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JAMES COOK UNIVERSITY AUSTRALIA

Developing intensive culture techniques for the tropical copepod *Parvocalanus crassirostris* as a live feed for aquaculture

Thesis Submitted by

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Statement of the Contribution of Others

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Abstract

Copepods are the most common metazoans in marine environment. The number of copepod species identified has reached about 11,500 species, composed of free-living pelagic, benthic and symbiotic copepods. Copepods are the most important secondary producers in marine environments and are the natural prey for larvae of most fishes. During the past decades, there has been increasing interest in the development of mass culture techniques for copepods as live food for aquaculture. Unlike traditional live feeds such as rotifers, copepods are rich with essential fatty acids, free amino acids and other essential micronutrients and their nutritional profiles meet the requirements of fish larvae. Moreover, the small size of copepod nauplii is vital for the first feeding larvae of fish species with small mouth gape sizes, such as groupers and snappers. Copepods also stimulate strong feeding responses in fish larvae through their distinctive swimming patterns. Despite multiple advantages of copepods as larval food, utilisation of copepods in aquaculture hatcheries is still sporadic, which is largely attributed to difficulties in their culture and, in particular, their low culture productivity. While recent improvements in intensive cultivation techniques for some copepod species had been achieved, sustainable and feasible mass culture methods for most species, particularly planktonic species, remain elusive. This thesis focused on filling the knowledge gaps by investigating improvements on intensive culture techniques, including several novel approaches, for calanoid copepods. The primary aim of this thesis was to develop intensive culture methods to overcome the current constraints hindering a reliable mass production of calanoid copepods, specifically improving culture productivity for the tropical calanoid *Parvocalanus crassirostris*.

The first chapter of this thesis reviews the current knowledge related to the advantages of copepods as larval feeds compared to traditional live feeds and discusses the current constraints and major hurdles in copepod cultivation. The subsequent experimental chapters can be grouped into three parts. The first part (Chapter 2 and 3) investigates the optimal algal diets for the cultivation of *P. crassirostris* and exploits the potential use of commercial algal pastes as an alternative diet. The second part (Chapter 4) focuses on identifying optimal culture density for *P. crassirostris.* Finally, the third part (Chapter 5 and 6) evaluates the efficacy of selective breeding and long-term cultivation (domestication) on improving culture productivity of *P. crassirostris*.

One of the major issues in the advancement of copepod mass cultivation is their species-specific dietary requirements. Generally, calanoid copepods feed on live microalgae. However suitable microalgal species for each copepod species needs to be determined, in addition, whether microalgae should be fed singly or in combination. Chapter 2 evaluated 9 different algal diets, including diet combinations and algal pastes, for the cultivation of *P. crassirostris*. The key parameters investigated were egg production, egg hatching success, survival and development of nauplii and copepodite stages, population growth and sex ratio of *P. crassirostris*. The results showed that the combination of two algae species, the Tahitian strain of *Isochrysis* sp. (T-Iso) and the diatom *Chaetoceros muelleri*, gave the overall best results. Meanwhile, a healthy culture could also be maintained using T-Iso only. Although, the use of commercial algae pastes is popular for other live feeds, yet, the results of Chapter 2 demonstrated that commercial algal pastes, including T-Iso pastes, have failed to support *P. crassirostris* culture. Moreover, culturing copepods using commercial pastes has been relatively unsuccessful in previous reports, with no clear explanation as to what may be the main cause of that failure. Therefore, chapter 3 investigated the potential causes that

led to the failure of commercial algae pastes in supporting productive copepod culture. A series of experiments were designed to test 3 hypotheses: I) Food availability was low in the water column of algae paste treatments due to the settlement of dead algal cells on the bottom; II) Adherence of the highly condensed cells of microalgal pastes to the swimming appendages and mouthparts of *P. crassirostris* significantly reduced their feeding and swimming efficiency; and III) the reproductive capacity and survival of *P. crassirostris* were negatively impacted due to lower digestibility of the pastes as well as lower levels of essential nutrient and/or other bio-active contents in dead algal cells. The results showed significantly high adherence to the mouthparts and swimming appendages of *P. crassirostris*, which was the primary reason behind the failure of algal pastes as a diet for copepod cultivation. Additionally, the lower digestibility of algal pastes is likely to contribute to that failure. The results suggest that the current method of producing commercial algal pastes are not suitable for calanoid copepod cultivation, which was demonstrated by the high adherence and low digestibility by copepods as compared to live algae. Therefore, new manufacture methods for algal paste production need to be developed to provide acceptable alternatives to live algae for calanoid copepod cultivation.

Despite increased research in recent years aimed at enhancing copepod culture techniques, the inability to achieve high culture density is still a major bottleneck in copepod culture, particularly for calanoid copepods. Sustainable culture densities were reported to be approximately two adults ml^{-1}, however, such a density would require a large volume of culture water to produce an adequate number for feeding fish larvae. *P. crassirostris* had been observed to sustain higher densities than those reported previously. Therefore, the aim of chapter 4 was to evaluate the effects of initial stocking density on a range of biological parameters affecting the culture productivity

of the calanoid copepod *P. crassirostris*. Five initial stocking densities of 1000, 3000, 5000, 7000 and 9000 adults L^{-1} were evaluated. The results demonstrated that *P. crassirostris* is a highly promising species as live prey for aquaculture hatcheries, and it can be stocked at a high initial density of 5000 adults L^{-1} without negatively impacting its culture productivity. Such high culture density is substantially higher than any other calanoid species documented so far.

Improving the reproductive capacity of calanoid copepods through optimizing diets and environmental conditions has their limitations. Therefore, chapter 5 and 6 explores the genetic improvements that could be made through selective breeding and domestication as new approaches in improving copepod cultivation. While selective breeding as a successful strategy to enhance the life history traits of aquaculture species had been well documented, it has never been tested for calanoid copepods. Chapter 5 was, hence, designed to determine if selective breeding is an effective way to improve the reproductive productivity of *P. crassirostris*. Using a family selection program combined with a circular mating strategy, *P. crassirostris* was cultured for five generations. Females were selected for their high reproductive capacity, and a control chosen by random selection was maintained alongside. The select line exhibited a positive response for selection with a significant increase in total egg production of 24.5% over the female lifespan as compared to the control line. The results provided the first clear evidence of the effectiveness and high potential of selective breeding in enhancing the reproductive capacity of calanoid copepods. Moreover, selective breeding can be employed as an effective approach for the improvement of other life history traits of copepods to enhance their culture productivity as well as other traits improving their quality as larval live food.

Finally chapter 6 investigated the effects of domestication on *P. crassirostris* culture. Domestication often leads to improved adaptability to culture environments of cultured species and the enhancement of traits related to culture productivity, however, this has never been tested for copepods. In this study, wild-caught *P. crassirostris* were compared to a long-held domesticated strain (>2 years) on a range of biological traits, including size of developmental stage, fecundity and lifespan of adult females, culture density, ingestion rates, faecal pellet production and fatty acid profiles. Overall, the results showed that the reproductive capacity and tolerance to the culture environment of *P. crassirostris* have improved significantly due to domestication. The results suggested that domestication could serve as a new approach to improve culture productivity of calanoid copepods.

Overall, the findings of this thesis confirm that *P. crassirostris* is a high potential species for aquaculture due to its superior performance on a range of parameters related to culture productivity. In contrast, *P. crassirostris* culture was relatively easy to maintain compared to other species, adding to its advantages. The thesis concludes with a general discussion and conclusions chapter (chapter 7), discussing the implications of major findings of the thesis in the wider context.

Table of Contents

List of Tables

List of Figures

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Copepods represent more than 80% of the zooplankton assemblages, and are considered to be the nutritional backbone for almost all marine organisms (Mauchline, 1998). The number of copepod species identified by Humes (1994) was 11,500 species, mostly composed of free-living pelagic, benthic and symbiotic copepods, and was forecasted to increase (Humes, 1994). Through the last five decades, copepods had been of interest both ecologically and biologically (Mauchline, 1998). Fish larvae were found to be significantly influenced by copepods horizontal and vertical migration patterns, which indicate their nutritional importance in such critical stage (Fox et al., 1999; Haslob et al., 2009; Möllmann et al., 2004).

Attempts to culture copepods in small-scale under laboratory conditions were first made to understand their dynamics and ecology, which underlined their importance as a limiting factor in ecosystems for early life stage survival as their important food source, a fact supported by numerous field studies (Castonguay et al., 2008; Drillet et al., 2011). The protocols produced to cultivate copepods in small-scale form the basis for aquaculturists to evaluate these organisms for further large-scale culture to provide a continuous food supply for fish larvae culture (Støttrup et al., 1986). The value of copepods resides within their nutritional profile, fundamentally the

presence of essential fatty acids (EFAs), such as highly unsaturated fatty acids (HUFAs); including eicosapentaenoic acid (EPA) 20:5n-3 and docosahexaenoic acid (DHA) 22:6n-3, which meet the requirements of larvae of most marine organisms and fish (McKinnon et al., 2003; Norsker and Støttrup, 1994; Watanabe, 1993). These characteristics inspired aquaculturists to intensively research the culture of copepods as a solution for an old problem, so called the “bottle neck” in aquaculture, which is the high mortality of marine fish larviculture during first feed (Conceição et al., 2010).

In terms of larval survival, copepods have demonstrated superiority over formulated feeds, as well as the traditional live feeds used in hatcheries, such as brine shrimp (*Artemia*) and rotifers (Payne et al., 2001; van der Meeren et al., 2008; Wilcox et al., 2006). Copepods are highly diverse (Boxshall and Halsey, 2004), which gives an advantage when selecting potential candidates to use in aquaculture hatcheries. However, this diversity can also be overwhelming and establishing a set of criteria for selection can help narrow down the list of free-living copepods available worldwide. When a species is selected, generally, a protocol of optimal diet, physical parameters and other ecological aspects need to be tested and recognized to produce a recommended culture regime for the species (Camus and Zeng, 2009; McKinnon et al., 2003; Milione and Zeng, 2008; Ohs et al., 2010a; Ohs et al., 2010b), yet in order to acquire evidence that copepods can support the demands of fish larviculture, these finding should be applied in aquaculture (Drillet et al., 2011).

This review discusses the opportunities and challenges of using copepods in aquaculture, such as their nutritional advantage, general criteria used in the selection of species and current techniques used to culture copepods. Finally, the potential of using copepods as food source for cultivation of ornamental fish larvae was discussed.

1.2 Nutritional advantages of copepods as larval foods

It has been well documented that live feeds in aquaculture are highly superior to artificial feeds, particularly for early larvae (Koedijk et al., 2010; Olivotto et al., 2009; Olivotto et al., 2008). The biochemical composition, enzymatic content and behavioral attraction of live prey to larvae, are the major components that rule in their favor, and in some cases makes it impossible to substitute them (Drillet et al., 2006a; Støttrup and McEvoy, 2003). In regards to nutrition values, ω-3 HUFAs are often considered to be one of the essential elements required by fish larvae, since growth, survival, metamorphosis and pigmentation are limited by their contents within their diet (Næss and Lie, 1998; Sargent et al., 1999; Shields et al., 1999; Yone and Fuji, 1975). Rotifers and *Artemia* are the only two live feeds routinely used in hatcheries. They are particularly easy to culture. However, although rotifers and *Artemia* are commonly used, they are originally low on the essential nutrients required by marine fish larviculture, and require a process of enrichment prior to their use (Sargent et al., 1999; Yone and Fuji, 1975), which is often labor intensive and costly. More importantly, a comparison of fatty acid (FA) composition (Table 1.1, HUFA and DHA/EPA), demonstrates that copepod nauplii surpass rotifers and *Artemia* nutritionally even after enrichment.

Copepods on the other hand have a very ideal biochemical profile (Table 1.2), which meets the requirements of most marine fish larvae (Evjemo et al., 2003; van der Meeren et al., 2008). The nutritional superiority of copepods is highlighted by their ability to synthesize essential fatty acids (EFAs) "*de novo*", where they can elongate and desaturate Alpha-linolenic acid (ALA) to EPA and DHA (Cutts, 2003; Norsker and Støttrup, 1994). Even in the case of some copepod species that contain relatively lower lipid content, such as *Pseudocalanus* sp., *Temora longicornis*, *Eurytemora* sp. and

Calanus finmarchicus, which have been used in the larval culture of Atlantic halibut and cod (Evjemo and Olsen, 1997; Evjemo et al., 2003; Fraser et al., 1989), their HUFA content is still twice as higher than rotifers and *Artemia*. Furthermore, their HUFA profile is noticeably skewed towards DHA, which is almost 60% of their total lipid content. Among HUFA, there is high emphasis on the significance of DHA in the diet of fish larvae, because DHA is a precursor to many compounds such as rohodopsin, which is a key protein in the pigmentation of fish, thus the importance of a viable DHA source in the diet (Nakamura et al., 1986; van der Meeren et al., 2008). DHA is also vital for the development of neural and visual systems in fish larvae, as its deficiency can lead to major problems including weakening the ability of prey capture (Sargent et al., 1999). Moreover, HUFAs originating from copepods are highly digestible by marine fish larvae in contrast to those of rotifers and *Artemia*, due to the nature and locality of HUFAs. In copepods a large fraction of HUFAs resides within phospholipids, which facilitate their absorption by the fish larvae's intestine, compared to HUFAs incorporated with neutral lipids (Conceição et al., 2010; Coutteau et al., 1997; Gisbert et al., 2005; Izquierdo et al., 2000).

In addition to fatty acids, free amino acids (FAA) are also present in very high quantities within copepods in comparison to rotifers and *Artemia* (van der Meeren et al., 2008). The high level of FAAs in copepods is believed to enhance the digestion process for fish larvae at their initial feeding stage, which can contribute to a higher growth rate and balanced energy partitioning (Conceição et al., 2003; Rønnestad et al., 2003). In addition, high levels of the carotenoid astaxanthin, vitamin C and vitamin E were also found in copepods, and these compounds serve as antioxidative agents and pigment enhancers in aquaculture (Ajiboye et al., 2011; McEvoy et al., 1998; Shields et al., 1999). There are other significant micronutrients that might play significant roles,

for example, the limited available information on iodine as an essential component for thyroid hormone is extraordinary, since evidence shows that copepods have 700-fold higher Iodine level than *Artemia*, which explains the high success in larval metamorphoses and low deformation rate of juvenile fish fed copepods (Moren et al., 2006; Solbakken et al., 2002). However, the concentrations of micronutrients in copepods have hardly been evaluated in most cases.

Table 1.1: Comparison of biochemical profiles of copepod's nauplii, enriched rotifers and enriched *Artemia.*

Organism	Protein	Lipid	Carbohydrate	HUFA[d]	DHA/EPA[d]	Reference
Enriched Rotifers[a]	28-63	9-28	10.5-27	3	1.7	(Lubzens and Zmora, 2003)
Enriched newly hatched *Artemia*[b]	56.2	17	3.6	4.3	1.4	(Garcia-Ortega et al., 1998)
Copepod nauplii[c]	30.3	8.6	-	4.9	2.8	(van der Meeren et al., 2008)

Note: values are in % DW.
[a] Enriched rotifers.
[b] *Artemia* newly hatched and enriched.
[c]Nauplii species belonged to *Eurytemora affinis*, *Centropages hamatus*, and *Acartia grani*.
[d] Data compiled from (Conceição et al., 2010).

Table 1.2: Comparison of Fatty acid composition of different copepod nauplii against rotifers and *Artemia*.

Fatty Acid	*Acartia grani, Centropages hamatus, and Eurytemora affinis*[d]	*Acartia tonsa*[c]	Rotifers[a]	*Artemia*[b]
14:0	1.3±0.8	3	6.7	1.7
16:0	13.7±2.5	15	19.7	14.9
16:1	1.8±1.4	1	9.2	4.8
18:0	3.9±1.0	6	3.9	5.0
18:1(n-9)	1.3±0.7	1	7.8	23.3
18:1(n-7)	2.0±0.5	5	4.9	5.5
18:2(n-6)	1.5±0.5	2	15.3	6.6
18:3(n-3)	1.5±0.9	13	1.2	12.2
18:4(n-3)	4.5±5.7	7	2.0	2.8
20:4(n-6)	0.6±0.3	0	1.9	2.0
20:5(n-3)	16.3±6.4	17	7.1	7.8
22:6(n-3)	40.5 ±2.4	23	12.4	10.6
EPA + DHA	56.8±6.8	40	19.4	18.4

Note: values are % of total lipid.
[a]rotifers grown on Rotimac and *Isochrysis galbana* algae.
[b]*Artemia* enriched with DC-DHA Selco.
[c]*Acartia tonsa* cultured on Rhodomonas baltica.
[d]*Acartia grani*, *Centropages hamatus*, and *Eurytemora affinis* are grown extensively in a pond.
[*]Data compiled from (Drillet et al., 2006a; van der Meeren et al., 2008).

1.3 Criteria for the selection of copepod species for aquaculture and the establishment of culture techniques for mass culture

Copepods vary significantly in their size and habitat, offering huge opportunities when searching for a candidate species. The broad range of size during copepod development is considered to be an advantage for larviculture, because it can serve multiple species of fish larvae, based on their mouth (gape) size (Cutts, 2003; Olivotto et al., 2006; Payne et al., 2001). To date, copepods used in aquaculture mainly come from three orders: Calanoida, Cyclopoida and Harpacticoida (Støttrup, 2006; Støttrup and McEvoy, 2003). Among them, the Calanoida are the most commonly used, due to their high abundance in pelagic coastal environments (Mauchline, 1998).

1.3.1 Copepod's size

Prey size is an essential attribute for larviculture of finfish, where first larval feeding is primitive, demanding a small prey to be ingested whole, without the need for any mechanical breakdown by the mouth (Cunha and Planas, 1999; McKinnon et al., 2003; Støttrup and Norsker, 1997; Støttrup et al., 1986). The size of copepod's nauplii is moderately diverse within one species, and highly diverse between species (McKinnon et al., 2003; Støttrup, 2006). For instance, the calanoids *Parvocalanus* sp., *Bestiolina* sp. and *Acartia* sp. nauplii (1st stage nauplii) can range between 60-70 μm (length), which is substantially smaller than the sizes of both rotifers and newly hatched *Artemia* nauplii, with a size range of 90-300 μm and 400-500 μm, respectively (Conceição et al., 2010; Lucas and Southgate, 2003; McKinnon et al., 2003; Shields et al., 2005; Støttrup and McEvoy, 2003). And are therefore ideal food for the red snaper *Lutjanus campechanus* and Baltic cod *Gadus morhua* larvae, who has small mouth gaps at the hatching (Kortner et al., 2011; Støttrup, 2006). Furthermore, the order Cyclopoida which is also pelagic, has nauplii ranging from 85-265 μm and therefore much larger than the other orders, which makes it ideal as food for shrimp larvae and fish larvae such as the grouper *Epinephelus coioides* (Toledo et al., 2005). It is important to highlight that feeding copepods to grouper larvae highly increased their survival and metamorphosis, where other feeds failed to match such success (Farhadian et al., 2008; Phelps et al., 2005; Su et al., 2005; Toledo et al., 2005). Epibenthic copepods in the order Harpacticoida (e.g. *Tisbe* sp.) produces nauplii around 80 μm, however due to their benthic nature, they dwell at the bottom of culture tanks, which is not preferable by pelagic fish larvae (Cutts, 2003; Fleeger, 2005), Nevertheless, these copepods had a positive effect on the survival and growth of benthic ornamental fish larvae, when combined with other live feeds (Olivotto et al., 2008).

1.3.2 Stocking density in culture

Copepods stocking density used in aquaculture is highly variable among different species (Conceição et al., 2010). Although high stocking densities of adult copepod over 1000 adult L^{-1} had been reported for *Paracyclopina nana* and *Acartia* spp (Lee et al., 2006; Schipp et al., 1999), recent studies on *Acartia sinjiensis* and *Acartia tonsa* demonstrated the constraints of high stocking densities for calanoid copepods, which affect fecundity, behavior and sex ratio (Camus and Zeng, 2009; Medina and Barata, 2004; Peck and Holste, 2006). On the other hand, Jepsen et al (2007) found that stocking densities of small sized calanoid copepods can be higher than 600 adult L^{-1}, with no effect on their egg production. Moreover, cyclopoid and harpacticoid copepods are well known to have densities over 10,000 adults L^{-1}, reaching a maximum of 40,000 adults L^{-1} in *Tisbe* sp. cultures (Cutts, 2003; Phelps et al., 2005; Shirgur, 1989). In general, cyclopoids and harpacticoids cultured at these high densities can maintain good survival and population growth as well as being simple to culture, which contrasts to results for calanoids, which require high maintenance and manipulation to be stabilized (Hernandez Molejon and Alvarez-Lajonchere, 2003; Souza-Santos et al., 2006; VanderLugt and Lenz, 2008). However, maintaining high stocking densities of copepods for long periods has been until now labor intensive and tedious, which emphasizes the difficulty of developing a reliable regime to sustain the high densities required by various aquaculture hatcheries (Payne and Rippingale, 2000a; Rajkumar and Kumaraguru vasagam, 2006).

1.3.3 Environmental conditions

Optimal environmental conditions are vital for a healthy copepod culture, because factors, such as salinity, temperature and light regime can all affect population growth and dynamics (Camus and Zeng, 2008; Holste and Peck, 2006; Milione and

Zeng, 2008; Rhyne et al., 2009; Santos et al., 1999). Moreover, since copepods feed on various live microalgae strains that require specific culture conditions, the nutritional values of algae used in cultivation also often be determined to some degree by environmental conditions (Rhyne et al., 2009). Among the most researched environmental factors are temperature (Rhyne et al., 2009), salinity (Ohs et al., 2010a), and photoperiod (Camus and Zeng, 2008). Setting the salinity at 14 to 20 psu and photoperiod at 16 to 20 hours of light during day was found to significantly increase the egg production and egg hatching success of the calanoid *Acartia tonsa* (Peck and Holste, 2006). In addition, temperature variation can affect the development time of many calanoid and cyclopoid copepods, as evidenced by most laboratory experiments where high temperature treatments yielded fast development, mainly related to faster metabolism (Peterson, 2001). Harpacticoids in general (e.g. *Tisbe* sp.) prefer a narrow range of salinity for an optimal population growth, however they can tolerate a wider range (Cutts, 2003; Souza-Santos et al., 2006). These factors also interact and the manipulation of salinity and temperature can be highly beneficial. For instance, Miliou (1996) found a minor interaction between salinity and temperature, and adjusting those factors can have a positive or negative effect on the final body size (adult) and development time of the harpacticoid *Tisbe holothuriae* even though salinity is the main influencing factor. However, the accelerated development by temperature had an inverse relationship with the lifespan of *Tisbe battagliai* (Williams and Jones, 1999). Implementing optimal environmental conditions for each copepod species, can result in maximizing its productivity (Camus and Zeng, 2008; Milione and Zeng, 2008; Ohs et al., 2010a; Rhyne et al., 2009), therefore researching on this field is vital to facilitate the use of copepods in aquaculture.

1.3.4 Feeds

Food quality and quantity play an essential role in copepods' population dynamics (Castro-Longoria, 2003) as well as their nutritional value (Støttrup, 2000). In recent year, increasing research has focused on evaluating the dietary requirements for the culture of potential copepods as live feeds for aquaculture, where the food type and concentration is essential to estimate the culture productivity, budget, labor, and demands of culture maintenance (Ajiboye et al., 2011; Camus and Zeng, 2010; Drillet et al., 2011; Gusmao and McKinnon, 2009b; Knuckey et al., 2005; Ohs et al., 2010b; Puello-Cruz et al., 2009). Currently, it is well established that the use of live microalgae to culture copepods determines their nutritional value and population growth rate (Farhadian et al., 2008; Knuckey et al., 2005; Ohs et al., 2010b), however the selection of microalgae species is limited to the copepod feeding habit and habitat (Støttrup and McEvoy, 2003). For instance, calanoid and cyclopoid copepods require planktonic microalgae, due to their pelagic nature (Støttrup, 2006), whereas benthic harpacticoids can feed on microalgae, but also on algal films, detritus, bacteria, yeast and inert food (Cutts, 2003; Støttrup and Norsker, 1997).

