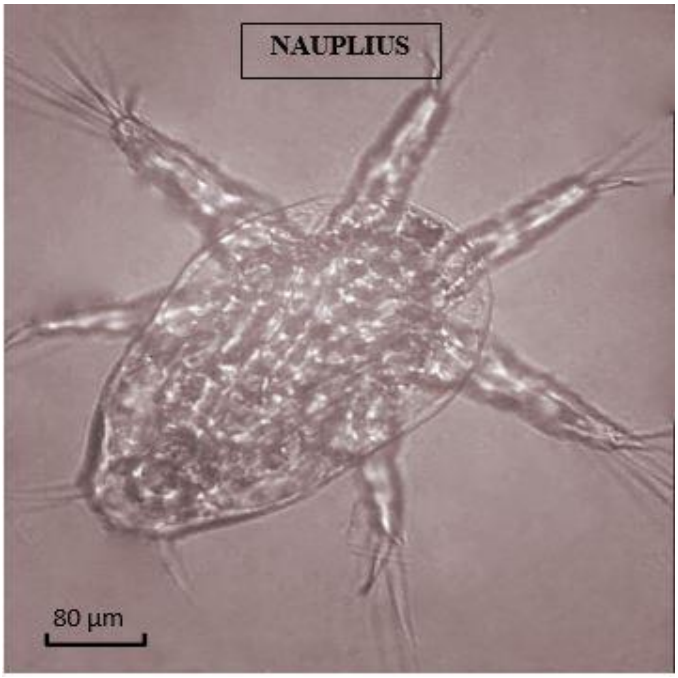


Fig. 8.4 Nauplius stage

**Taxonomy of this species**

| | | |
|---|---|---|
| Kingdom | : | Animalia |
| Phylum | : | Arthropoda |
| Subphylum | : | Crustacea Pennant, 1777 |
| Class | : | Maxillopoda Dahl, 1956 |
| Subclass | : | Copepoda Milne Edwards, 1840 |
| Order | : | Cyclopoida Burmeister, 1834 |
| Family | : | Cyclopinidae Sars, 1913 |
| Genera | : | Cyclopina Claus, 1862 |
| Species | : | *Cyclopina kasignete* Karanovic, 2008 |

(Karanovic, 2008)

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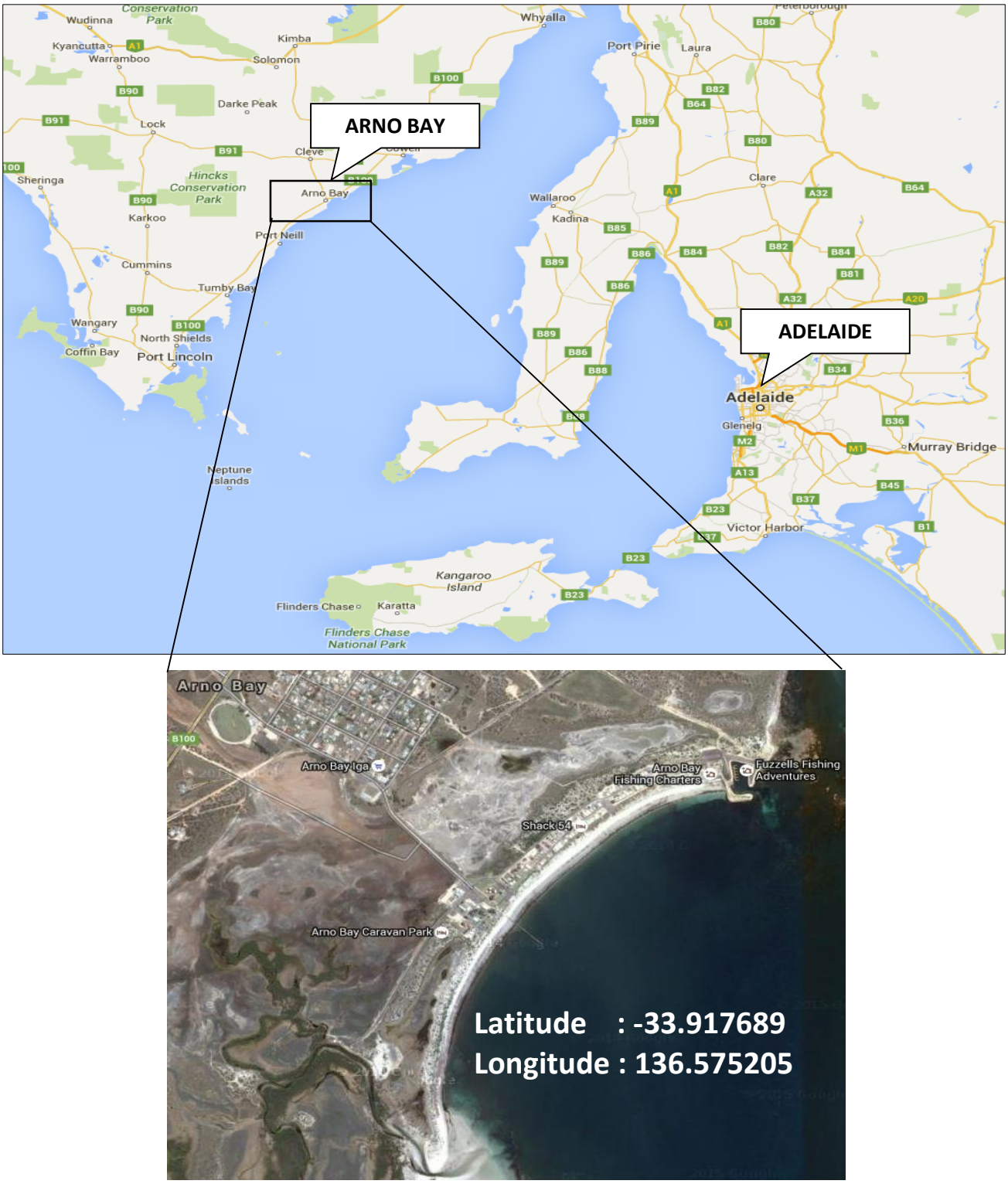


Fig. 8.5 Location of the sampling site