Another important factor in the feeding of copepods is whether to use one or more algal species. Many studies have recommended the use of mixed algal diets rather than mono diets to balance the nutritional uptake of copepods, with evidence of a higher egg production and egg hatching success for the calanoids *Bestiolina similis* and *Pseudodiaptomus pelagicus* when fed mixed diets (Camus and Zeng, 2010; Chapman, 1981; Ohs et al., 2010b; Støttrup, 2000). However, not in all cases a mixed diet is the best option, as found for the calanoid *Temora stylifera* who maintained as good productivity on mono-algal diets as in mixed algae diets (Buttino et al., 2009). Furthermore, when culture copepods with the intention of to be used in aquaculture,

algal species whose culture is less time consuming and more cost efficient are highly preferable (Buttino et al., 2009). The use of cheap food such as pieces of lettuce and macroalgae (*Ulva sp.*), although lacking in EFAs, has been proposed for harpacticoids because they have the ability of FA's elongation (see nutritional facts) (Kahan et al., 1982). Another alternative food source is dissolved glucose (Chapman, 1981), nevertheless water quality is highly affected by this food introducing heterotrophic bacteria and other risks (Drillet et al., 2011).

1.3.5 Egg production

Copepod egg production can occur in two ways, either by egg broadcasting or via egg sacs that rupture at egg maturation releasing nauplii (Boxshall and Halsey, 2004). The mode of egg production and egg production capacity can determine the choice of particular copepod species and their application. For example, broadcasted eggs are usually negatively buoyant, hence it is easy to harvest from the bottom in a giving culture, rather than using tedious methods to collect nauplii from a large culture (Støttrup, 2006). Broadcasting calanoids, particularly *Acartia* sp., can produce an average of 11-50 eggs (female^{-1} day^{-1}), from a single fertilization (Mauchline, 1998). Where, *Parvocalanus crassirostris* and *Bestiolina similis*, which are calanoids belonging to a different family can produce 31 and 48 eggs (female^{-1} day^{-1}) respectively (Table 1.3), these relatively large numbers of eggs produced by these species indicate their suitability for mass culture (McKinnon et al., 2003). It is also worth noting that Camus (2010) found that the calanoid *Bestiolina similis* can produce an average of 156 eggs female^{-1} during life span. Unlike calanoids, cyclopoid copepods carry their eggs in two egg sacs until they hatch (Huys and Boxshall, 1991). On an individual basis, the cyclopoid *Paracyclopina nana* can produce an average of 17 eggs (female^{-1} day^{-1}), and 267 eggs female^{-1} life span (Lee et al., 2006). An other example of Cyclopoida

Apocyclops panamensis, a commonly used cyclopoid in intensive and extensive systems for red snapper larvae rearing, was harvested at an incredibly high number with an average of 18,000 L^{-1} nauplii (Phelps et al., 2005). In contrast to calanoids and cyclopoids, harpacticoids are the most efficient egg producers during $female^{-1}$ life span (Cutts, 2003). In a continuous system an average of half a million *Tisbe holothuriae* nauplii was harvested daily (Støttrup, 2006; Støttrup and Norsker, 1997). Although there are some difficulties in harvesting harpacticoid nauplii due to their benthic nature, simple or even complex harvesting systems can be created to adapt to such issues (Cutts, 2003; Kahan et al., 1982; Olivotto et al., 2008; Støttrup and Norsker, 1997).

Table 1.3: Egg/nauplii production of different copepod species.

Species	Production (egg/nauplii) ($female^{-1}$ day^{-1})	Feeds	Reference
Calanoida			
Gladioferens imparipes	25	*Isochrysis galbana*	(Payne and Rippingale, 2001a).
Temora stylifera	37.5±17.3	*Rhodomonas baltica*	(Buttino et al., 2009)
Bestiolina similis	44.1	*Isochrysis* sp., *Tetraselmis chuii* and *Pavlova50*	(Camus et al., 2009)
Parvocalanus crassirostris	31	*Heterocapsa niei*	(McKinnon et al., 2003)
Acartia sinjiensis	17.6±1.7	*Isochrysis* sp., *Tetraselmis chuii*	(Camus and Zeng, 2008)
Acartia tonsa	24.4±7.0	*Rhodomonas reticulata*	(Zhang et al., 2013)
Cyclopoida			
Paracyclopina nana	17.2±1.5	*Tetraselmis suecica* and *Phaeodactylum tricornutum*	(Lee et al., 2006)
Apocyclops royi	8	*Chaetoceros muelleri*	(Su et al., 2005)
Harpacticoida			
Euterpina acutifrons	19.5±1.7	*Tetraselmis chuii* and *Chaetoceros muelleri*	(Camus and Zeng, 2012)
Tisbe holothuriae	25	*DunalielIa tertiolecta*, *Skeletonema costatum* and grains of dehydrated mantle- tissue of *Mytilus edulis*	(Zhang and Uhlig, 1993)

1.3.6 Egg storage: viability and composition

A favorable characteristic of some copepod species, is the production of dormant eggs that can be induced to hatch when needed (Marcus, 1996). Copepod's dormant eggs are similar to those of rotifers and *Artemia*, however they have highly variable hatching success and postembryonic survival, which is problematic when considered to be used in aquaculture (Marcus, 1996). Dormant copepod eggs are sometimes available in high densities (> $1x10^6$ m^2) in the bottom sediment, where they can be induced to hatch by environmental triggers like temperature, salinity and photoperiod (Marcus, 1996; Støttrup and Norsker, 1997). Copepod dormant eggs can be in two states; *quiescence*, where the eggs resume development upon favorable conditions (i.e. limited oxygen level); and *diapause*, which is an extreme state of dormancy that does not resume under favorable conditions only, but is limited to a refractory period, during which the egg metabolism ceases (Grice and Marcus, 1981; Marcus, 1996). Recently, copepod egg dormancy was the focus of much research (Drillet et al., 2007; Drillet et al., 2006b; Højgaard et al., 2008; Holmstrup et al., 2006; Marcus and Murray, 2001; Ohs et al., 2009) since the ability to store and induce dormant eggs on demand for marine fish larviculture is the ultimate goal. There is substantial evidence that even after long-term storage eggs remain viable. For example, eggs of *Centropages hamatus* stored for 17 months still yielded high hatching success of around 80% (Marcus and Murray, 2001). Furthermore, the use of antibiotics can enhance the shelf life and hatching success of the eggs as found for the calanoid *Acartia tonsa* (Drillet et al., 2007). More recently, (Ohs et al., 2009) investigated the viability of eggs beyond the exposure of cryoprotectants and hypersaline water, and found promising results that indicated a none significant change on the egg viability from such exposure, which provides a future option for the use of cryopreservation.

However, more research is required to establish the right cues to jump-start embryonic development during dormancy (Holmstrup et al., 2006). There is a biochemical cost of storing copepod eggs (Table 1.4) due to a slight decay in EFAs (Drillet et al., 2006a; Støttrup et al., 1999), which is insignificant compared to the profit gained by the aquaculture industry.

Table 1.4: Effect of cold storage on fatty acid composition of *Acartia tonsa* eggs and nauplii, after different storage periods.

	ng egg^{-1}					g 100g^{-1}		
Fatty Acid	3 months	6 months	11 months	12 months	20 months	NHa1	NHa4	NHa12
14:1	0.07	0.05±0.02	0.06	0.03±0.02	0.00	-	-	-
16:1(n-7)	0.37±0.34	0.17±0.07	0.10	0.25±0.21	0.02	1.5	1.6	1.5
18:1(n-9)	0.19±0.10	0.47±0.19	0.39	0.16±0.14	0.07±0.01	0.9	3.9	1.6
18:1(n-7)	0.30±0.11	0.25±0.10	0.22	0.41±0.34	0.54±0.05	3.8	4.5	4.2
18:2(n-6)	0.26±0.06	0.31±0.20	0.11	0.38±0.31	0.09±0.01	2.9	2.8	2.3
18:3(n-3)	0.65±0.08	0.85±0.62	0.43	1.17±0.96	0.50±0.04	7.4	9.2	12.3
18:4(n-3)	0.46±0.07	0.34±0.24	0.46	0.43±0.35	0.32±0.02	5.6	4.0	7.2
20:4(n-6)	0.08±0.02	0.05±0.02	0.02	0.06±0.05	0.03	0.6	0.9	1.1
20:5(n-3)	1.54±1.10	0.79±0.29	0.51	0.92±0.76	0.61±0.07	15.5	13.0	13.8
22:6(n-3)	0.84±0.16	0.84±0.34	0.42	1.03±0.84	0.64±0.07	34.8	28.0	23.3
Total HUFA	4.17	3.45	2.16	4.24	2.45	68.5	61.2	63.5

[8] NHa = Newly hatched nauplii after 1,4 and 12 weeks.
[*] Data compiled from (Drillet et al., 2006b; Støttrup et al., 1999).

1.3.7 Extensive culture system

The use of copepods in extensive culture systems for fish larviculture is global, where ponds or lagoons are inoculated with natural zooplankton dominated by copepods of a known size range, depending on the cultured fish species (Conceição et al., 2010; Støttrup, 2000). Grouper, red snapper, flounder and turbot are some of the fish species that have been produced extensively on a copepod diet (Ogle et al., 2005; Støttrup and McEvoy, 2003; Su et al., 2005; Toledo et al., 2005). However, within a region each system is dominated by specific copepod genera depending on availability. For instance, (Terje, 1991) reared turbot larvae on natural zooplankton with *Acartia*, *Centropages* and *Temora* as dominant copepods, and based on prey size selectivity of fish larvae these copepods may not necessarily be ideal to use as feeds.

A disadvantages of extensive culture requires collecting copepods from the wild which carries many problems (Su et al., 2005). Problems like viruses and parasites had been associated with extensive culture systems of zooplankton, causing high mortality rates and major stock losses (Carvajal et al., 1998; Støttrup and McEvoy, 2003; Su et al., 2005). Nonetheless, the low-cost of extensive systems based on natural zooplankton (>80% copepods) have a high yield production when equipped with the correct copepod composition (van der Meeren and Naas, 1997).

1.3.8 Intensive culture system

The intensive cultivation of copepods is still largely in the research phase, yet various studies (Table 1.5) show promising results (Payne and Rippingale, 2001b; Payne et al., 2001; Schipp et al., 1999; Støttrup and McEvoy, 2003; Sun and Fleeger, 1995). Harpacticoid copepods have high potential for intensive mass culture systems, as evidenced by the harpacticoid *Amphiascoides atopus* which produced an average of $1x10^6$ individuals (day^{-1}), and can be used in many aquaculture applications (Sun and Fleeger, 1995). Furthermore, methods to avoid the benthic nature of harpacticoids have been successfully implemented, such as using floating baskets to increase their availability to fish larvae (Kahan et al., 1982). The nauplii of calanoid copepods are naturally more available to fish larvae, hence less manipulation is needed than hapacticoids. The calanoid *Gladioferens imparipes* was cultured intensively in 500 L vessels with an automated nauplii collection system, and found to provide 878 ± 46 nauplii (culture $vessel^{-1}$ day^{-1}) over a year, these results can not match the production of harpacticoids, however the culture protocol used is unique and produced sustainable high numbers than other techniques proposed, thus can be applied for other calanoid species to maximize their production (Payne and Rippingale, 2001a). In addition, extensive research on *Acartia tonsa* produced a culture protocol, able to provide a

sustainable ration of nauplii based on the culture setup demands (Marcus and Wilcox, 2007). Furthermore, various cyclopoid copepod species are currently used in intensive batch systems, such as *Apocyclops panamensis* that produces $8.2x10^6$ (nauplii and copepodite L^{-1}) from an initial stocking density of 2560 adults (Phelps et al., 2005). Although copepods intensive cultivation varies production-wise, the previous results indicated that intensive cultivation is possible and requires more research to establish a suitable culture protocol for potential species.

Most research stops at only recommending copepod species for mass cultivation without an extensive evaluation of required attributes for large scale culture (i.e. high stocking density), mainly due to limited resources (McKinnon et al., 2003). However, significant data can only be acquired through a larger commercial scale trial, which currently is recommended to meet the demands of larviculture (Ajiboye et al., 2011).

Table 1.5: Various intensive culture systems for the mass cultivation of copepods.

Species	Vessel	Production (indv.$^{-1}$ vessel^{-1} day^{-1})	Density	Feeds	Reference
Calanoida					
Gladioferens imparipes	500 L	878 ± 46 (nauplii)	1 mL^{-1}	*Isochrysis galbana* or mixed *Isochrysis galbana* and *Rhodomonas baltica*	(Payne and Rippingale, 2001a).
Pseudodiaptomus pelagicus	1800 L	>1x10^{6} (nauplii)		*Isochrysis galbana* and *Thalassiosira weissflogii*	(Cassiano, 2009)
Acartia tonsa	200 L (2)	213 (nauplii)	100 L^{-1}	*Isochrysis galbana* and *Rhodomonas baltica*	(Støttrup et al., 1986).
Cyclopoida					
Apocyclops panamensis	40 L (bag)	17,873 ± 654 (after 4 days)	5,120 L^{-1}	*Isochrysis galbana*	(Phelps et al., 2005).
Harpacticoida					
Tigriopus japonicus	210000 L	42x10^{8} (nauplii)	10-22 mL^{-1}	*Chlorella minutissima* and **ω**-yeast	(Fukusho, 1980)
Tisbe holothuriae	5 L (trays)	3x10^{5} (nauplii)	8 mL^{-1}	*Rhodomonas baltica*	(Støttrup and Norsker, 1997).
Amphiascoides atopus	1,440 L	5x10^{6}	Not measured	*Chaetocerous muelleri*	(Sun and Fleeger, 1995).

1.4 Thesis aims and outline

Copepod as larval diets are highly recommended to meet the requirements of many aquaculture hatcheries, and over the last few decades, much progress has been made toward a sustainable production system, featuring many species of copepods mainly from three orders; calanoida, cyclopoida and harpacticoida (Støttrup and McEvoy, 2003). Though copepod intensive cultivation is not yet perfected, current copepod applications have been successful in several cases, such as grouper larval feeding. Therefore, the primary aim of this thesis was to develop intensive culture

methods to overcome the current constraints in the way of developing a reliable mass culture. Specifically, the current research concentrated on improving culture productivity for the tropical calanoid *Parvocalanus crassirostris*.

The calanoid species *P. crassirostris* (Dahl, 1894) is highly abundant in tropical coastal waters (Hopcroft and Roff, 1998; Lenz, 2012; McKinnon and Thorrold, 1993; McKinnon et al., 2005) and is small compared to other calanoid copepods (Al-Yamani and Prusova, 2003). *P. crassirostris* is an omnivorous species that feeds on a range of phytoplanktons including diatoms and other protozoans (Eneida Eskinazi, 2013; McKinnon and Ayukai, 1996). *P. crassirostris* is sexually dimorphic with females being larger than males (450 μm and 350 μm prosome length respectively) and of different body shape (Al-Yamani and Prusova, 2003). Adult males are non-feeding due to their reduced feeding appendages, therefore, have a short lifespan compared to females (Lawson and Grice, 1973). Females broadcast eggs continuously in the water column as a mode of production, producing a batch of four eggs at once (Lawson and Grice, 1973). Embryonic development is very rapid and hatching occurs within 6 to 12 hours (Yang, 1977). Early hatched nauplii are non-feeding (NI-NII), and starting from NIII stage feeding begins with little known about the feeding habits of early nauplii stages in small copepods (Hopcroft and Roff, 1998; Mauchline, 1998). Generally, the lifespan of tropical calanoid species is short (weeks – month) compared to temperate species (months – years) with faster development (Mauchline, 1998). *P. crassirostris* develops from first naupliar stage to adult stage within 6 days at 27 °C (McKinnon et al., 2003). As one of the most abundant taxa found in tropical costal waters, *P. crassirostris* is considered to be a major contributor to the inshore zooplankton assemblage (Duggan et al., 2008; McKinnon and Ayukai, 1996; McKinnon and Thorrold, 1993; McKinnon and Klumpp, 1997b; Uye and Liang, 1998). Measured egg

production rates for *P. crassirostris* were found to be within 5-7 eggs female^{-1} day^{-1} (McKinnon and Ayukai, 1996; McKinnon and Klumpp, 1997b), however, laboratory results demonstrated higher egg production rates of 31 eggs female^{-1} day^{-1} (McKinnon et al., 2003).

The thesis has three parts. The first part (Chapter 2 and 3) investigated the diet requirements and alternative diets of *P. crassirostris*, to develop an optimal diet. The second part (Chapter 4) focused on optimizing culture density for *P. crassirostris*, to evaluate maximum functional density. Finally, the third part (Chapter 5) evaluates the effects of selective breeding and long-term cultivation (domestication) on *P. crassirostris* life history traits. The following outlines the thesis chapters:

Chapter 1 reviews copepods attributes in relation to other live food in aquaculture and current copepod application.

Part 1: Diet requirements and alternative diets

A fundamental step towards evaluating copepod species for the use as live food in aquaculture is to determine their diet requirements, thus, chapter 2 evaluated different algal diets at different compositions. *P. crassirostris* egg production, hatching success, survival, postembryonic development, population growth and sex ratio were the key parameters of that investigation.

Chapter 3 synthesises the information gathered from chapter 2 and available literature to determine the obstacles facing the use of alternative diets for calanoid copepods. Although, the use of commercial algae pastes is popular for other live feeds, yet, algae pastes fails to sustain a healthy copepod culture. Thus, the chapter examines the underlying reasons behind this failure.

Part 2: Culture density

The inability to culture calanoid copepods at high stocking density is a major constraint. Chapter 4 evaluates the maximum density attained by *P. crassirostris* to optimize culture productivity. The chapter tested higher densities than those explored previously for other calanoid copepods, using egg production, hatching success, adult mortality rate, population growth and sex ratio as parameters for the investigation.

Part 3: Selective breeding and domestication

Improving the reproductive capacity of calanoid copepods through optimizing diets and environmental conditions has limitation. Therefore, chapter 5 and 6 explores the genetic improvements that could be made through selective breeding and domestication as a new approach in copepod culture techniques. While, selective breeding as a successful strategy to enhance the life history traits of aquaculture species had been well documented, such a strategy has never been reported for calanoid copepods. Thus, chapter 5 was designed to determine if selective breeding is an effective way to improve the reproductive productivity of *P. crassirostris*.

Chapter 6 investigates the effects of domestication after years of isolating *P. crassirostris* from the wild. Particularly, the chapter tests the hypothesis that domestication often leads to improved adaptability to culture environments of cultured species and the enhancement of traits related to culture productivity. Such, is virtually none existing, hence, an opportunity to develop an understanding about this subject.

Chapter 7 discusses the main finding of this thesis and points towards future directions.

CHAPTER 2

EVALUATION OF MICROALGAL DIETS FOR THE INTENSIVE CULTIVATION OF THE TROPICAL CALANOID COPEPOD, *PARVOCALANUS CRASSIROSTRIS*[1]

This chapter evaluates different algal diet treatments for the cultivation of *P. crassirostris*. The treatments used were either in singular or combinations of live algae strains, in addition, to commercial algal pastes. The chapter examined the effect of those diets over key biological traits to determine an optimal diet. The information produced by this chapter serves as a reference for the diet required by this species in current research as well as future studies.

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2.1 Introduction

Despite recent improvements in intensive cultivation techniques for some copepod species (Payne and Rippingale, 2001b; Støttrup and McEvoy, 2003), sustainable and feasible mass culture methods for most species, particularly planktonic species, remain elusive (Ajiboye et al., 2011). One of the major issues in the advancement of copepod mass cultivation is the varied dietary requirements of different species (Buttino et al., 2009). Generally, calanoid copepods feed on live microalgae. However, selecting suitable microalgal species is important, as is determining whether microalgae should be fed singly or in combination. Among the considerations taken when selecting microalgae species are particle size that should be within an acceptable range for cultured copepods (Berggreen et al., 1988), and the nature of selected microalgae. For instance, previous studies indicated that diatom diets reduced development rates and increased mortality in the copepod *Temora stylifera* (Carotenuto et al., 2002), which is related to their biochemical compositions (Paffenhöfer et al., 2005). However, these observed effects vary among species (Hassett, 2004; Paffenhöfer et al., 2005).

Evidence have shown significant impacts of algal diet on egg production (Camus and Zeng, 2010; McKinnon et al., 2003; Ohs et al., 2010b), egg hatching success (Milione and Zeng, 2007) and postembryonic survival and development of copepods (Carotenuto et al., 2002). An alternative to live microalgae is algal paste or frozen algae, which provide off the shelf convenience and have been used successfully for rotifer culture. This option has been trialed for the culture of the calanoid *Acatia sinjiensis*, but was not successful (Milione and Zeng, 2007).

The paracalanid copepod *Parvocalanus* has been recommended for use as live feed in aquaculture due to its small size, short generation time, excellent nutritional

profile and relative high culture density (McKinnon et al., 2003; Shields et al., 2005; Støttrup, 2006). McKinnon *et al.* (2003) listed *P. crassirostris* as one of the tropical copepods with the highest potential as live prey for hatchery larval culture. The small size of the species suggests that their early copepodites might also be suitable as the diet for first feeding larvae (McKinnon et al., 2003; Støttrup, 2000). Thus, further investigation into improving its culture productivity is warranted. The present chapter aimed at investigating the effects of various live microalgal species, fed singly or in different combinations, as well as algal paste, on a range of important biological parameters related to the culture productivity of the species.

2.2 Materials and methods

2.2.1 Microalgae culture

The selection of microalgal species was based on common availability, relative ease of culture and biochemical composition. All the algal species used were originally obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) microalgae supply service, Australia. The live algal species used, the Tahiti strain of *Isochrysis* sp. (T-Iso; CS-177), *Pavlova* 50 (Pav; CS-50) and the diatom *Chaetoceros muelleri* (Cht; CS-176), were cultured in a temperature-controlled room, using 20 L polycarbonate carboys filled with 1 µm filtered, autoclaved and UV-irradiated seawater of salinity 35 ± 1 ‰. All microalgae were cultured using f/2 medium (Guillard and Ryther, 1962), with silicates added for the cultures of the diatom *Chaetoceros muelleri*. All cultures were maintained at a temperature of 25 ± 1 ºC with vigorous aeration (0.2 µm filtered air). The photoperiod was set at a light/dark cycle of 16:8 h with a light intensity of approximately 65 µmol m^{-1} s^{-1} (fluorescent). The algal cultures were in their exponential growth phase when used for feeding copepods. The

paste of *Isochrysis* sp. (P-Iso) and *Thalassiosira weissflogii* (P-TW) were commercial products purchased from Instant Algae® (Reed Mariculture Inc., California, USA) and were prepared according to the production manual.

2.2.2 Copepod stock culture

Parvocalanus crassirostris was initially collected from plankton tows in the coastal waters of Townsville, Northern Queensland, Australia, in August, 2011. *P. crassirostris* was cultured in a laboratory of James Cook University, where it was isolated from other zooplankton and gradually scaled up. The culture was maintained in 20 L polycarbonate carboys filled with 1 µm filtered UV-irradiated seawater (36 ±1‰) with gentle aeration (Fig. 2.1). Culture temperature was kept at 26 ± 1 °C and a photoperiod of 16:8 h light:dark was maintained. Partial water changes were performed weekly with the removal of detritus by siphoning the bottom of the carboys. To ensure that adult copepods would not be siphoned out, this was done through a 100 µm sieve attached to the end of the siphoning tube. The filtered water was collected and sieved through a 25 µm mesh and re-suspended in a 1 L beaker to avoid the loss of eggs or nauplii. The beaker was left to settle for two days. Then the water was gently poured on a 25 µm mesh, leaving the detritus at the bottom of the beaker. Culture salinity increase due to evaporation was adjusted by adding distilled water if needed. The copepods were fed a combination of the Tahiti strain of *Isochrysis* sp. every second day at 1000 µg C l^{-1}. When culture density in a carboy reached 4−5 individuals per mL^{-1}, the carboy was split to initiate a new culture, and through this procedure, a continuous growing stock was made available.

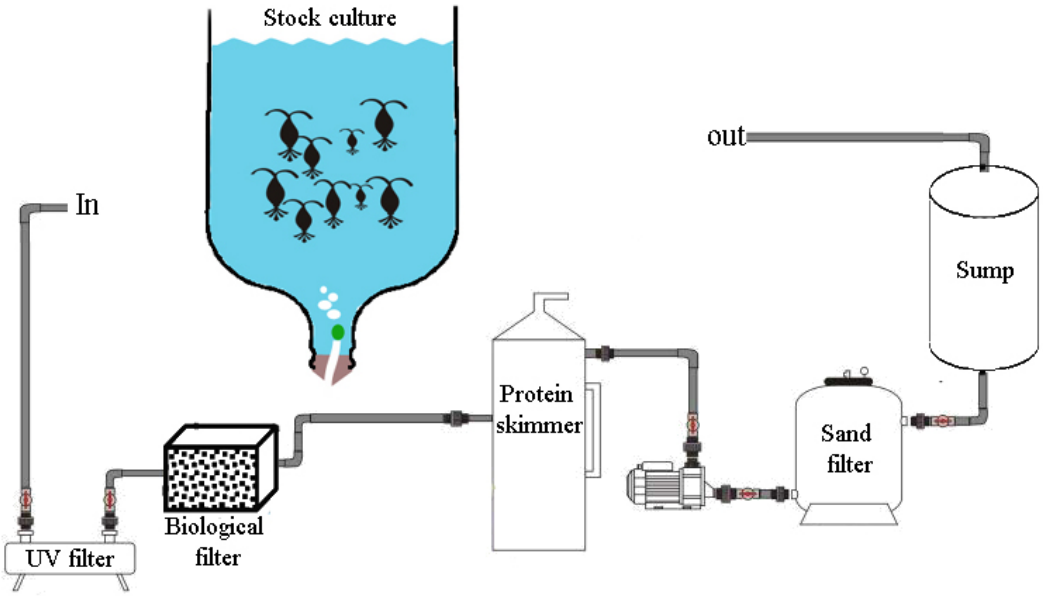

Figure 2.1: Illustration demonstrating *P. crassirostris* culture system. Carboys used to culture copepods were filled with water from a recirculating system designed by the Marine and Aquaculture Facility Unit (MARFU).

2.2.3 Experimental design and setup

A total of 9 diet treatments, including 7 live algal treatments, comprised of both mono and mixed microalgae, and 2 mono-algal paste treatments were set up. The details of treatments are shown in Table 2.1. Prior to all experiments, *P. crassirostris* was transferred from the stock culture to 1 L beakers (n = 3) to precondition them to respective diet treatment for at least one generation. This procedure should have eliminated potential effects of previous diets on the copepods used for the experiment (Mayzaud et al., 1998). The 1 L beakers were filled with 1 µm filtered seawater (36 ± 1‰) and supplied with gentle aeration concretely through a fine-tipped glass pipette. The preconditioning stocks were placed under the same temperature and photoperiod as the stock culture, which also applied for all experiments.

Table 2.1: Microalgal diets used for feeding *P. crassirostris*.

		Algal Species	Cell size	Cell number equivalency (mL^{-1})
Live microalgae	**Mono**	Tahiti strain of *Isochrysis* sp. (T-Iso)	5 µm	$8x10^4$
		Pavlova sp. (Pav)	4.5 µm	$1.2x10^4$
		Chaetoceros muelleri (Cht)	6 µm	$6x10^4$
	Mixed*	T-Iso + Pav		
		T-Iso + Cht		
		Pav + Cht		
		T-Iso+ Pav + Cht		
Microalgae paste	**Mono**	*Thalassiosira weissflogii* (P-TW)	12 µm	$1.5x10^3$
		Isochrysis sp. (P-Iso)	5 µm	$8x10^4$

*Ratio at 1:1 for binary algal treatments and 1:1:1 for the tri-algal treatment.

For the feeding of different algal diets to *P. crassirostris*, cell concentrations of microalgal cultures were calculated daily using a haemocytometer under a microscope, and then converted to carbon concentration for each species (Strathmann, 1967). Carbon equivalents used were 12.6 pg C per cell for T-Iso, 8.6 pg C per cell for Pav, 15.8 pg C per cell for Cht and 75.8 pg C per cell for P-TW. During both the preconditioning period and the experiments, *P. crassirostris* was fed a same ration of 1000 µg C l^{-1} for all treatments. For the mixed algal diets, a ratio of 1:1 for binary algal treatments and 1:1:1 for the tri-algal treatment applied.

2.2.4 Egg production

To evaluate the effects of diets on daily and total egg production over live span of female *P. crassirostris*, stage five copepodite (C5) females were randomly collected from the preconditioning stocks by siphoning small amounts of water onto a 100 µm mesh suspended in seawater. The females were identified under a dissecting microscope using available keys (Al-Yamani and Prusova, 2003; Lawson and Grice,

1973), and then placed individually into 30 ml petri dishes with covers to reduce evaporation. The same procedure was performed for males using a 90 µm mesh. When a C5 female was found to have moulted and became a sexually mature adult (C6) during daily observations, it was transferred to a 75 mL container and fed the same microalgal diet it was conditioned on with a male assigned to it. Every 24 h, the content of the container was gently poured through a 90 µm and then a 25 µm mesh suspended in seawater to collect the adult pair of *P. crassirostris* and the eggs they produced, respectively. The adult pair was immediately placed back into a new container containing fresh food and seawater. This procedure continued until the female died. The eggs produced in each replicate were then counted using a Sedgewick-Rafter counter and a dissecting stereo-microscope. The mean daily and total egg production over female life span was calculated by averaging 5 replicate pairs of *P. crassirostris* (n = 5) for each diet treatment.

2.2.5 Egg hatching success and naupliar and copepodite survival

To determine egg hatching success, 12 h prior to the experimentation, the bottoms of each preconditioning beaker were thoroughly siphoned to remove existing eggs. This was done to ensure that the eggs collected for the experiment were laid within 12 h. The newly produced eggs for each treatment were then siphoned out and collected on a 25 µm mesh. The number of eggs was counted using a Sedgewick-Rafter counter and 30−50 eggs were stocked into a covered 30 ml petri dish. There were 5 replicate petri dishes for each diet treatment, and the eggs in each replicate were checked every 6 h over a 48 h period for any newly hatched nauplii, which were transferred out using a broad mouth pipette to a new petri dish containing the designated feed and fresh seawater for subsequent naupliar and copepodite survival experiment. Hatching success over 48 h was estimated for each microalgal diet based

on the difference between the number of eggs introduced and the number of unhatched eggs (Camus and Zeng, 2009). Eggs that did not hatch beyond a 48 h observation period were considered not viable.

The newly hatched nauplii transferred to petri dishes were subsequent monitored daily with mortalities recorded to assess overall survival during naupliar stage. Newly moulted copepodites observed during the daily checking were also recorded and then transferred to new petri dishes for estimating copepodite survival. During the experiment, fresh algal diet was added daily to each replicate and towards the end of the experiment, any individuals found during daily checking to have reached adulthood were immediately removed.

Overall survival for naupliar and copepodite stage was calculated use the following formula:

- Naupliar survival (%) = [(Number of copepodites found/Number of nauplii initially stocked)] x 100%
- Copepodite survival (%) = [(Number of adult copepods found/Number of copepodites initially stocked)] x 100%

2.2.6 Naupliar and copepodite development

To evaluate the effects of microalgal diets on the development of *P. crassirostris*, the same procedure described previously was performed, except that only a single egg was introduced and incubated in a 30 mL petri dish. This was done to enable accurate determination of developmental duration for each naupliar and copepodite stage. For this experiment, post-embryonic development was monitored every 6 h until an individual reached C6 stage. Identification of each stage was based on the development key for *P. crassirostris* by Lawson and Grice (1973). The

cumulative development time to each post-embryonic stage was averaged from 5 replicates for each treatment.

2.2.7 Population growth and sex ratio

To assess the population growth of *P. crassirostris* over a 15 day culture period when fed different algal diets, 100 adults randomly collected from the preconditioning stocks were stocked into 1 L beakers and cultured for 15 days. Sex ratio was maintained at 4:1 female:male sex ratio, as this was the normal range for the stock culture population. Each treatment was triplicated with the same feeding and maintenance procedure for preconditioning adopted during the experiment. After 15 days of culture, the beaker contents were drained onto a 25 μm mesh. Nauplii, copepodites and adults collected from each replicate were fixed and then counted. All adults were then sexed to obtain the sex ratio data.

2.2.8 Statistical analysis

Data are presented as mean ± standard error (SE). All data were tested for normality and homogeneity of variance prior to mean comparison procedures. Population composition and sex ratio data were square root transformed before analysis. Treatment effects were compared using one-way ANOVA. When significant differences ($p < 0.05$) were found, Tukey's multiple comparison test was used to determine specific differences among treatments. All statistical analyses were conducted using Statistica, version 7.

2.3 Results

2.3.1 Egg production

The daily egg production of *P. crassirostris* females fed live algae showed a similar pattern over their life span: i.e. having a low egg production (ranging from 1.4

to 8.2 eggs female^{-1} day^{-1}) on the first day of their adulthood (i.e., newly moulted to C6), but increased considerably on day 2 with a peak production period between day 2 to 4 (Table 2.2). The highest two daily egg productions were both recorded from the T-Iso + Cht treatment on days 3 and 4, with an average of 29.6 ± 1.2 and 28.2 ± 1.0 eggs female^{-1} day^{-1}, respectively, which is followed closely by 27.4 ± 0.9 eggs female^{-1} day^{-1} produced by the T-Iso treatment on day 3. Egg production decreased noticeably from day 5 onward with the only exception of the T-Iso + Cht treatment, which still produced reasonable number of eggs (22.8 ± 0.9 eggs female^{-1} day^{-1}) on day 5. Eventually, egg production ceased in all treatments after day 8 of becoming adults (Table 2.2). In comparison to live algal treatments, daily egg productions of algal paste treatments were substantially inferior with consistent low numbers recorded (<1.5 eggs female^{-1} day^{-1}), which did not lasted beyond day 3 (Table 2.2).

Table 2.2: Daily egg production during *P. crassirostris* female lifespan when fed different microalgal diets.

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
P-Iso	0.8±0.4^{a}	1.0±0.3^{c}	0.4±0.2^{c}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
P-TW	1.4±0.5^{ab}	1.4±0.2^{c}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
T-Iso	6.6±0.7^{c}	21.2±0.9^{bd}	27.4±0.9^{de}	25.4±1.6^{c}	7.8±2.1^{b}	0.8±0.4^{a}	0.0±0.0
Pav	2.2±0.9^{ab}	16.2±1.4^{a}	13±1.1^{ab}	14.6±0.9^{b}	5.0±2.3^{ab}	0.4±0.4^{a}	0.0±0.0
Cht	6.2±0.7^{cd}	19.8±0.6^{ab}	18.8±0.9^{bd}	14.4±0.9^{b}	8.2±0.5^{b}	4.8±1.6^{ab}	1.0±0.5
T-Iso + Pav	2.2±0.4^{ab}	16.0±1.1^{a}	11.2±4.7^{ab}	4.0±1.8^{a}	0.8±0.5^{a}	0.0±0.0	0.0±0.0
T-Iso + Cht	8.2±0.7^{c}	24.8±1.1^{d}	29.6±1.2e	28.2±1.0^{c}	22.8±0.9^{c}	5.8±1.9^{b}	1.6±0.8
Pav + Cht	1.4±0.5^{ab}	8.8±1.6^{e}	8.2±1.6^{ac}	1.0±0.6^{a}	0.0±0.0	0.0±0.0	0.0±0.0
T-Iso + Cht + Pav	3.6±0.5^{bc}	17.8±1.0^{ab}	13.4±1.3^{ab}	5.0±1.7^{a}	1.8±0.8^{a}	0.0±0.0	0.0±0.0

Data are presented as mean±S.E (n = 5). Superscript letters indicate significant differences (p <0.05).

Total egg production over the female lifespan differed significantly between treatments (p <0.001). The T-Iso + Cht treatment produced significantly higher number of eggs than all other treatments, reaching a mean of 121 ± 5.9 eggs female^{-1}. This was followed by the T-Iso (89.2 ± 3.5 eggs female^{-1}) and Cht (73.2 ± 1.9 eggs female^{-1}) diet

treatments, which were significantly superior than the remaining treatments (p <0.001). The lowest total egg production among live algal treatments was obtained from Pav + Cht (19.4 ± 2.9 eggs female^{-1}) treatment, but it was still substantially higher than the two algal paste treatments (P-Iso: 2.2 ± 0.6 eggs female^{-1} and P-TW: 2.8 ± 0.7 eggs female^{-1}) (Fig. 2.2).

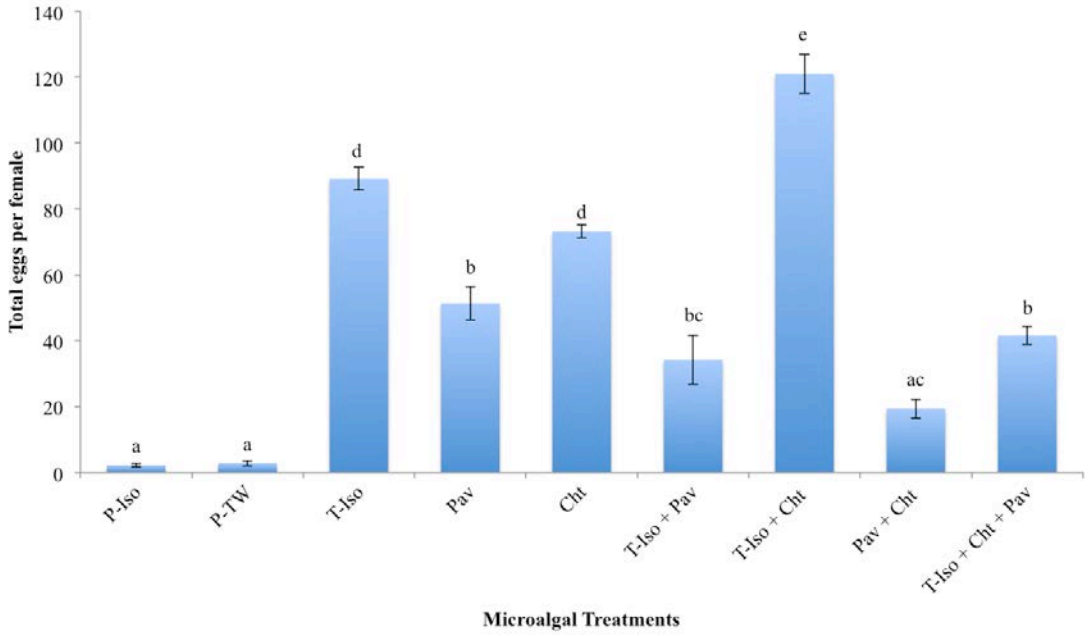

Figure 2.2: Mean total egg production over the lifespan of *P. crassirostris* females fed different microalgal diets. Data are presented as mean ± standard error (n = 5). Different letters on the tops of bars indicate significant differences (p <0.05).

2.3.2 Egg hatching success and naupliar and copepodite survival

Egg hatching success was very high, ranging between 90−100%, among all live algal treatments and differences between these treatments were statistically insignificant (p >0.05). However, egg hatching rates for the two algal paste treatments were lower, between 60−70%, which were significantly inferior than all live algal treatments (p <0.05; Fig. 2.3). It is worth noting that for all treatments, no hatching was observed beyond 12 h of incubation.

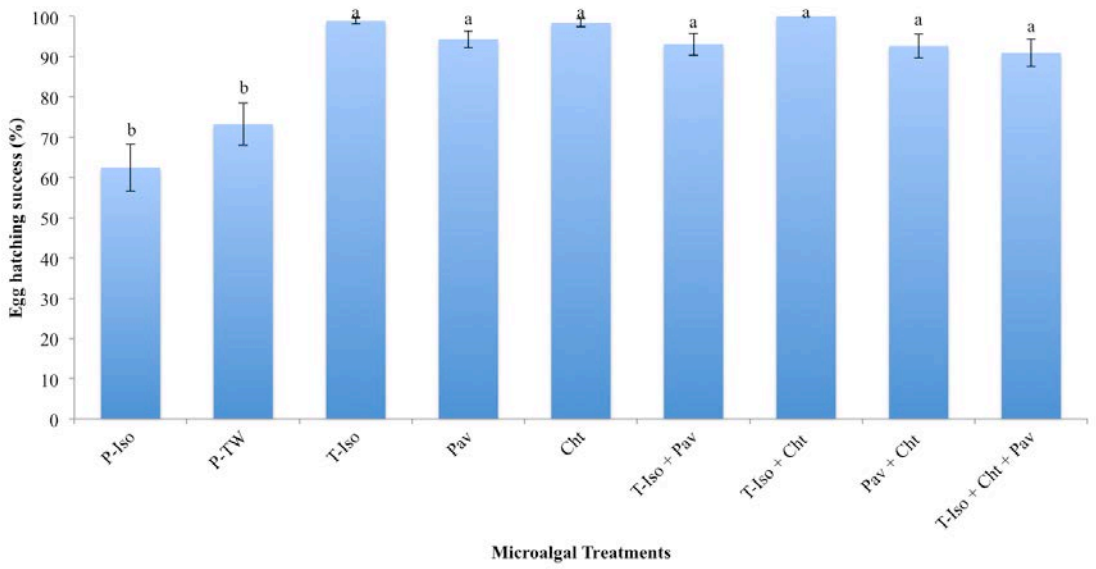

Figure 2.3: The percentage of egg hatching success of *P. crassirostris* fed different microalgal diets. Data are presented as mean ± standard error (n = 5). Different letters on the tops of bars indicate significant differences (p <0.05).

Overall survival of the naupliar stage was significantly greater (p <0.001) when *P. crassirostris* were fed Cht (89.4 ± 2.1%), T-Iso + Cht (83.1 ± 3.2%) and T-Iso (80.5 ± 1.2%). At the other end of spectrum, the two algal paste treatments, P-Iso (40.8 ± 2.6%) and P-TW (27.2 ± 3.1%) produced the lowest survival, which is significantly lower than all live algal treatments except between T-Iso+Pav and P-Iso (Fig. 2.4a). Again, the best copepodite survival were from the treatments of T-Iso + Cht (89.0 ± 1.6%), T-Iso (84.5 ± 2.7%) and Cht (78.6 ± 1.5%), which is significantly higher than other treatments (p <0.05). In contrast, the lowermost survival came from the P-TW (34.9 ± 10.0%) and P-Iso (33.9 ± 4.9%) treatments (Fig. 2.4b).

The overall survival from hatching to adult stage was similarly significantly higher (p <0.05) from treatments of T-Iso + Cht (73.9 ± 3.0%), Cht (70.2 ± 2.3%) and T-Iso (68.1 ± 3.0%) while the lowest survival came from the P-Iso (14.2 ± 2.7%) and P-TW (9.1 ± 2.1%) treatments (Fig. 2.4c).

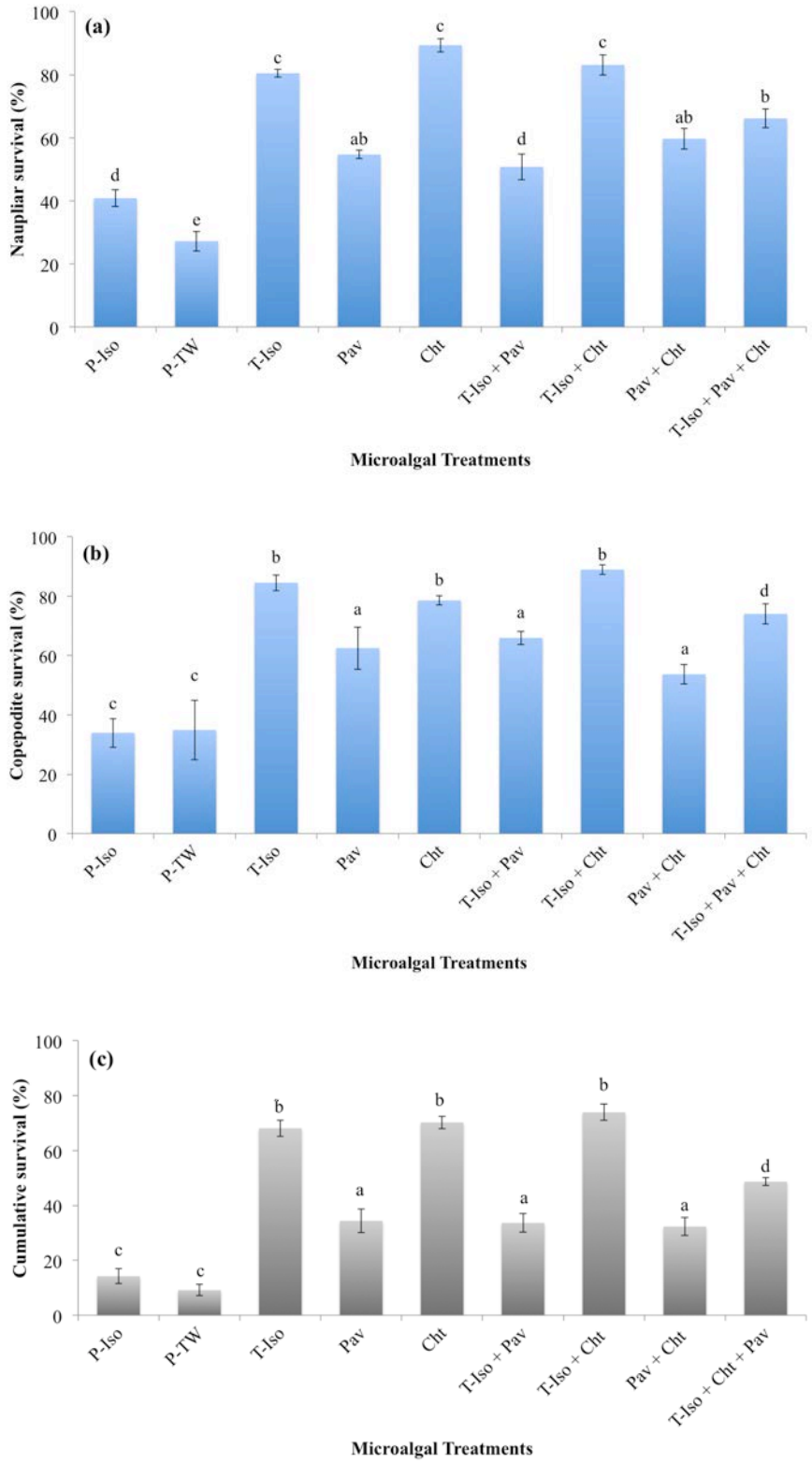

Figure 2.4: The percentage survival of naupliar (a), copepodite (b) stages and cumulative survival from hatching to adult stage (c) of *P. crassirostris* fed different microalgal diets. Data

are presented as mean ± standard error (n = 5). Different letters on tops of bars indicate significant differences (p <0.05).

2.3.3 Naupliar and copepodite development

Cumulative development time from newly hatched nauplii to C6 stage of *P. crassirostris* was significantly different between treatments (p <0.001) (Table 2.3). The T-Iso treatment engendered the shortest total development time (6.2 ± 0.1 days), which was significantly shorter than other diet treatments (p <0.05), excepting the T-Iso + Cht (6.6 ± 0.1 days) and Cht (6.7 ± 0.1 days) treatments (p >0.05).

Table 2.3: Cumulative development time (days) of postembryonic stages of *P. crassirostris* fed different microalgal diets.

Treatment	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	C6
P-Iso	0.5 ± 0.1^{bc}	0.7 ± 0.0^{bc}	1.0 ± 0.0^{bc}	1.6 ± 0.1^{ab}	2.1 ± 0.1^{abc}	2.4 ± 0.1^{abc}	3.0 ± 0.1^{abc}	3.5 ± 0.1^{abc}	4.4 ± 0.1^{ab}	5.4 ± 0.1^{ab}	6.1 ± 0.1^{ab}	7.1 ± 0.1^{ab}
P-TW	0.5 ± 0.0^{c}	0.8 ± 0.0^{c}	1.0 ± 0.0^{c}	1.7 ± 0.1^{b}	2.2 ± 0.1^{c}	2.5 ± 0.1^{c}	3.1 ± 0.1^{a}	3.6 ± 0.1^{a}	4.5 ± 0.1^{a}	5.5 ± 0.1^{a}	6.2 ± 0.1^{a}	7.3 ± 0.1^{b}
T-Iso	0.3 ± 0.0^{a}	0.5 ± 0.0^{a}	0.8 ± 0.0^{a}	1.3 ± 0.1^{a}	1.8 ± 0.1^{a}	2.1 ± 0.1^{a}	2.6 ± 0.1^{d}	2.9 ± 0.1^{d}	3.6 ± 0.1^{c}	4.5 ± 0.1^{c}	5.2 ± 0.1^{c}	6.2 ± 0.1^{d}
Pav	0.3 ± 0.1^{ab}	0.6 ± 0.1^{ab}	0.8 ± 0.0^{ab}	1.4 ± 0.1^{a}	2.0 ± 0.1^{abc}	2.3 ± 0.1^{abc}	3.0 ± 0.1^{abcd}	3.5 ± 0.1^{abc}	4.2 ± 0.1^{abd}	5.2 ± 0.1^{abd}	6.0 ± 0.1^{abd}	7.0 ± 0.1^{abc}
Cht	0.3 ± 0.0^{a}	0.5 ± 0.0^{a}	0.8 ± 0.0^{a}	1.4 ± 0.1^{a}	1.9 ± 0.1^{ab}	2.1 ± 0.1^{ab}	2.7 ± 0.1^{bcd}	3.2 ± 0.1^{bcd}	3.9 ± 0.1^{bcd}	4.9 ± 0.1^{bcd}	5.7 ± 0.1^{bcd}	6.7 ± 0.1^{acd}
T-Iso + Pav	0.4 ± 0.1^{abc}	0.7 ± 0.1^{abc}	0.9 ± 0.1^{abc}	1.5 ± 0.1^{ab}	2.2 ± 0.1^{bc}	2.4 ± 0.1^{bc}	3.1 ± 0.1^{a}	3.6 ± 0.1^{a}	4.4 ± 0.1^{a}	5.4 ± 0.1^{a}	6.2 ± 0.1^{a}	7.1 ± 0.2^{ab}
T-Iso + Cht	0.3 ± 0.0^{a}	0.5 ± 0.0^{a}	0.8 ± 0.0^{a}	1.3 ± 0.1^{a}	1.9 ± 0.1^{ab}	2.1 ± 0.1^{ab}	2.6 ± 0.1^{bd}	3.1 ± 0.1^{bd}	3.9 ± 0.1^{cd}	4.9 ± 0.1^{cd}	5.6 ± 0.1^{cd}	6.6 ± 0.1^{cd}
Pav + Cht	0.4 ± 0.1^{abc}	0.7 ± 0.1^{abc}	0.9 ± 0.1^{abc}	1.6 ± 0.1^{ab}	2.2 ± 0.1^{c}	2.5 ± 0.1^{c}	3.1 ± 0.1^{ac}	3.6 ± 0.1^{ac}	4.4 ± 0.1^{ab}	5.4 ± 0.1^{ab}	6.1 ± 0.1^{ab}	7.1 ± 0.1^{ab}
T-Iso + Cht. + Pav	0.3 ± 0.0^{a}	0.5 ± 0.0^{a}	0.8 ± 0.0^{a}	1.4 ± 0.1^{ab}	2.0 ± 0.1^{abc}	2.3 ± 0.1^{abc}	3.0 ± 0.1^{abc}	3.5 ± 0.1^{abc}	4.4 ± 0.1^{ab}	5.4 ± 0.1^{ab}	6.1 ± 0.1^{ab}	7.1 ± 0.1^{abc}

Data are presented as mean±S.E (n = 5). Superscript letters indicate significant differences ($p < 0.05$).

35

2.3.4 Population growth and sex ratio

The total number of post-embryonic *P. crassirostris* individuals harvested at the end of 15 days of culture differed significantly between treatments (Fig. 2.5) (p <0.001). The T-Iso + Cht treatment yielded the highest number of individuals (1192.0 ± 8.1 individuals), which was significantly more than all the other treatments except the second highest T-Iso treatment (1042.0 ± 8.5 individuals). On the other end, Pav (400.7 ± 36.7 individuals), T-Iso + Pav (344.0 ± 11.5 individuals) and Pav + Cht (206.0 ± 17.7 individuals) had the lowest population growth among live algal treatments. The population of the two algal paste treatment actually declined at the end of the culture period as P-Iso only had 76.7 ± 12.7 individuals while P-TW had 46.7 ± 5.2 individuals. There was no significant difference (p >0.05) between the T-Iso, T-Iso + Cht and Cht treatments in terms of percentage compositions of naupliar, copepodite and adult male and female of the final populations (Fig. 2.4).

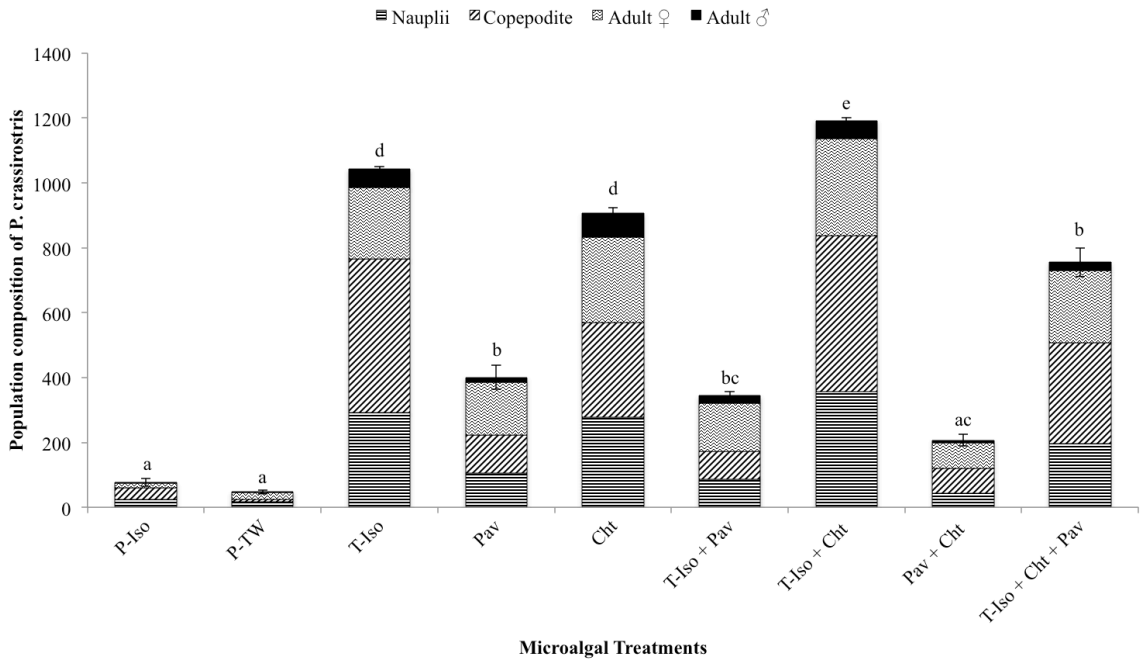

Figure 2.5: The final mean population compositions of *P. crassirostris* (n = 3) following 15 day culture of an initial 100 adults on different microalgal diets. Data are presented as mean ± standard error (total population mean). Different letters on tops of bars indicate significant differences (p <0.05).

Unlike other parameters examined, no significant differences in sex ratio were detected between all treatments (p >0.05), and females were highly dominant, ranging from 84.4 ± 3.7% (T-Iso + Cht) to 96.0 ± 4.0% (P-TW) of the adult populations.

2.4 Discussion

The results of the present study clearly demonstrate that microalgal diet significantly affected major parameters, such as egg production, survival and development of nauplii and copepodites, that are crucial to culture productivity of *P. crassirostris*. However, a few parameters, such as sex ratio and hatching rate appear to be less sensitive.

The microalgae used in the present experiment are from species that are commonly available and relatively easy to culture in the tropics. Some microalgae, for instance, *Rhodomonas* sp., although reportedly a good diet for *P. crassirostris* (McKinnon et al., 2003), is not widely available, and is known to be intolerant to temperature beyond 26 °C and prone to culture crash. Therefore, might not be the best choice for the culture of tropical copepod species (Ohs et al., 2010b).

Egg productivity is obviously of paramount importance when considering utilizing of any copepod species for aquaculture as egg production is directly linked to the production of nauplii, which are considered the crucial prey for first feeding fish larvae with small mouth gaps (Ajiboye et al., 2011; McKinnon et al., 2003). In the past studies, egg production of copepods were normally estimated for only one or a few days but not the total egg production over female lifespan (Calliari and Tiselius, 2005; Støttrup and Jensen, 1990). Our results demonstrated that daily egg production of female *P. crassirostris* varied substantially over their lifespan and it generally followed a clear pattern. Hence, the estimation of total egg production over female lifespan provides more accurate and reliable information. Moreover, the daily egg production

over female lifespan presents a more complete picture of the egg production capacity of potential copepod species for aquaculture, enabling effective comparison among different species, therefore can be used as a key criteria for species screening and selection.

Our result showed clear evident that diet quality dramatically impacted egg production of *P. crassirostris*. The binary algal diet T-Iso + Cht was identified as the best diet as it produced significant higher number of eggs over female lifespan than any other treatments. When T-Iso and Cht were fed alone, although the total egg production were less than when they were combined (i.e. T-Iso + Cht treatment), still, significant higher numbers of eggs were produced when compared to the rest of treatments, including mixed algal treatments. Similar positive effects of these two algae on the culture of calanoid *Gladioferens imparipes* have been reported, including naupliar production, survival and development (Payne and Rippingale, 2000b; Støttrup and Jensen, 1990). The further improvement in egg production when these two algae were fed in combination might relate to their complementary EFAs profiles (Camus et al 2009). The highly unsaturated fatty acids (HUFAs) and DHA:EPA ratio have been suggested to have significant implications for calanoid copepod egg production (Jónasdóttir and Kiørboe, 1996; Payne and Rippingale, 2000b).

Egg hatching success is another important parameter required for the estimation of copepod nauplii production since eggs might fail to hatch due to variable factors, and one of which is food quality (Evjemo et al., 2008) as egg hatching success had been closely linked to maternal nutrition (Jónasdóttir and Kiørboe, 1996). Interestingly, no significant differences in *P. crassirostris* egg hatching success were found among live algal diets tested with very high hatching rates (>90%) recorded for all of them. In contrast, significant variability in egg hatching success has been observed in the

tropical calanoid copepods *A. sinjiensis* and *B. similis* (Camus et al., 2009; Milione and Zeng, 2007) when fed different live microalgal diets, suggesting a species-specific response. However, it is worth noting that T-Iso as a mono-algal diet has consistently performed well in supporting the highest egg hatching success in all three tropical species of *A. sinjiensis, B. similis* and *P. crassirostris* (Camus et al., 2009; Milione and Zeng, 2007).

There are contradictory reports on whether diatoms are suitable diets for copepods (Ianora et al., 1995; Ianora et al., 2003; Miralto et al., 1999; Paffenhöfer et al., 2005). Generally, more negative effects of diatoms were reported, particularly for calanoid species, which include lower fecundity and hatching success in the calanoid *Temora stylifera* (Ianora et al., 1995), as well as in 16 calanoid copepods selected as representatives of various environments (Ban et al., 1997). However, Puello-Cruz et al. (2009) did report greater productivity using Cht as a mono-algal diet as opposed T-Iso or a mixture of the two for the culture of the calanoid copepod *Pseudodiaptomus euryhalinus*. Camus and Zeng (2012) also found that *Chaetoceros muelleri* was one of the best diets for the naupliar production of the harpacticoid copepod, *Euterpina acutifrons*. Species-specific diet requirements are likely the explanation for such difference and it highlights the need for diet experiments to be carried out for each copepod species that is considered as a potential species for aquaculture use.

Both naupliar and copepodite survival are significantly affected by diets and Cht, T-Iso + Cht and T-Iso diets were significantly superior to the others. Naupliar survival has been suggested to be affected by maternal nutrition as well (Knuckey et al., 2005) since calanoid nauplii only initiate their feeding when mandibles are well developed (Mauchline, 1998). It is generally believed that naupliar feeding is non-selective (Swadling and Marcus, 1994) while copepodites are capable of selective

feeding (Mauchline, 1998). However, evidence showed that nauplii are able to select a particle size to ingest (Paffenhöfer and Lewis, 1989; Smith et al., 2008), for example the preference of *Acartia tonsa* nauplii to feed on *Euplotes* sp., which considerably a larger prey than others (Ismar et al., 2008).

All successful hatching was observed to occur within 12 h under the experimental condition for *P. crassirostris*. This is agreeable to the embryonic development of *P. crassirostris* as described by Yang (1977), who suggested that hatching occurs after 3 h of incubation. In fact, the incubation time of *P. crassirostris* is substantially shorter and hatching is more synchronized than various tropical species, including calanoid *A. sinjiensis* and *B. similis* (Camus et al., 2009; Milione and Zeng, 2007). Short incubation time is a positive attribute for intensive copepod cultivation, since it allows a faster generation turnover and population growth.

Evidence demonstrated that among other factors (e.g. temperature) controlling development time of naupliar and copepodite stages, food quality has a major role (Huntley et al., 1987; Koski et al., 1998). For instance, faster development time was achieved using *Rhodomonas* sp. as a diet for multiple calanoid species (Ismar et al., 2008; Knuckey et al., 2005; Koski et al., 1998). The naupliar and copepodite development duration of *P. crassirostris* was also significantly affected by diets in the present study, as T-Iso, T-Iso+Cht and Cht treatments produced the shortest development durations. In contrast, Ohs et al. (2010b) reported a slower development when using T-Iso as a diet for the calanoid *Pseudodiaptomus pelagicus* compared to *Thalassiosira weissfloggi*. Tropical calanoid copepods generally have a short generation turnover time as compared to temperate species (Mauchline, 1998). Therefore, given their ability to reach high culture densities in a shorter time (Schipp et al., 1999). Our results show that *P. crassirostris* has a relatively shorter development

time even among tropical copepod species (Mauchline, 1998; McKinnon et al., 2003; Ohs et al., 2010b), which is a clear advantage.

Sex ratio in copepods is linked to their reproductive strategy (Barthélémy, 1998). For example, copepod species belonging to the superfamily Centropagoidae requires multiple copulation due to the lack of a seminal receptacle, suggesting the necessity of a 1:1 sex ratio (Barthélémy, 1998). This was demonstrated by *Centropages typicus* population in the northwestern Mediterranean, as the sexes were almost equal (Kouwenberg, 1993). However, the skewed sex ratio towards females in the family Paracalanidae is the result of many factors (Kouwenberg, 1993; Ohtsuka and Huys, 2001). For instance, the presence of a seminal receptacle indicates the requirement of a single successful copulation through female's lifespan, hence, fewer males are required (Barthélémy, 1998). Additionally, *P. crassirostris* non-feeding males have a short lifespan (Ohtsuka and Huys, 2001), which could lead to an underestimation of sex ratio in a mixed population. Sex ratio was heavily skewed towards females in all treatments, ranging from 84% to 96% of the adult populations. This indicates that food quality did not significantly impact the sex ratio of *P. crassirostris*. Similar observation on consistent sex ratio under different culture conditions in other copepods (Arnott et al., 1986; Camus and Zeng, 2010; Ohs et al., 2010b). Gusmão and McKinnon (2009a) has argued that a female-skewed sex ratio is a result of sex change due to food limitation in *Acrocalanus gracilis*. Whether or not factors controlling sex ratio is endogenous or exogenous, the highly skewed sex ratio toward female in *P. crassirostris* denotes high egg productivity, hence another positive attribute for cultivation.

A better balanced nutrition is generally believed to be achieved through feeding copepods with multiple algal species, hence leads to positive outcomes on their culture productivity. In fact, tri-algal diets used for the culture of *A. sinjiensis* and *B. similis*

were found to enhance their overall performance than various binary and mono-algal diets (Camus and Zeng, 2010; Camus et al., 2009; Milione and Zeng, 2007; Ohs et al., 2010b). Interestingly, it is not the case in the present study. Despite containing both T-Iso and Cht, the tri-algal diet treatment performed mediocrely in the present study with many parameters significantly inferior to the binary diet T-Iso+Cht and monoalgal diets of T-Iso or Cht. A likely reason is that the inclusion of Pav in the tri-algal diet had negative effects. Pav has similar cell size as T-Iso, a more balanced fatty acid profile than T-Iso and was found to be a good diet for the culture of both *A. sinjiensis* and *B. similis* (Camus and Zeng, 2010; Camus et al., 2009; Milione and Zeng, 2007; Ohs et al., 2010b). However, in the present study, it performed poorly as mono-algal diet and so as for all mixed diets included the algae species. While the underling reasons for the poor performance of Pav would need further research, such variable performance of a same algal species for different species indicates that copepod feeding habits and nutritional demands are highly species-specific (Koski and Klein Breteler, 2003).

Compared to live algal treatments, the two algal paste treatments (P-Iso and P-TW) produced substantially inferior outcomes in virtually all parameters tested. Similar poor results were obtained when paste of two different algal species (*Nannochloroposis* sp. and *Tetreselmis chuii*) were used for feeding *A. sinjiensis* (Milione and Zeng, 2007). There are several possible explanations for the failure of the algal pastes for copepod culture: 1) settlement of dead algal cells; 2) lack of enzymatic and/or other bio-active contents in dead algal cells (Milione and Zeng, 2007); 3) adherence of dead algal cells to feeding and swimming appendages of copepods (Puello-Cruz et al., 2009); and 4) selective feeding of copepods avoiding dead cells. Clearly, future research is warranted to find out why algal pastes do not work for copepods while they are now routinely

used for rotifer culture since algal paste would offer major advantages of off-the-shelf convenience and as back-up for algal culture crash.

In summary, our results demonstrated that *P. crassirostris* is a promising species for the use as live feed in aquaculture. In additional to the small size, fully planktonic life cycle, short generation time, good nutritional profile and high culture productivity of *P. crassirostris* (McKinnon et al., 2003), its relatively simple algal dietary requirement is another major advantage. Based on our results, the binary diet of T-Iso + Cht is recommended for maximizing its culture productivity; yet a mono-algal diet of T-Iso can also be used to maintain a healthy culture.

2.5 Summary

This chapter investigated the effects of algal diets on a range of productivity related parameters, including egg production, hatching success, naupliar and copepodite survival, post-embryonic development time, population growth and sex ratio of a small sized tropical copepod, *P. crassirostris*, which has been identified as a high potential species for aquaculture. The diets tested included three live algal diets: *Isochrysis* sp. (T-Iso), Pavlova 50 (Pav) and the diatom *Chaetoceros muelleri* (Cht), two algal paste diets: *Isochrysis* sp. (P-Iso) and *Thalassiosira weissflogii* (P-TW), as well as four mixed algae treatments composed of three binary algae diets (T-Iso + Pav, T-Iso + Cht, and Pav + Cht) and a tri-algal diet (T-Iso + Pav + Cht).

All parameters exanimated except sex ratio were significantly affected by the diets. The binary algal diet T-Iso + Cht produced significant higher total egg production over female life span (121.0 ± 5.9 eggs) than all other treatments (p <0.05). Survival during naupliar and copepodite stage was significantly superior when *P. crassirostris* were fed either the mixed diet of T-Iso + Cht or the monoalgal diets of Cht or T-Iso (p

<0.05) than other diets. These three treatments also had the fastest time of development to adults (ranging from 6.2 to 6.7 days). With an initial 100 adults, the T-Iso + Cht treatment produced the highest population growth over a 15 day cultivation period, which was significantly higher than other treatments (p <0.001). In contrast, the algal paste treatments, including (P-Iso) produced overall the worst results for various parameters examined. However, the sex ratio of *P. crassirostris* was not significantly affected by diets and was always heavily skewed towards females (>84 % were females) across all treatments. Based on the results of this study, T-Iso + Cht is recommended for intensive cultivation of *P. crassirostris*.

CHAPTER 3

COMMERCIAL ALGAL PASTE AS A DIET FOR THE INTENSIVE CULTIVATION OF CALANOID COPEPODS: CAUSES OF FAILURE AND POTENTIAL SOLUTION

In the previous chapter live and commercial algae paste diets were investigated, however, a remarkable difference was observed between those diets. The general performance using paste diets was extremely poor compared to their live form. Therefore, this chapter tests multiple hypotheses in the attempt to identify the underlying factors leading to the failure of algal pastes as an alternative diet for calanoid copepods.

3.1 Introduction

Various microalgal species has been identified as appropriate diets for different calanoid copepods (Alajmi and Zeng, 2015; Camus and Zeng, 2010; Camus et al., 2009; Ohs et al., 2010b; Pan et al., 2012; Zhang et al., 2013). However, culturing these microalgae species not only requires specific facilities and skilled personnel that carries substantial operational costs, but also brings a constant risk of culture contamination or crash (Hemaiswarya et al., 2011). Over the past two decades, commercial microalgae pastes have been developed and used successfully for the cultivation of rotifers and bivalve larvae in aquaculture hatcheries (Nunes et al., 2009). Such microalgae paste products provide off-the-shelf convenience while effectively avoid the risk of culture loss as well as reducing the costs of algal culture facilities and labor, hence are becoming increasingly popular in aquaculture hatcheries. However, unfortunately the past investigations on the use of commercial algae pastes as alternatives to live microalgae for culturing various calanoid copepod species have been met with persistent failures (Alajmi and Zeng, 2015; Milione and Zeng, 2007; Puello-Cruz et al., 2009). The striking difference between algal pastes as an alternative to live algae for copepod cultivation compared to the success in rotifers and bivalve larvae rearing has prompted several hypotheses being proposed attempting to explain the potential causes for the failure in copepods culture. These hypotheses were: I) Poor copepod culture productivity was largely attributed to low food availability caused by settlement of dead algae cells of pastes (Milione and Zeng, 2007); II) Copepod low reproductive capacity and survival were due to inefficient feeding caused by fouling algae cells of pastes to their body, particularly, to their swimming and mouth appendages (Puello-Cruz et al., 2009); and III) copepod inferior performance was a result of the low digestibility and nutritional value of dead algae cells (Milione and Zeng, 2007). The

present study was set out to test these hypotheses in the attempt to identify the underlying factors leading to the failure of algal pastes as a diet for calanoid copepods. Such knowledge will be crucial for developing alternative off-the-shelf algal products to replace current available algal pastes.

3.2 Materials and methods

3.2.1 Copepod and microalgal diet culture

The copepod stock cultures were fed the Tahitian strain *Isochrysis* sp. (T-Iso) every second day at 1000 µg C l^{-1} (Alajmi and Zeng, 2015). When the culture density in a carboy reached 4-5 individuals ml^{-1}, the carboy content was split to initiate a new culture and through this procedure, a continuous growing stock was maintained. Temperature, salinity and photoperiod were maintained as described for the stock culture in section 2.2.2.

3.2.2 Testing hypothesis I: Poor copepod culture productivity was largely attributed to low food availability caused by settlement of dead algae cells of pastes

To test this hypothesis, two culture methods were used to culture *P. crassirostris.* In the first method, 500 ml beakers filled with 1 µm filtered seawater (36 ±1) and provided with gentle aeration were used. This setup was used previously in copepod culture experiments with algal pastes (Alajmi and Zeng, 2015; Milione and Zeng, 2007). In the second method, screw capped culture vessels were attached to a plankton wheel rotating at a speed of 1 rpm (round per minute) to ensure the constant

suspension of algal cells in the water column. *P. crassirostris* cultured using both methods were fed two diets; live T-Iso (Iso-l) and commercial T-Iso paste (Iso-p) (Instant Algae®, Reed Mariculture, Campbell, CA, USA) at a concentration of 1000 µg C l^{-1}. Prior to the experiment, *P. crassirostris* was transferred to 5 1-L beakers for each treatment to precondition them to respective diet treatment for at least one generation. That procedure ensured no residual effects from their previous feeding history (Alajmi and Zeng, 2015; Mayzaud et al., 1998). At the beginning of the experiment, each culture vessel of both methods was stocked with 10 adult pairs of *P. crassirostris* that were randomly collected from preconditioning cultures. For each treatment there were 10 replicates (n =10). Population growth was evaluated after a 15 days culture period. Culture conditions of temperature, salinity and photoperiod were maintained as described for the stock cultures in section 3.2.1. During the 15-day culture period, daily water changes (20-40%) were carried out using a small tube with an attached 25-µm mesh to avoid the removal of any eggs or nauplii. A 100% water exchange was also performed on day 7. To maintain a food concentration above 1000 µg C l^{-1} in each replicate, algal cells were counted daily to determine the amount of food required. After 15 days, the content of each container was filtered through a 25-µm mesh and fixed in 10% formalin. All postembryonic stages were counted to assess the population growth (%) after the 15-day culture period. Nauplii, copepodites and adults were counted using a Bogorov counting chamber under a dissecting stereo-microscope (Nikon SMZ645).

3.2.3 Testing hypothesis II: Copepod low reproductive capacity and survival were due to inefficient feeding caused by fouling algae cells of pastes to their body

Because no significant difference was detected between the beaker culture and plankton wheel methods (p >0.05; see section 3.3.1) used in the previous experiment, the beaker culture was adopted as the standard culture method for all subsequent experiments.

To test hypothesis II, T-Iso in four different forms were used to feed *P. crassirostris* at an identical concentration equivalent to a 1000 µg C l^{-1} (Table 3.1). These four forms were: 1) live T-Iso (Iso-l) harvested from microalgae cultures held at the Marine and Aquaculture Research Facilities Unit, James Cook University; 2) the commercial T-Iso paste (Iso-p) (Instant Algae®, Reed Mariculture) prepared according to manufacturer's instruction; 3) blended commercial T-Iso paste (Iso-bp) prepared by blending Iso-p with a conventional blender for 3 seconds (Fig. 3.1a and 3.1b); and 4) cold stored T-Iso (Iso-c) prepared from live T-Iso culture. Iso-c was prepared by harvesting 2 L of live T-Iso culture at exponential growth phase (day 3) and then stored in a refrigerator at 4±1 °C for 7 days. The algal cells were checked under a microscope before use and they appeared non-motile and non-clumped similar to suspended live T-Iso (Fig. 3.1c). Throughout the subsequent experiments, *P. crassirostris* individuals were preconditioned (see section 3.2.2) and maintained at culture conditions described in section 2.2.2.

Table 3.1: Treatments used for feeding *P. crassirostris*.

Treatment	Description
Iso-l	Live T-Iso.
Iso-p	Commercially produced T-Iso paste.
Iso-bp	Commercially produced T-Iso paste broken down to single cells using a blender.
Iso-c	Cold stored T-Iso obtained from the suspended microalgae culture.

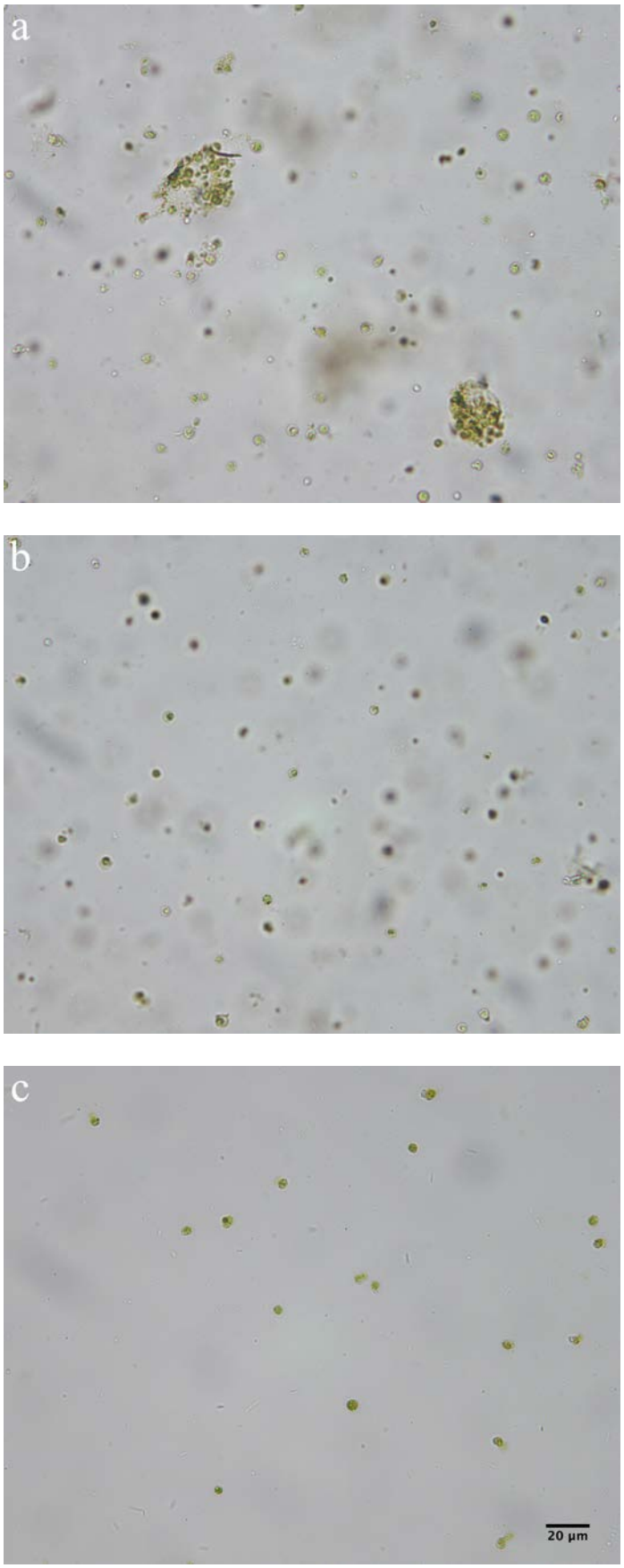

Figure 3.1: The apperance of a) paste (Iso-p), b) blended paste (Iso-bp) and c) cold stored (Iso-c) T-Iso.

To evaluate the magnitude of algal cell adherence to mouthparts and swimming appendages of *P. crassirostris* for each treatment a fouling index was developed. To estimate the fouling index, stage V copepodite (CV) females were collected from preconditioned cultures and then cultured in 30 mL petri dishes until they moulted into sexually matured adults. Ten recently matured adult females were then randomly selected from each treatment and cultured for 24 h. After 24 h, all *P. crassirostris* were sampled and the adherence of algal cells to both feeding (mandibles, maxillule and maxilla) and swimming appendages (antennule, swimming legs and caudal rami) was observed under a microscope for each treatment. A score of either 1 or 0 was given to each of above mentioned appendages based on whether there was algal cells adhered to them. The scores for each individual were then accumulated to have a score scale ranging from a minimum of 0 (no fouling to all appendages and caudal ramus) to a maximum of 7 (all appendages fouled). The scoring was performed using a Leica DM LB microscope and an Olympus DP25 camera with captured photos analysed using Olympus DP2-BSW image analysing software.

The daily faecal pellet production was evaluated over 8 consecutive days for each treatment. Faecal pellet production was evaluated to determine feeding efficiency (Besiktepe and Dam, 2002). Recently matured adult *P. crassirostris* female (n =10) was cultured in 500 mL beakers containing previously described treatments. Every day, the faecal pellet production of each replicate was assessed by gently filtering the content of each beaker through a 74 μm mesh suspended in seawater to collect the adults followed by a 25 μm mesh to collect the produced faecal pellets. The adults retained on the 74 μm mesh were then immediately transferred to a new beaker containing seawater and fresh food while collected faecal pellets were counted under a

microscope to obtain daily production. The daily egg production was collected similarly to faecal pellet production, however using recently matured pairs (n =10).

To determine naupliar and copepodite survival, 12 h prior to the experimentation, the bottoms of each preconditioning beaker were thoroughly siphoned to remove existing eggs. This was done to ensure that the eggs collected for the experiment were laid within 12 h. The newly produced eggs form each preconditioning beaker were then collected on a 25 μm mesh and stocked in separate 500 mL beakers pending hatching. One hundred newly hatched nauplii were then randomly selected and transferred to a 30 mL petri dish filled with seawater and designated diet (n=10). The survival of nauplii and their development to copepodite stage were monitored daily for each replicate. Any newly appeared copepodites found during daily observations were recorded and then transferred to a separate petri dish for estimating copepodite survival. During the experiment, daily water changes (50%) were carefully done using a syringe and small tube with an attached 25-μm mesh to avoid the removal of any individual. In addition, fresh algal diet was added daily to each replicate to maintain a food concentration above 1000 μg C l^{-1}. Towards the end of the experiment, any individuals found during daily observation to have reached adulthood, apparent by enlarged genital somite for females and long fifth leg for males, were immediately removed (Lawson & Grice 1973). Survival for naupliar and copepodite stages were calculated based on the following formulae (Alajmi and Zeng, 2015):

$$\text{Naupliar survival (\%)} = \left[\frac{\text{Number of copepodites found}}{\text{Number of nauplii initially stocked}}\right] \text{X100}$$

$$\text{Copepodite survival (\%)} = \left[\frac{\text{Number of Adults found}}{\text{Number of copepodites initially stocked}}\right] \text{X100}$$

3.2.4 Testing hypothesis III: Copepod inferior performance was a result of the low digestibility and nutritional value of dead algae cells

The digestibility of different forms of T-Iso was assessed by randomly selecting 50 faecal pellets from each replicate of each treatment (n = 10). Each pellet was observed under a Leica DM LB microscope with an Olympus DP25 camera. The captured photos were then analysed using ImageJ software (version 1.47v, Wayne Rasband, National Institutes of Health, USA) to determine the volume of each faecal pellet. The volumes (μm^3) of faecal pellets were calculated assuming them to be cylindrical and T-Iso cells ellipsoid. Each faecal pellet was then dissected to enumerate the number of undigested T-Iso cells, which were defined as intact cells. Digestibility was then calculated using the formula:

$$\text{Digestibility (\%)} = \left(\frac{\text{Total volume of faecal pellet} - \text{Undigested cells volume}}{\text{Total volume of faecal pellet}}\right) \times 100$$

Scanning electron microscopy (SEM) was used to observe faecal pellet samples and confirm digestibility results. To collect faecal pellets with minimum handling, circular glass covers were placed at the bottom of culture vessels of each treatment (n = 3). After 24 hours, glass covers with adhering faecal pellets were dehydrated through a graded ethanol series (70-100%) for 1 hour in each concentration. Samples were then air-dried and mounted afterwards. Mounted samples were then gold coated and examined under SEM.

The fatty acid profiles of *P. crassirostris* fed the four different forms of T-Iso was analysed and compared. To obtain an adequate sample for fatty acid analysis, *P. crassirostris* were cultured in 250 L tanks and fed the four different forms of T-Iso, respectively. Culture conditions were maintained as described in section 2.2.2. After 7 days of culture, *P. crassirostris* were harvested by filtering the content of the culture tanks through a 150 µm mesh followed by a 25 µm mesh. This process ensured the collection of only second generation of eggs, nauplii and early copepodites (I-III; $\bar{x}$ = $4.5x10^5$). This size fraction represented the dominant component of the cultured population and included the desired size range (71 to 112 µm) commonly captured by fish larvae with small mouth gaps at first feeding (Payne and Rippingale, 2001b; Sampey et al., 2007). The collected copepod samples (n = 3) were then flushed three times with 10‰ saltwater to remove salt before being transferred to 2 ml vials for freeze drying.

For fatty acid analysis of the copepod samples, fatty acid methyl esters (FAMEs) were prepared by transesterification using 14% BF, (boron trifluoride/methanol, w/w*)*, following the method of Morrison and Smith (1964). FAMEs were quantified by an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a flame ionization detector, with an Omegawax 320 fused silica capillary column (30 m x 0.32 mm; Supelco, Bellefonte, PA). The injector and detector temperature was maintained at 260 °C. The column temperature was initially set to 60 °C, then increased at a rate of 50 °C min^{-1} to 170 °C, followed by an increase at a rate of 2 °C min^{-1} to 180 °C, and was then held for 2 min. The temperature was further increased at the same rate of 2 °C min^{-1} to 230 °C and held for 1 min before another increase at a rate of 1 °C min^{-1} to the final temperature of 240 °C, until all FAMEs had been eluted. The total time was 46.2 min. The carrier gas was helium with

a flow velocity of 25 cm s^{-1}. Peaks were identified by comparing retention times with known standards (Nu-Chek-Prep Inc, Elysian, MN). Fatty acid contents were expressed as a percentage of a particular fatty acid to the total fatty acids (%).

3.2.5 Statistical analysis

Data are presented as mean ± standard error (SE) except for the fatty acid composition data, which were shown as mean ± standard deviation (SD). All data were tested for normality and homogeneity of variance prior to mean comparison procedures, and data expressed in percentages were arcsin-transformed prior to analysis. The treatment effects on population growth, naupliar and copepodite survival, T-Iso digestibility and *P. crassirostris* fatty acid composition were compared using one-way ANOVA, while egg and faecal pellet production data were analysed with repeated measures ANOVA. When significant differences (p <0.05) were found, Tukey's multiple comparison test was performed to determine specific differences among treatments. Fouling index was analysed using Kruskal-Wallis test. All statistical analyses were conducted using Sigmaplot, version 11.

3.3 Results

3.3.1 Testing hypothesis I: Poor copepod culture productivity was largely attributed to low food availability caused by settlement of dead algae cells of pastes.

The population growth (%) after a 15-day culture period of the two different culture methods and diet treatments are shown in Fig. 3.2. No significant difference was detected between the two methods used whether *P. crassirostris* were fed live T-Iso (Iso-l) or the commercial algal paste (Iso-p) (p >0.05), rejecting the first hypothesis.

However, when the results of two different forms of T-Iso were compared within the same culture method of either beaker culture or plankton wheel culture, significant differences were detected for both methods (p <0.001). When the plankton wheel was used, the percentage increase in population fed Iso-l and Iso-p was 2780.0 ± 182.2% and 26.0 ± 19.3 %, respectively while for the beaker culture, the increase was 2693.0 ± 99.6% and 25.0 ± 16.9%, respectively The results further confirm unsuitability of the commercial algal paste for culturing calanoid copepods.

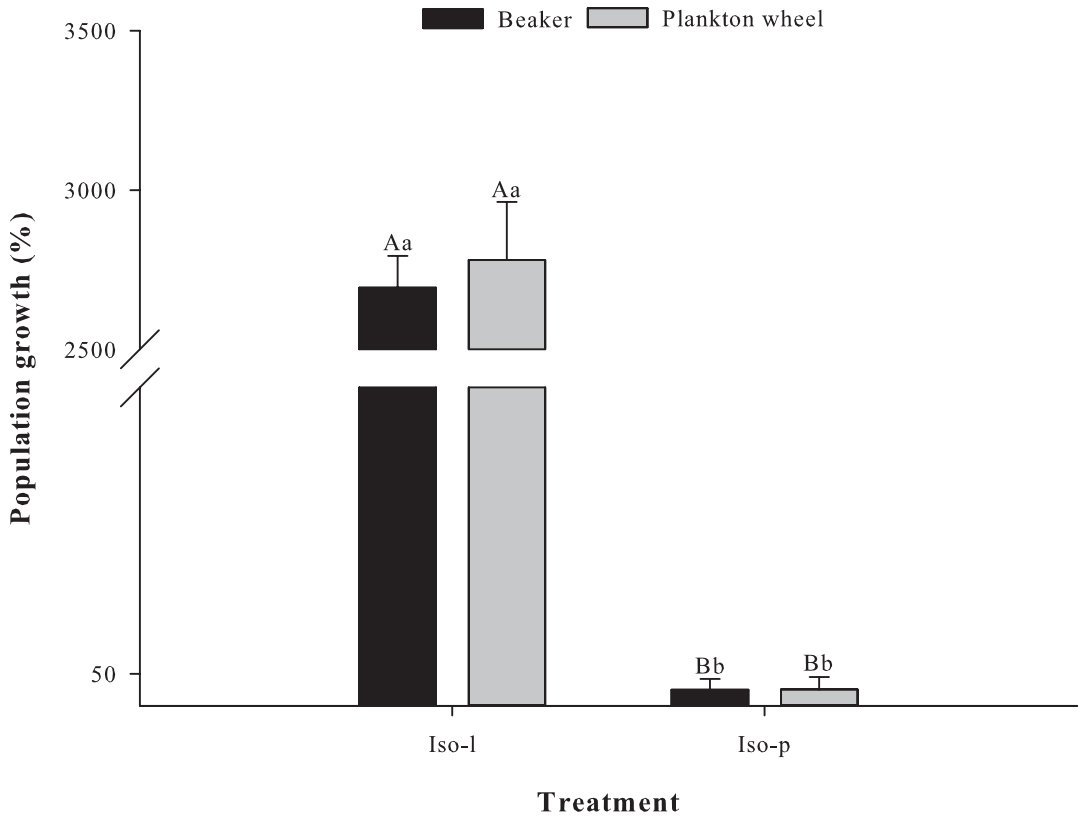

Figure 3.2: Population growth (%) of *P. crassirostris* after 15 day culture fed live and commercial paste *Isochrysis* sp. using two different culture methods (n = 10). Data are presented as the mean ± standard error. The different letters on the tops of the bars indicate significant differences (p <0.05).

3.3.2 Testing hypothesis II: Copepod low reproductive capacity and survival were due to inefficient feeding caused by fouling algae cells of pastes to their body.

The fouling index indicated a degree of algal cell adhesion to the copepod body parts that was significantly different among four diet treatments (p <0.001; Fig 3.3). Significantly lower levels of fouling to *P. crassirostris* body parts were observed for the live T-Iso (Iso-l) and cold stored T-Iso (Iso-c) treatments with similar fouling index at 1.1 ± 0.1 and 0.9 ± 0.1, respectively, while substantially high levels of fouling found in the commercial T-Iso paste (Iso-p) and branded commercial T-Iso paste (Iso-bp) treatments, for which fouling index was 3.0 ± 0.1 and 2.9 ± 0.1, respectively.

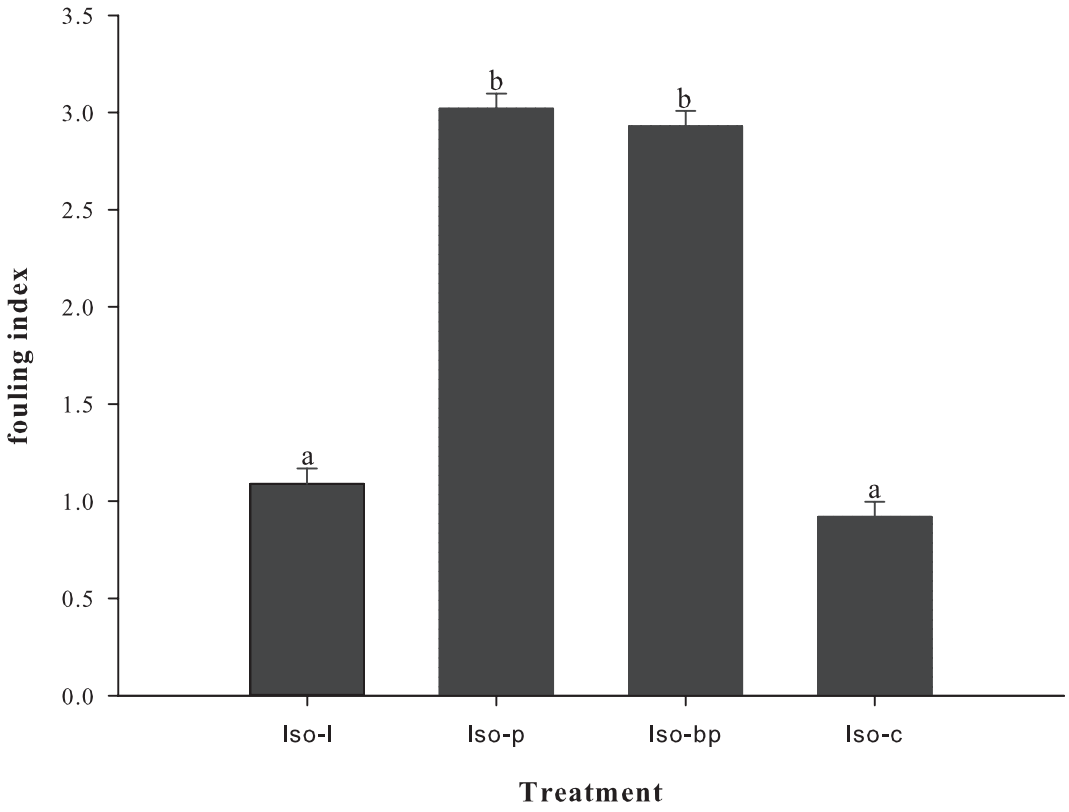

Figure 3.3: Fouling index of algal cells from different T-Iso forms to *P. crassirostris* after 24h culture period (n = 10). Data are presented as the mean ± standard error. The different letters on the tops of the bars indicate significant differences (p <0.001).

The mean daily faecal pellet production of adult females (female^{-1} day^{-1}) over 8 days was significantly different among four treatments in which *P. crassirostris* were fed different forms of T-Iso (p <0.001; Fig. 3.4). The mean daily faecal pellet production was significantly higher in the Iso-l (32.9 ± 3.2 female^{-1} day^{-1}) and Iso-c (41.6 ± 1.9 female^{-1} day^{-1}) treatments when compared to that of the Iso-p (24.3 ± 0.6 female^{-1} day^{-1}) and Iso-bp (25.0 ± 1.7 female^{-1} day^{-1}) treatments (p <0.001). However, no significant difference was detected between both Iso-p and Iso-bp treatments (p >0.05).

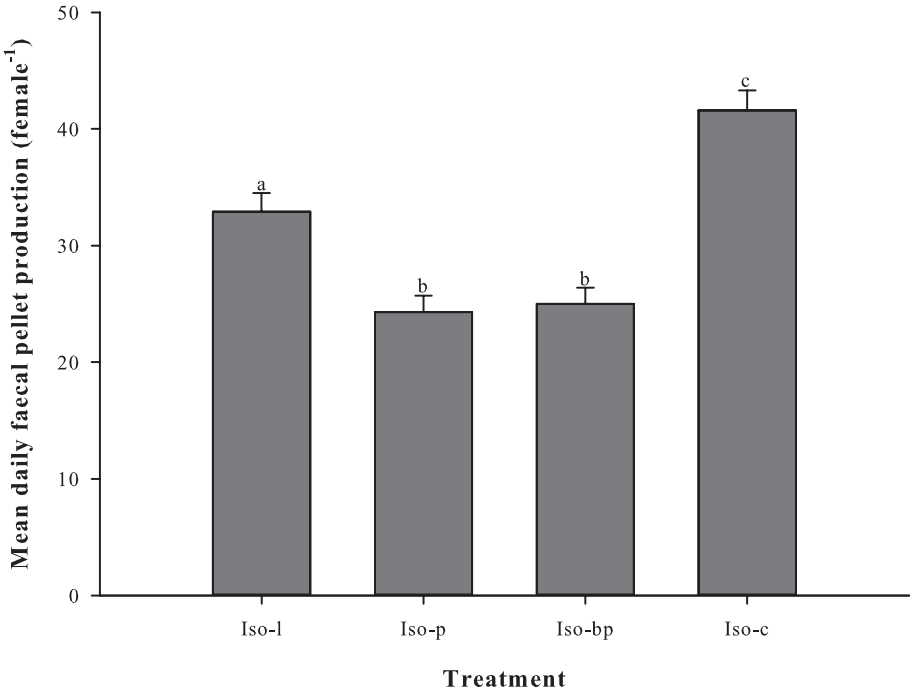

Figure 3.4: Mean daily faecal pellet production of *P. crassirostris* fed different forms of T-Iso (n = 10). Data are presented as the mean ± standard error. The different letters on the tops of the bars indicate significant differences (p <0.001).

Both daily and total egg production of *P. crassirostris* females over 8 days were significantly different when they were fed different forms of T-Iso (p <0.001; Fig. 3.5). Among four treatments, daily egg production of the Iso-l and Iso-c treatments showed a similar pattern: a low egg production on the first day of sexual maturation with a

significant increase on day 2 and a peak production between day 2 and 4 followed by a drop in production on day 5 onwards (Fig. 3.5a). The highest daily egg production recorded on day 2, 3 and 4 were 20.2 ± 1.0, 26.3 ± 0.8 and 22.7 ± 1.2 eggs female^{-1} day^{-1} respectively for the Iso-l treatment, which was followed closely by the Iso-c treatment at 19.0 ± 1.0, 25.0 ± 1.2 and 22.4 ± 1.0 eggs female^{-1} day^{-1} (p >0.05). In contrast, the daily egg production of the Iso-p and Iso-bp treatments was significantly inferior (p <0.001), with consistent low numbers recorded throughout the 8-day experimental duration. In fact, the highest daily egg production (female^{-1} day^{-1}) was only 5.8 ± 1.0 (female^{-1} day^{-1}) on day 3 and 9.0 ± 1.4 eggs (female^{-1} day^{-1}) on day 4 for the Iso-p and Iso-bp treatment, respectively. The total egg production over 8 days of the Iso-l (86.9 ± 2.0 eggs female^{-1}) and Iso-c (82.9 ± 2.4 eggs female^{-1}) treatments were 2.5 to 3.3 times higher than that of Iso-p (26.2 ± 2.2 female^{-1}) and Iso-bp (33.5 ± 2.1 female^{-1}) treatments (p <0.001) (Fig. 3.5b).

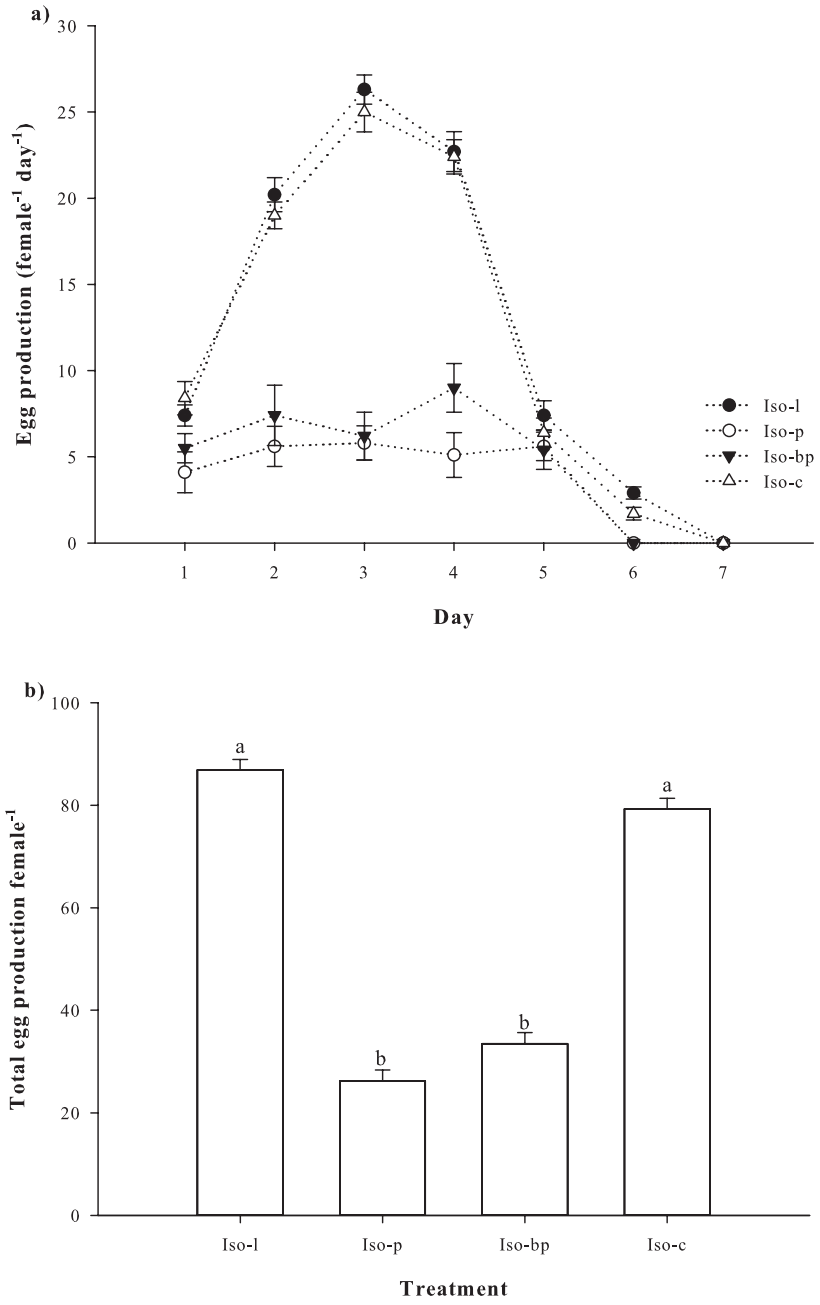

Figure 3.5: Egg production of *P. crassirostris* fed different forms of T-Iso (n = 10). a) Mean daily egg production female^{-1} over 8 days; b) mean total egg production female^{-1} over 8 days. Data are presented as the mean ± standard error. The different letters on the tops of the bars indicate significant differences (p <0.05).

Naupliar survival was significantly higher when *P. crassirostris* were fed Iso-l (79.8 ± 1.1%) and Iso-c (80.7 ±1.3%) as compared to those fed Iso-p and Iso-bp (51.8 ± 2.0% and 48.6 ± 2.2%, respectively) (p <0.001; Fig 3.6). Similarly, the differences between copepodite survival of the Iso-l and Iso-c treatments (91.0 ± 2.0% and 85.4 ± 1.8%, respectively) were even higher compared to the Iso-p and Iso-bp treatments (23.5

± 3.0% and 22.9 ± 3.3%, respectively) (p <0.001). The overall survival from nauplii to adult stage was 5 to 6 times higher in Iso-l and Iso-c treatments (72.5 ± 1.0% and 68.8 ± 1.2%, respectively) than that of the Iso-p (12.1 ± 1.6%) and Iso-bp (10.8 ± 1.3%) treatments (p < 0.001).

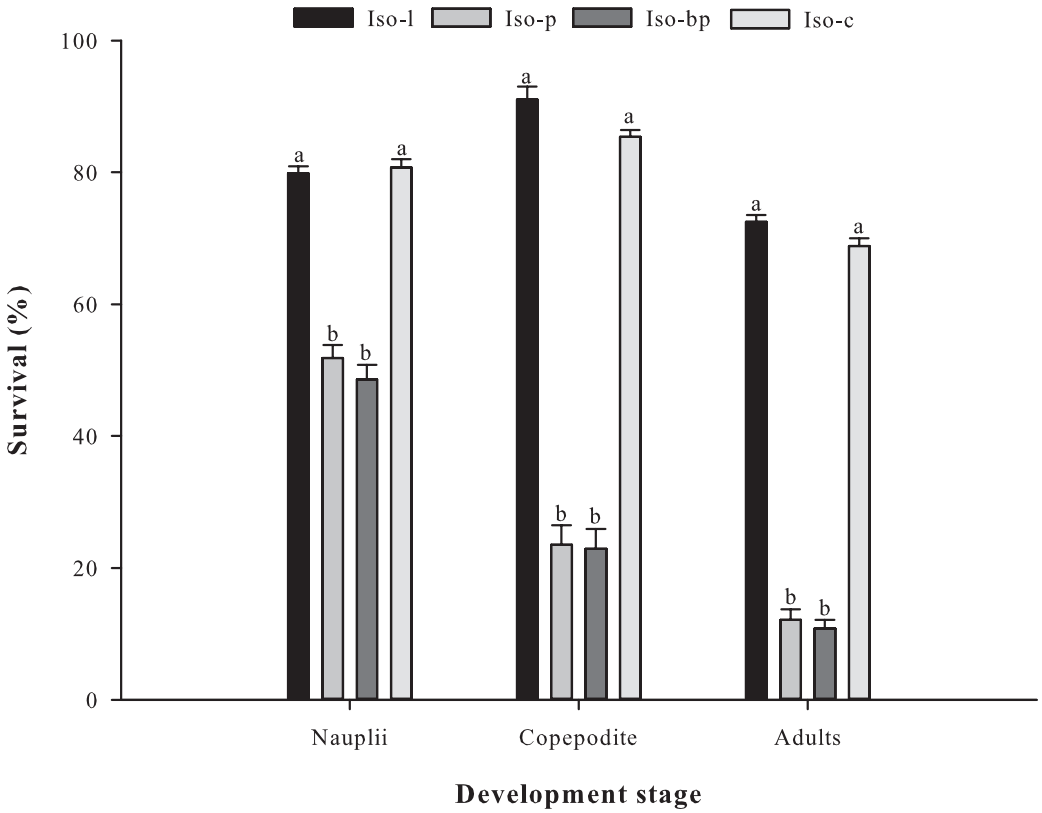

Figure 3.6: The percentage survival of naupliar, copepodite and adult stages of *P. crassirostris* fed different forms of T-Iso. Data are presented as mean ± standard error (n = 10). Data are presented as the mean ± standard error. The different letters on the tops of the bars indicate significant differences (p <0.001).

3.3.3 Testing hypothesis III: Copepod inferior performance was a result of the low digestibility and nutritional value of dead algae cells

As indicated by the number of intact algal cells contained in faecal pellets of adult female *P. crassirostris*, the digestibility of four different forms of T-Iso was significantly different (p <0.001; Fig 3.7). The highest digestibility was found for Iso-l (68.5 ± 0.7%), which was significantly higher than Iso-c (58.1 ± 0.7%; p <0.001). The lowest digestibility was recorded for Iso-p (31.8 ± 0.9%), which was significantly lower than both Iso-l and Iso-c (p <0.001). Unfortunately, no data was obtained for the Iso-bp treatment due to the initial presence of many damaged cells.

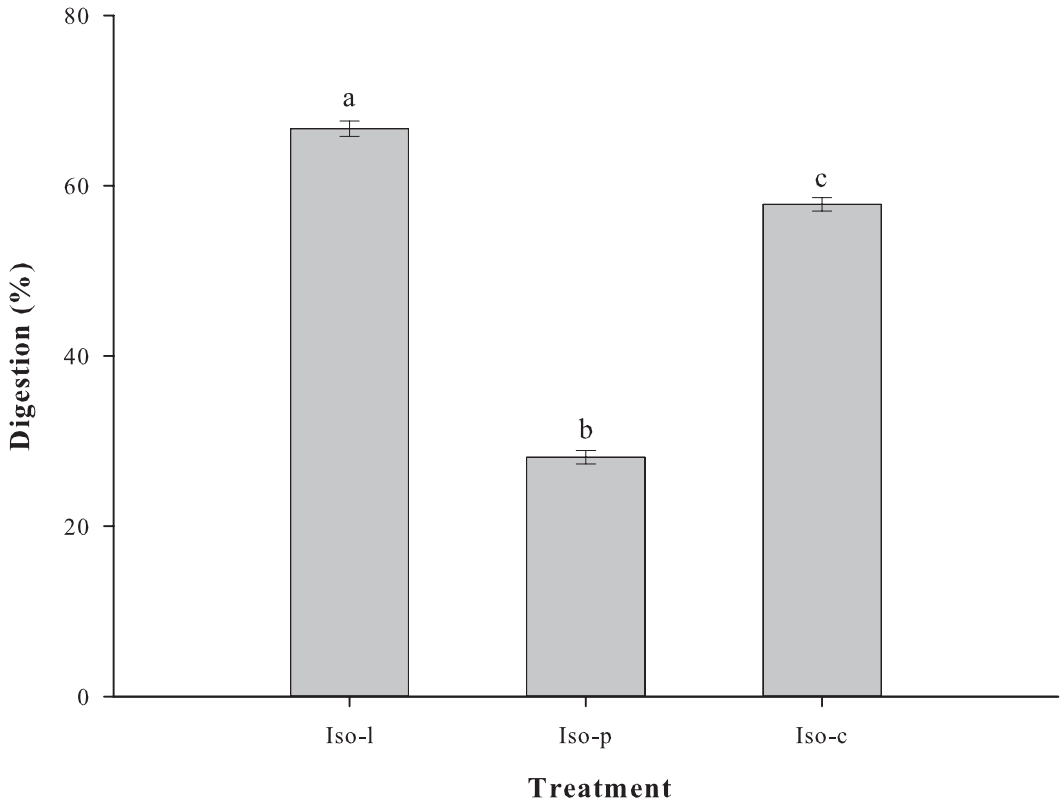

Figure 3.7 Mean digestion (%) of *P. crassirostris* fed different forms of T-Iso (n = 10). Data are presented as the mean ± standard error. The different letters on the tops of the bars indicate significant differences (p <0.001).

Due to poor survival of *P. crassirostris* fed Iso-p and Iso-bp, a sufficient sample could not be acquired for the fatty acid analysis for the two treatments. Table 3.2 shows the fatty acid composition of *P. crassirostris* fed Iso-l and Iso-c. *P. crassirostris* fed Iso-l had significant higher levels of essential fatty acids of linoleic acid (LA; C18:2ω6; 4.7±0.1%) and linolenic acid (LNA; C18:3ω3; 3.3±0.1%) than those fed the Iso-c treatment (3.2±0.0 and 2.3±0.1%, respectively). Iso-l fed *P. crassirostris* also had significantly higher levels of the two most important long chain polyunsaturated fatty acids (LC-PUFA), docosahexaenoic acid (DHA; 20:5ω3) and eicosapentaenoic acid (EPA; 20:5ω3) (22.3 ± 0.3% and 5.4 ± 0.1%, respectively) than those fed Iso-c (17.1 ± 0.1% and 0.5 ± 0.0%, respectively). However, no significant difference was detected for arachidonic acid (ARA; C20:4ω6). Overall, the total LC-PUFA level was significantly higher in the Iso-l fed *P. crassirostris* (36.2 ± 0.4%) compared to those fed Iso-c (23.7 ± 0.1%; p <0.001).

Table 3.2: Fatty acid composition of *P. crassirostris* fed live and cold stored *Isochrysis* sp. (mean % of total fatty acids ± SD)

Fatty acid	Treatment		ANOVA (*p* value)
	Iso-l (*n*=3)	Iso-c (*n*=3)	
C14:0	8.8±0.1	11.2±0.1	.000
C15:0	0.6±0.1	0.7±0.1	.223
C16:0	16.5±0.1	17.3±0.1	.001
C18:0	5.2±0.1	5.1±0.1	.192
C22:0	0.4±0.0	6.7±0.1	.000
C23:0	0.5±0.0	0.6±0.0	.006
C24:0	0.4±0.0	6.6±0.3	.000
C14:1ω7	1.0±0.0	0.6±0.1	.000
C16:1ω7	4.5±0.1	5.8±0.2	.000
C16:1ω5	0.5±0.0	0.4±0.0	.013
C18:1ω9	7.1±0.1	5.2±0.1	.000
C18:1ω7	3.5±0.1	3.5±0.1	.125
C20:1ω9	0.1±0.0	0.3±0.0	.016
C20:1ω7	0.2±0.0	0.2±0.0	.439
C22:1	0.2±0.0	0.4±0.0	.002
C16:2ω4	0.7±0.0	1.6±0.1	.000
C16:3ω4	1.2±0.0	0.7±0.0	.000
C18:2ω6 (LA)	4.7±0.1	3.2±0.0	.000
C18:3ω6	0.2±0.0	0.5±0.0	.001
C18:3ω3 (LNA)	3.3±0.1	2.3±0.1	.000
C18:3ω4	6.6±0.2	5.1±0.1	.000
C18:4ω3	0.3±0.0	0.1±0.0	.000
C20:2ω6	0.2±0.0	0.3±0.0	.000
C20:3ω6	0.7±0.0	1.1±0.1	.006
C20:4ω6 (ARA)	0.5±0.1	0.5±0.0	.871
C20:3ω3	0.4±0.0	1.1±0.1	.000
C20:4ω3	1.0±0.0	0.2±0.0	.000
C20:5ω3 (EPA)	5.4±0.1	0.5±0.0	.000
C22:2ω6	0.5±0.0	0.3±0.0	.000
C22:5ω3	2.1±0.1	0.3±0.0	.000
C22:6ω3 (DHA)	22.3±0.3	17.1±0.1	.000
ΣSFA	32.4±0.3	48.1±0.1	.000
ΣMUFA	2.2±0.0	16.4±0.3	.000
ΣPUFA	50.2±0.5	35.0±0.2	.000
ΣLC-PUFA	36.3±0.4	23.7±0.1	.000
DHA/EPA	4.1±0.1	34.2±2.2	.000

3.4 Discussion

Through a series of experiments, the present study comprehensively tested various hypotheses that have been put forward to explain the failure of commercial algae pasts as alternative diets for live algae for culturing calanoid copepods. The results rejected the hypothesis that the failure of algal pastes was due to low food availability in the water column for calanoid copepods due to high settlement rate of dead algal cells from pastes, since in previous experiments copepods were cultured in beakers with gentle aeration (Alajmi and Zeng, 2015). The use of a plankton wheel rotating at 1 rpm to culture *P. crassirostris* ensured that algal cells were suspended in the water column even though they were dead cells. However, the use of the plankton wheel did not improve the poor population growth of *P. crassirostris* when algal paste was used as the feed and the result clearly demonstrated that food availability in the water column was not the cause leading to poor result of algal pastes. Meanwhile, the simultaneously cultured *P. crassirostris* using live T-Iso as the diet for both beaker and plankton wheel methods yielded very similar results with population growth rates more than 100 time higher than those using the T-Iso paste, confirming unsuitability of pastes as the feed for calanoid copepods as well as beaker culture as an appropriate culture method.

The second hypothesis speculating that fouling of body parts by algal cells from pasts significantly reduced copepod feeding efficiency, leading to low reproductive capacity and survival was tested by comparing results of feeding *P. crassirostris* on four different forms of T-Iso. In order to obtain better insights, in addition to the treatments in which *P. crassirostris* were fed live T-Iso (Iso-l) and T-Iso paste prepared according to instruction (Iso-p); two further treatments using T-Iso paste blended before use to reduce algal clumps (Iso-bp) and a cold stored T-Iso (dead cells) (Iso-c)

were also included. To quantify the degree of fouling, a fouling index was developed which measured the severity of adherence of algal cells to copepod's swimming and feeding appendages. The result clearly showed that both algal paste treatments, even when blended beforehand to reduce algae clumping (Iso-bp), had significantly high levels of fouling than both live and cold-stored T-Iso despite the latter were also all dead cells. Obviously, severe fouling to body parts, particularly swimming and feeding appendages by algae cells (Fig. 3.8) could critically impact the swimming ability as well as feeding efficiency of copepods, leading to significantly lower egg production and survival as confirmed by the faecal pellet and egg production as well as survival experiments. Although adherence of some live algae cells to *P. crassirostris* was also observed in the present experiment, Zhang et al. (2013) also reported that live microalgae *Tetraselmis suecica* formed filamentous structures that adhered to *Acartia tonsa* after 13 days of culture. However, copepods are able to self-clean their appendages to maintain optimal sensory performance (Costello et al., 1990). One of the significant finding from these experiments is the similarity in results between *P. crassirostris* fed live algae (Iso-l) and cold stored T-Iso (Iso-c), depicted by the faecal pellet and egg production as well as naupliar and copepodite survival. Suggesting, that there is still high potential for utilizing highly condensed commercial algal products that were cold stored for copepod culture, however, the current methods of producing commercial algae pastes needs to be modified. In fact, recently developed technique for culturing microalgae as biofilms could provide a potential solution, were algae cultured on biofilms are harvested in the form of densely packed cells that would not require further processing (Naumann et al., 2013) .

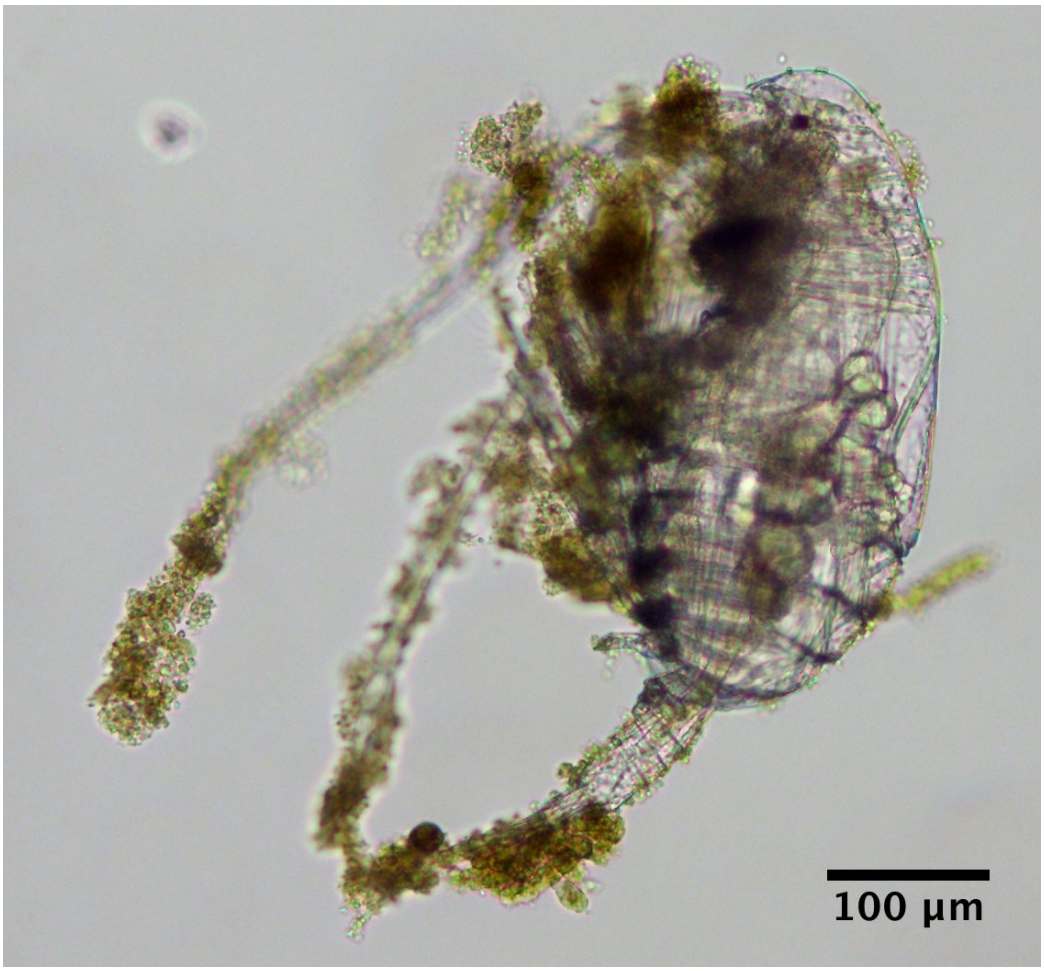

Figure 3.8: The observed fouling of commercial algae paste on *P. crassirostris*, showing an adult female after a 24 h culture period with many cells adhering to swimming and feeding appendages.

Aside from feeding efficacy, digestibility and nutritional values of algae diets could also significantly impact copepod performance, which was basically what hypothesis III was based on. In fact, our knowledge on the digestion process in calanoid copepods is relatively limited despite knowing that digestion in copepods depends on multiple factors and one of those key factors is the quality of their food (Freese et al., 2012; Knotz et al., 2006; Kreibich et al., 2011; Mayzaud et al., 1998). In the present investigation, the commercial T-Iso paste was not digested well, evidenced by the presence of significantly more intact cells in the faecal pellets than the live and cold-stored T-Iso treatments (Fig. 3.9). Interestingly, the digestion of cold stored T-Iso

(Iso-c) was also significantly poorer than the live T-Iso (Iso-l), which could be attributed to the fact that Iso-c contained only dead algae cells, thus, could explain the high faecal pellet production observed for Iso-c as compared to Iso-l treatment. The observed poorer digestion of Iso-c may be explained by missing essential components from dead algal cells that aids copepods in the digestion process (Koski et al., 1998; Tirelli and Mayzaud, 2005).

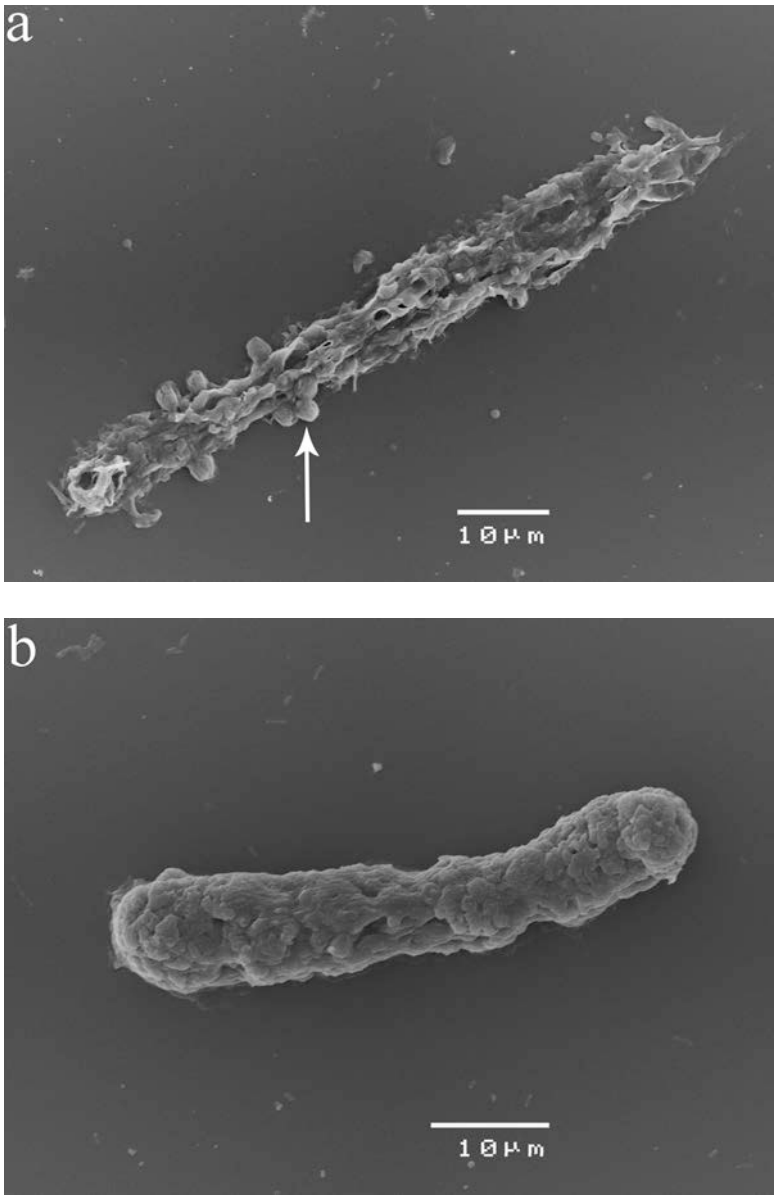

Figure 3.9: Faecal pellets of adult *P. crassirostris* fed (a) live and (b) paste T-Iso (scanning electron microscope images). Arrow indicates intact cell.

Despite stored at low temperatures, algal pastes contains reduced nutritional contents as compared to live algae (Ponis et al., 2003; Ponis et al., 2008). For example, lower levels of essential fatty acids, which are believed to be vital for reproduction in copepods, were reported after a period of storage (Ponis et al., 2003; Ponis et al., 2008; Støttrup and Jensen, 1990). Shin et al. (2003) has demonstrated a positive correlation between the reproductive capacity of *Acartia omorii* and essential fatty acid content of their diet. Although low levels of essential fatty acids within calanoid copepods diets may be compensated by the ability of copepods to desaturate and elongate precursor fatty acids (Desvilettes et al., 1997; Parrish et al., 2012), the fatty acid profile of *P. crassirostris* fed live T-Iso was clearly superior than those fed cold-stored T-Iso (Iso-c), particularly the most important LC-PUFAs. Nevertheless, the Iso-c fed *P. crassirostris* contained an abundant amount of LC-PUFAs as compared to traditional hatchery larval prey of rotifer and *Artemia* (van der Meeren et al., 2008). This suggests that commercial algal products should still present good alternatives to live algae for culturing calanoid copepods, particularly, considering their off-the-shelf convenience and practicality as backups for live algal culture. The issue of lower nutritional values of dead algae in the commercial algal products could also be mitigated by a short period of enrichment with live algae prior to use.

3.5 Summary

To explain the failure of algal pastes, a series of experiments tested the following hypotheses: I) poor copepod culture productivity was largely attributed to low food availability caused by settlement of dead algae cells in pastes; II) copepod low reproductive capacity and survival were due to inefficient feeding caused by fouling algae cells of pastes to their body; and III) copepod inferior performance was a

result of the low digestibility and nutritional value of dead algae cells. Hypothesis I was rejected as an experiment showed that the population growth of *P. crassirostris* after a 15-day culture period on the commercial paste *Isochrysis* sp. (Tahitian strain, T-Iso) was not significantly different between the beaker and plankton wheel culture methods, despite the latter method ensuring constant suspension. Hypothesis II and III were tested with 4 forms of T-Iso: live (Iso-l), cold stored (Iso-c), paste prepared according to instruction (Iso-p) and blended paste in order to reduce algal clumps (Iso-bp). The results showed that after a 24 h culture period, fouling of algal cells to body appendages of *P. crassirostris* was significantly higher in the Iso-p and Iso-bp treatments than Iso-l and Iso-c treatments denoted by a fouling index. Meanwhile, the highest mean daily faecal pellet production (female^{-1} day^{-1}) was observed in Iso-l (32.9 ± 3.2) and Iso-c (41.6 ± 1.9) treatments compared to Iso-p (24.3 ± 0.6) and Iso-bp (25.0 ± 1.7) treatments. The total egg production over 8 days of Iso-p (26.2 ± 2.2 female^{-1}) and Iso-bp (33.5 ± 2.1 female^{-1}) treatments were significantly lower than Iso-l (86.9 ± 2.0 female^{-1}) and Iso-c (82.9 ± 2.4 female^{-1}) treatments (p <0.05). Survival from nauplii to adults fed Iso-p (12.1 ± 1.6%) and Iso-bp (10.8 ± 1.3%) was also significantly inferior to Iso-l (72.5 ± 1.0%) and Iso-c (68.8 ± 1.2%) treatments (p <0.001). Digestibility, calculated by the number of undigested algal cells in faecal pellets was significantly low in the Iso-p treatment (31.8 ± 0.9%; p <0.001) compared to Iso-l and Iso-c treatments (68.5 ± 0.7 and 58.1 ± 0.7%, respectively). Fatty acid analysis of *P. crassirostris* fed Iso-l and Iso-c indicated significantly higher levels of long-chain polyunsaturated fatty acids in the Iso-l. The results suggest that the low reproductive capacity and survival of *P. crassirostris* were due to fouling algae cells of pastes to their swimming and feeding appendages, which lead to poor feeding, egg production and survival. Therefore, fouling was the main reason for the failure of algal pastes in

copepod culture while low digestibility and nutrition value of dead cells also contribute to the failure.

CHAPTER 4

THE EFFECTS OF STOCKING DENSITY ON KEY BIOLOGICAL PARAMETERS INFLUENCING CULTURE PRODUCTIVITY OF THE CALANOID COPEPOD, *PARVOCALANUS CRASSIROSTRIS*[2]

To optimize culture productivity, culture density must be assessed. In this chapter density treatments ranging from 1000 to 9000 adults mL^{-1} were evaluated. The effects of density treatments were examined over key biological parameters linked to culture productivity (i.e. egg production and daily mortality rate). Data produced by this chapter is essential for the mass production of *P. crassirostris* in aquaculture.

[2]Published as: Alajmi, F., Zeng, C., 2014. The effects of stocking density on key biological parameters influencing culture productivity of the calanoid copepod, *Parvocalanus crassirostris*. Aquaculture. 434, 201-207.

4.1 Introduction

In recent years, the failure of traditional live prey in rearing larvae of various commercially valuable fish species, such as groupers (e.g., *Epinephelus coioides*) and snappers (e.g., *Lutjanus campechanus*), has generated renewed interest in developing intensive culture techniques for copepods as the alternative prey for the larvae of marine fish and crustaceans (Buttino et al., 2012; Payne and Rippingale, 2001a; VanderLugt et al., 2009). As a result, successful intensive production of copepods has been reported for a number of species (Støttrup, 2006). As the most abundant copepod taxonomic group (Mauchline, 1998), calanoid copepods have been considered the most promising candidates for hatchery larval prey (Conceição et al., 2010; Evjemo et al., 2003). However, planktonic calanoid copepods are considerably more difficult to culture compared to benthic copepods (Støttrup, 2006). They typically demanding a large water volume, robust management of water quality and a food supply of mainly live microalgae (Payne and Rippingale, 2001a; Puello-Cruz et al., 2009).

Despite increased research in recent years aimed at enhancing copepod culture techniques, the inability to achieve high culture density is still a major bottleneck in copepod culture, particularly for calanoid copepods (Ajiboye et al., 2011; Camus and Zeng, 2009; Drillet et al., 2011; Jepsen et al., 2007; Medina and Barata, 2004; VanderLugt and Lenz, 2008). For instance, *Acartia tonsa* and *Acartia sinjiensis* are two calanoid species that were been identified as good candidates as live prey and have received much attention recently (Camus and Zeng, 2009; Jepsen et al., 2007; Milione and Zeng, 2007; Peck et al., 2008; Zhang et al., 2013). Their sustainable culture densities were reported to be generally approximately two adults mL^{-1} (Camus and Zeng, 2008, 2009; Medina and Barata, 2004), which is considered a good culture density for calanoid copepods. However, such a density would require a large volume

of culture water to produce an adequate number for feeding fish larvae. More recently, encouraging results have emerged for smaller paracalanid copepods, such as *Parvocalanus* sp. and *Bestiolina similis*, with substantially higher sustainable culture densities and peak densities reaching 20 to 30 ind. mL^{-1} (Shields et al., 2005; Støttrup, 2006).

The paracalanid copepod, *P. crassirostris*, is an abundant species in the coastal waters of Northern Queensland, Australia (McKinnon and Klumpp, 1997a) and has been identified to have high potential for use as live feed in aquaculture (McKinnon et al., 2003). *P. crassirostris* is known to have one of the smallest sized nauplii among calanoid copepods, a short generation time and an excellent nutritional profile that meets the requirements of fish larvae (Alajmi and Zeng, 2015; McKinnon et al., 2003). Meanwhile, *P. crassirostris* has also been shown to be relatively easy to maintain in a stable culture, and it responds well to selective breeding (Alajmi et al., 2014). Because culture density is another key parameter determining the suitability of a copepod species as potential larval prey in aquaculture, the present study was designed to investigate the effects of initial stocking density on a range of important biological parameters affecting the culture productivity of *P. crassirostris*, which should provide useful information for developing culture techniques for this species.

4.2 Methods

4.2.1 General procedures

Five initial stocking density treatments of 1000, 3000, 5000, 7000 and 9000 adults L^{-1} were set up for all experiments. These density treatments were selected based on our observation and previous reports that species from the genus *Parvocalanus* could attain substantially higher peak culture densities (up to approximately 30 ind.

mL^{-1}) than other calanoid species (Schipp et al., 1999; Shields et al., 2005; Støttrup, 2006). To avoid the difficult task of isolating thousands of adult copepods to stock replicates, new cultures were initiated only with eggs collected from the bottoms of stock cultures. Although the sinking rate of *P. crassirostris* eggs was not measured, eggs were found at the bottom of culture containers 3 hours post siphoning the bottom. Because these cultures came from eggs collected at the same time, their development were closely synchronised. When the majority of these cultures achieved the final copepodite stage (i.e., fifth copepodite stage or C5), they (C5) were collected and stocked to replicate vessels because it is known that C5 copepodites develop into adults within 24 h (Alajmi and Zeng, 2015). The identification of developmental stages was based on published keys for the species (Al-Yamani and Prusova, 2003; Lawson and Grice, 1973).

There were six replicates for each treatment. For each replicate, *P. crassirostris* was cultured at a designated density inside a 200-mL vessel submerged in a 1 L beaker. A 74-μm mesh was attached to the bottom of the vessel to ensure that the adults remained inside the vessel while eggs produced by the females were allowed to pass through the mesh and accumulate at the bottom of the beaker for later counting. This design also reduced the stress of daily water changes, which were performed by simply lifting the vessel out of the old beaker and placing it inside a new beaker with fresh seawater and food every 24 h. The eggs that accumulated at the bottom of the old beakers were counted with a Bogorov counting chamber and a dissecting stereo-microscope (Nikon SMZ645).

Throughout the experiments, *P. crassirostris* were fed the Tahitian strain of *Isochrysis* sp. and the diatom *C. muelleri* at 1000 μg C l^{-1} daily at a ratio of 1:1 (cell volume; Strathmann, 1967). This amount was sufficient for all densities tested because

it was observed at the highest stocking density of 9000 adults L^{-1} that there were still algae that remained after 24 h. Temperature, salinity and photoperiod were maintained as described for the stock culture in section 2.2.2.

4.2.2 Egg production experiment

Because the final stage copepodites (C5) of *P. crassirostris* should develop into adults within 24 h (Alajmi and Zeng, 2015), the experiment was commenced 24 h after C5 were stocked into replicate vessels to ensure that all C5 stocked had become adults before the experiment started. The daily egg production of *P. crassirostris* in each replicate was subsequently assessed over seven consecutive days. On each of these days, each replicate vessel with a mesh bottom was moved to a new beaker retaining adults within while eggs and recently hatched nauplii passed through the mesh bottom and remained in the old beaker, which were then filtered through a 25-μm mesh and collected. The eggs and nauplii that were collected were fixed in 10% formalin for subsequent counting. The mean daily egg production (eggs $female^{-1}$ day^{-1}) of *P. crassirostris* was calculated based on the number of surviving females in each replicate each day. The number of surviving females was estimated by the daily deduction of dead individuals found.

4.2.3 Egg hatching success experiment

To evaluate the hatching success of eggs produced by *P. crassirostris* cultured under different densities, 100 eggs were collected for three consecutive days (day 3 to 5) from each replicate of a treatment as the highest egg productions over a female lifespan of *P. crassirostris* were found on these days (Alajmi and Zeng, 2015; Alajmi et al., 2014). On each of these days, adults in a replicate were removed to a new vessel 12 h prior to egg collection (as described in 4.2.1); this process was carried out to ensure that all eggs collected for the hatching success experiment were laid within 12 h. After

the newly laid eggs were collected from a replicate, they were incubated in a 30 mL petri dish for 24 h under the same conditions as for the adults. After 24 h, the content of each petri dish (nauplii and unhatched eggs) were fixed in 10% formalin for later counting to calculate the hatching rate. The hatching success of eggs was calculated as the average of the hatching rates of eggs collected over 3 consecutive days with the highest egg production (collected on day 3 to 5) for each treatment.

4.2.4 Mortality rate experiment

The cumulative mortality rates of adult *P. crassirostris* under different stocking densities were estimated by collecting dead copepods daily from the bottom of each submerged vessel through gentle siphoning. The siphoning was performed using a 3 mm tube attached to a 5 mL syringe. Any dead *P. crassirostris* found were then counted and sexed while any live *P. crassirostris* collected accidently were gently returned to the vessel. The daily mortality rate percentages of adults were calculated as:

Daily mortality % = [(No. of dead copepods found on a particular day/ Total surviving adults of the previous day)]*100%

4.2.5 Population growth experiment

The population growth of *P. crassirostris* under 5 initial stocking densities was evaluated over a culture period of 15 days. The initial set up was similar to that described in section 4.2.1 except that 1 L beakers (n = 6) were used for this experiment. During the 15 day culture period, daily siphoning of the bottom of each beaker accompanied by a 20 to 30% water exchange was carried out using a small tube with an attached 25-µm mesh to avoid the removal of any eggs or nauplii. Siphoning was done using a 50 mL syringe attached to the siphon tube. A gentle siphon was done to reduce the stress that might accompany the water exchange process. A 100% water exchange

was also performed on day 7. Food was added daily as described in 4.2.1. However, to maintain a food concentration above a 1000 µg C l^{-1} algal cells, daily subsamples were collected from replicates and algae cells were counted under a microscope using a haemocytometer to determine the amount of food required. After 15 days, the content of each beaker was filtered through a 25-µm mesh and fixed in 10% formalin for late enumeration as well as sex determination for adults. The counting of eggs, nauplii, copepodites and adults were performed with a Bogorov counting chamber under a dissecting stereo-microscope (Nikon SMZ645).

4.2.6 Statistical analysis

Data are presented as the mean ± standard error (SE). All data collected were tested for normality and homogeneity of variance prior to mean comparison procedures, and data expressed in percentages were arcsin-transformed prior to analysis. The treatment effects on population growth, sex ratio and egg hatching success data were compared using one-way ANOVA, while mortality rate and egg production data were analysed with repeated measures ANOVA. When significant differences (p <0.05) were found, Tukey's multiple comparison test was performed to determine specific differences among treatments. All statistical analyses were conducted using Sigmaplot, version 11.

4.3 Results

4.3.1 Egg production

Figure 4.1 shows the mean daily and total egg production per adult female *P. crassirostris* over seven days of their lifespan across different initial stocking densities. The differences in mean daily egg production per female were highly significant among different density treatments (p <0.001) and two very different patterns were

demonstrated. The first pattern was characterised by a low mean egg production per female on the first day but increased sharply on day 2 with a peak production between day 2 to 5. A steep decrease followed on day 6 and 7 although egg production was still found on day 7. This pattern was found at lower initial stocking densities of 1000, 3000 and 5000 adults L^{-1}. The second pattern is featured by a steady and substantially lower mean egg production per female from day 1 to 4, followed by a significant drop on day 5 and the cessation of all egg production after day 6. This pattern was assumed by the two high density treatments of 7000 and 9000 adults L^{-1} (Fig. 4.1a).

Mean total egg production per female over 7 days was also significantly different across the different stocking densities (p <0.001; Fig. 4.1b). The initial stocking density treatment of 5000 adults L^{-1} produced significantly more total eggs per female than all other treatments, yielding a mean of 22.5 ± 0.1 eggs $female^{-1}$, which was followed by the 1000 and 3000 adults L^{-1} treatments at 21.0 ± 0.1 and 20.0 ± 0.1 eggs $female^{-1}$, respectively. The high initial stocking densities of 7000 and 9000 adults L^{-1} produced substantially lower total eggs at only 6.2 ± 0.1 and 4.1 ± 0.0 eggs $female^{-1}$, respectively (Fig. 4.1b).

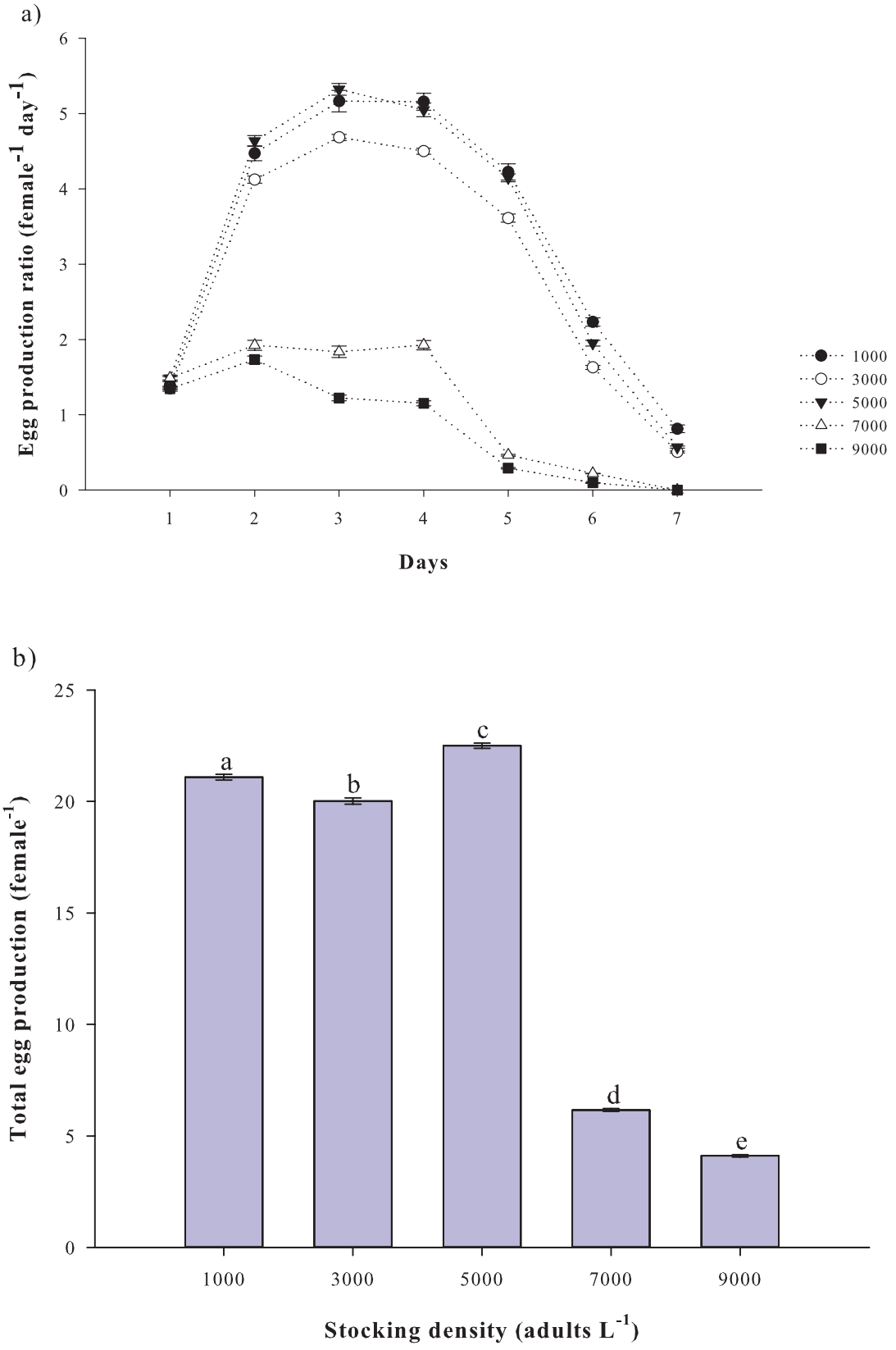

Figure 4.1: Egg production of *P. crassirostris* under different initial adult stocking densities (n = 6). a) Mean daily egg production per female over 7 days; b) mean total egg production per female over 7 days. Data are presented as the mean ± standard error. The different letters on the tops of the bars indicate significant differences (p <0.01).

In terms of total daily egg production by all females in a treatment, except on day 1, the 5000 adults L^{-1} treatment consistently produced the highest number of eggs over the seven-day period and the grand total of eggs produced during the seven days was nearly double that of the second highest total egg production by the 3000 adults L^{-1} treatment. Interestingly, although there were substantially more females in the higher density treatment of 7000 and 9000 adults L^{-1}, the total egg production over the 7 day period by all females of the two treatments was only approximately 1/3 and 1/4 of that of the 5000 adults L^{-1}, respectively (Table 4.1).

Table 4.1: The mean daily total egg production of *P. crassirostris* over seven days and mean seven day total egg production with different initial stocking

Initial stocking density	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Total
1000	1355.0±38.1^{a}	4268.2±52.8^{a}	4875.7±88.1^{a}	4713.7±45.0^{a}	3749.8±45.8^{a}	1688.5±38.5^{a}	438.5±11.3^{a}	21089.3±126.9^{a}
3000	4039.7±46.3^{b}	12299.3±130.1^{b}	13941.5±139.6^{b}	13336.7±109.0^{b}	10621.0±135.0^{b}	4537.0±71.9^{b}	1271.0±35.3^{b}	60046.2±426.9^{b}
5000	7393.7±116.7^{c}	22973.2±335.0^{c}	26246.7±351.6^{c}	24801.2±415.2^{c}	19952.2±228.3^{c}	8751.0±142.3^{c}	2367.8±52.5^{c}	112485.7±605.9^{c}
7000	9232.7±194.3^{d}	10554.0±380.4^{d}	10014.7±329.0^{d}	10042.5±338.7^{d}	2301.8±40.2^{d}	998.0±9.5^{d}	0.0±0.0	43143.7±436.2^{d}
9000	9959.0±226.2^{e}	11126.7±240.5^{d}	7296.5±206.0^{e}	6564.5±194.8^{e}	1575.0±49.1^{e}	438.0±28.1^{e}	0.0±0.0	36959.7±453.5^{e}

Data are presented as mean ± standard error (n = 6). Data with different superscript letters within a same column indicate significant differences (p <0.01).

83

4.3.2 Egg hatching success

Egg hatching success was also significantly affected by initial stocking density (p <0.001) (Fig. 4.2). High and very similar rates of hatching success (91.8 – 92.3%) were observed for the treatments with an initial density ≤ 5000 adults L^{-1} (p >0.05). High stocking densities of 7000 and 9000 adults L^{-1} had significantly lower hatching rates of 73.2 ± 2.0% and 54.8 ± 1.9%, respectively (p <0.001).

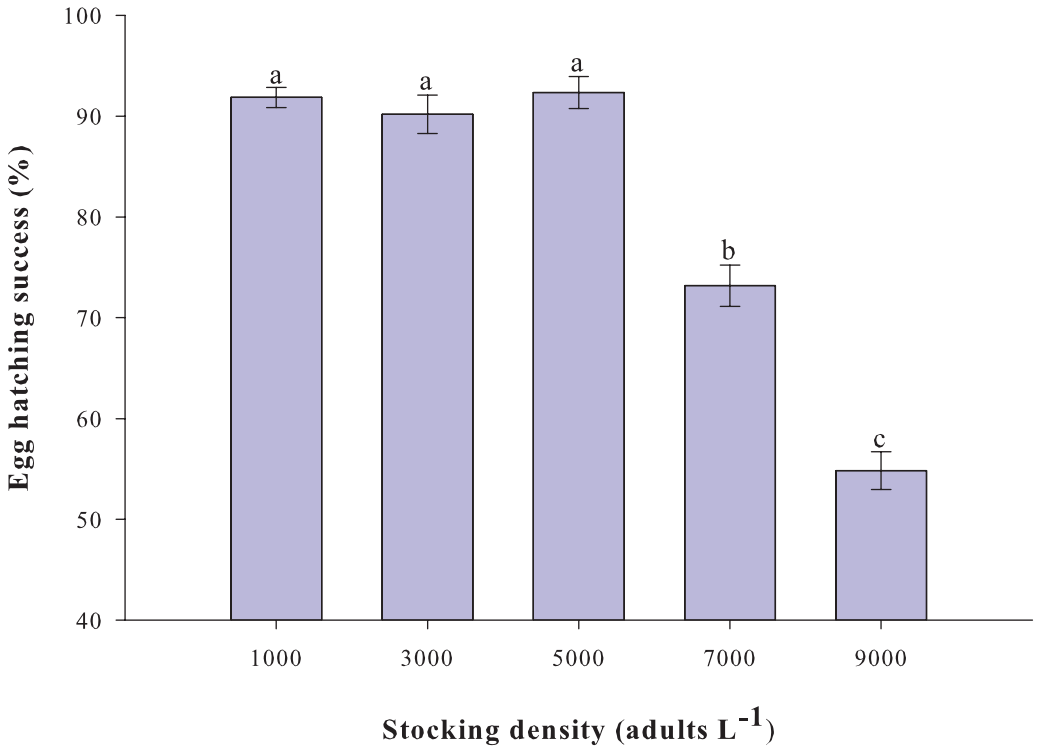

Figure 4.2: Egg hatching success (%) of *P. crassirostris* cultured at different initial adult stocking densities (n = 6). Data are presented as the mean ± standard error. The different letters on the tops of the bars indicate significant differences (p <0.01).

4.3.3 Mortality rate

The daily mortality rate was significantly different among the different density treatments (p <0.001) (Table 4.2). During the first 5 days, the daily mortality rates remained very low for the 3000 and 5000 adults L^{-1} treatments and no significant

difference was detected between them (p >0.05), resulting in `a cumulative mortality < 12% for both treatments by day 5 (Table 4.2; Fig. 4.3a). In contrast, under high densities of 7000 and 9000 adults L^{-1}, dramatically higher daily mortality rates were recorded for the first 2 days, leading to a cumulative mortality of 33.2 ± 1.4% and 40.1 ± 0.6%, respectively (Fig. 4.3a). Daily mortality rates of the 3 lowest density treatments increased from day 6 onward although the cumulative mortality of the 3000 and 5000 adults L^{-1} treatments was still below 20% by day 7. However, it is worth noting that regardless of the initial density, no adults in any of the treatments survived beyond day 8 (Table 4.2; Fig. 4.3a).

Interestingly, when the mortality rate of male adults was examined separately, it showed an overall higher percentage male mortality for the first few days in all density treatments (Fig. 4.3b). However, the 7000 and 9000 adults L^{-1} treatments had a significantly higher male mortality rate than did the other densities (p <0.001), with a loss of approximately 50% of the adult males on day 1 alone (41% and 53%, respectively). The loss of the adult males was comparatively lower for the three lower initial stocking density treatments of 1000, 3000 and 5000 adults L^{-1} on day 1, ranging from 20% to 26% (Fig. 4.3b).

Table 4.2: The mean daily adult mortality rate (%) of *P. crassirostris* over eight days at different initial stocking densities.

Initial stocking density (Adults L^{-1})	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
1000	4.8±0.7^{a}	4.0±0.9^{a}	3.0±0.4^{a}	6.1±0.6^{a}	6.0±0.7^{a}	17.2±0.9^{a}	32.9±1.9^{a}	100
3000	2.3±0.3^{b}	2.2±0.3^{ab}	1.9±0.2^{b}	2.5±0.2^{b}	1.9±0.2^{b}	5.9±0.2^{b}	10.9±0.6^{b}	100
5000	2.4±0.1^{b}	2.0±0.2^{b}	2.4±0.2^{ab}	2.5±0.2^{b}	3.2±0.1^{bc}	7.5±0.4^{c}	8.1±0.4^{c}	100
7000	17.8±0.6^{c}	18.7±1.0^{c}	7.3±0.2^{c}	5.9 0.4^{c}	6.2±0.5^{c}	11.0±0.6^{bc}	12.2±0.5^{c}	100
9000	25.4±0.7^{d}	19.6±0.3^{c}	13.1±0.4^{d}	6.3±0.3^{bc}	6.4±0.3^{c}	20.2±0.5^{d}	14.9±0.7^{c}	100

Data are presented as mean ± standard error (n = 6). Data with different superscript letters within a same column indicate significant differences (p <0.01).

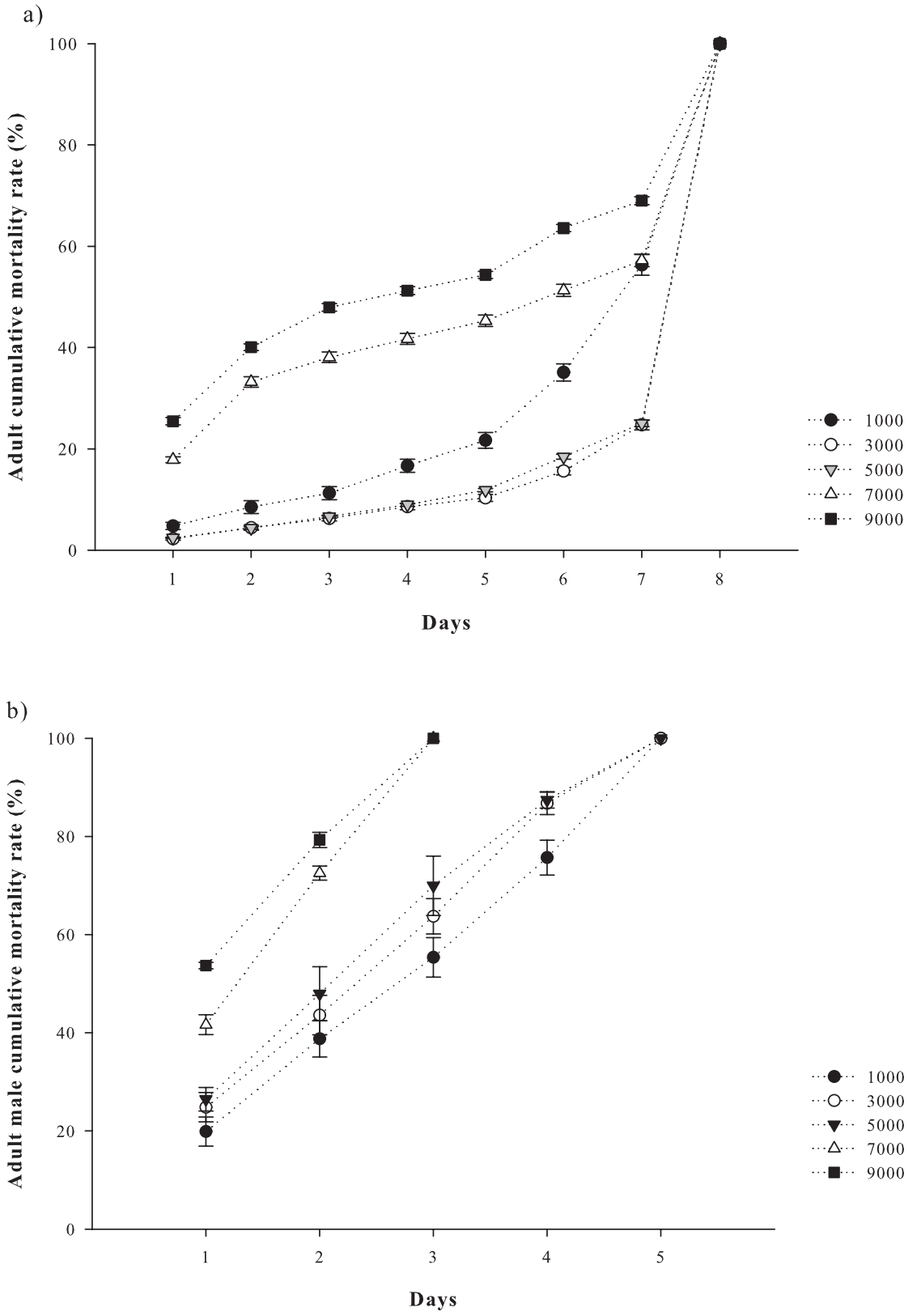

Figure 4.3: Mean cumulative daily mortality rate (%) of *P. crassirostris* under different initial adult stocking densities (n = 6). a) Cumulative mortality rate (%) of all adults; b) cumulative mortality rate (%) of male adults. Data are presented as the mean ± standard error.

4.3.4 Population growth

The effect of initial stocking density on the population growth over a 15 day culture period is shown in Fig. 4.4. The greatest mean total number of all postembryonic stages of *P. crassirostris* harvested at the end of the culture period was from the 5000 adults L^{-1} treatment (6429.4 ± 202.2 ind L^{-1})(Fig. 4.4a). However, the population growth expressed as the percentage increase over the initial stocked adult number was the highest for the lowest density treatment of 1000 adults L^{-1} (319.4 ± 20.0%) (p <0.001), which was followed by the 3000 and 5000 adults L^{-1} treatment (53.4 ± 5.9% and 25.6 ± 9.9%, respectively) (Fig. 4.4b). In contrast, at the high density treatments of 7000 and 9000 adults L^{-1}, the populations showed negative growth, with a reduction of -41.95 ± 3.7% and -54.57 ± 3.1%, respectively, over the 15 day culture period (Fig. 4.4b).

The population composition of all post-embryonic stages at the end of the culture period for each treatment is summarised in Table 3. Except adult females, the percentage compositions of nauplii, copepodites and adult males were all significantly different among the treatments (p <0.001). In general, significantly higher percentages of nauplii and copepodites were recorded in the lower density treatments of 1000, 3000 and 5000 adults L^{-1} compared to the higher densities at 7000 and 9000 adults L^{-1} (p <0.001) (Table 4.3).

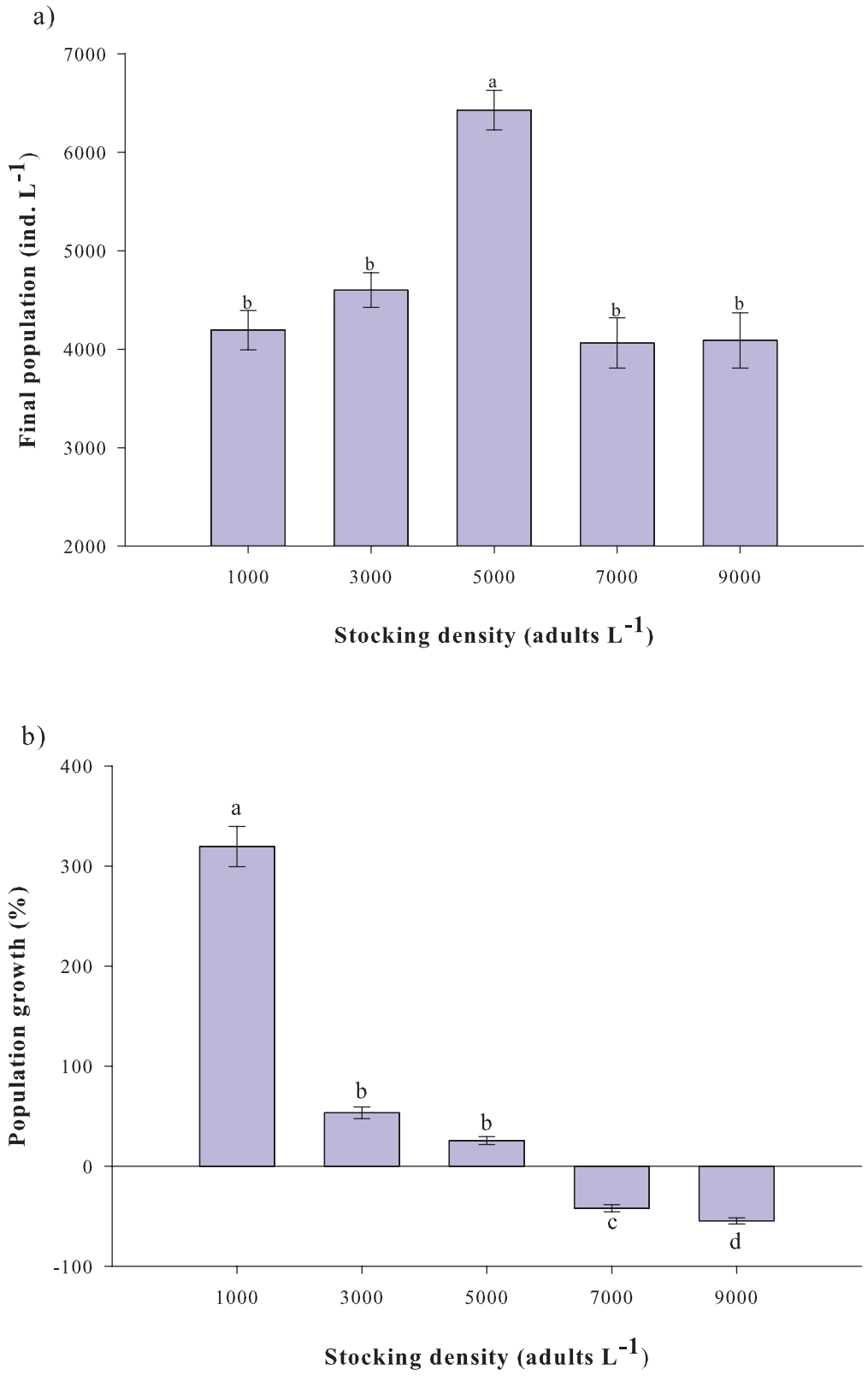

Figure 4.4: Population growth of *P. crassirostris* after 15 day culture with different initial adult stocking densities (n = 6). a) Final population density (ind. L^{-1}) at the end of 15 day culture, including all postembryonic stages; b) percentage population growth over the initial population. Data are presented as the mean ± standard error. The different letters on the tops of the bars indicate significant differences (p <0.01).

Table 4.3: The final mean population composition of *P. crassirostris* after a culture period of 15 days from different initial stocking densities.

	Population Composition (%)			
Initial stocking density (Adults L^{-1})	Nauplii	Copepodites	Females	Males
1000	43.7±1.1[a]	26.3±0.9[a]	26.4±0.9[a]	3.6±0.3[a]
3000	46.2±1.2[a]	26.0±1.3[a]	24.3±1.0[a]	3.4±0.2[a]
5000	43.0±1.7[a]	26.7±1.8[a]	26.7±1.1[a]	3.7±0.2[a]
7000	28.4±2.6[b]	22.1±2.4[ab]	31.2±2.4[a]	18.3±2.1[b]
9000	32.7±3.0[b]	16.3±2.1[b]	28.1±2.6[a]	22.9±2.2[b]

Data are presented as mean ± standard error (n = 6). Data with different superscript letters within a same column indicate significant differences (p <0.01).

Conversely, the percentages of adult males in the final population were significantly greater at the higher densities of 7000 and 9000 adults L^{-1} than the lower densities of 1000, 3000 and 5000 adults L^{-1} (p <0.001). This trend led to significant differences in the sex ratio of the final population among treatments (p <0.001) (Fig. 4.5). While in the low stocking densities of 1000, 3000 and 5000 adults L^{-1}, the sex ratio remained highly skewed with approximately 85% to 90% of adults being female, which was within the normal range observed for this species. However, under high densities the sex ratio was altered substantially with only 63% and 55% of adults being female for the treatments of 7000 and 9000 adults L^{-1}, respectively, reaching a near 1:1 sex ratio.

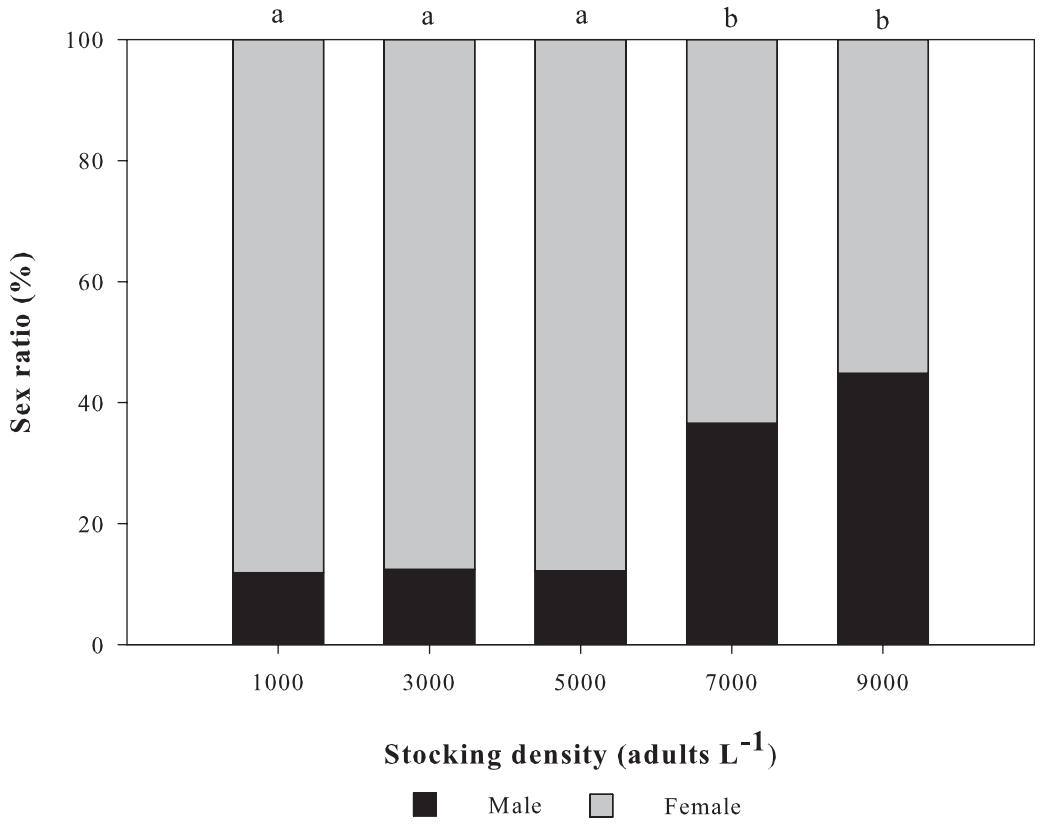

Figure 4.5: Mean sex ratio (%) of *P. crassirostris* at the end of 15 days of culture at different initial adult stocking densities (n = 6). The different letters on the tops of the bars indicate significant differences (p <0.05).

4.4 Discussion

Culturing copepods at high density within species-specific tolerable range maximises culture productivity. However, overly high culture density can negatively impact various life history traits and the behaviour of copepods and potentially lead to culture crash. For instance, high stocking densities have been reported to reduce female reproductive capacity, the egg hatching rate and delays in post-embryonic development, as well as enhanced cannibalism rates of adults on nauplii (Camus and Zeng, 2009; Lee et al., 2012b; Medina and Barata, 2004; Peck and Holste, 2006).

While it is true that data on copepod stocking density from past literature are inconsistent in a range of factors such as setups, population composition and climate

origin between tropical and temperate species, nevertheless, presents a fundamental guideline. The present study demonstrated that among a wide range of initial adult stocking densities tested, *P. crassirostris* culture productivity was generally not significantly affected by adult density as high as 5000 ind. L^{-1}. In contrast, in other calanoid copepod species that have been considered as promising species for aquaculture, generally a maximum culture density of 2000 ind. L^{-1} or lower was reported (Camus and Zeng, 2009; Medina and Barata, 2004). The substantially higher maximum adult stocking density of *P. crassirostris* without negative effects indicates that this species is a highly promising candidate species that can be cultured as larval prey in aquaculture hatcheries.

P. crassirostris female reproductive capacity was significantly affected when the adult density increased to 7000 and 9000 adults L^{-1}, with a significant drop in both the daily and total egg production when compared to lower densities. This finding showed that similar to other calanoid copepods (Camus and Zeng, 2009; Medina and Barata, 2004), *P. crassirostris* egg production is sensitive to overly high stocking density. The stress caused by overcrowding is likely to be the explanation for the reduced egg production as well as the higher mortality rates observed under high densities (Zhang and Uhlig, 1993). Clearly, under overcrowded conditions the competition over space is intense with copepods being more likely to collide (Miralto et al., 1996). The substantially increased hydrodynamic disturbance caused by many individuals swimming nearby might also have negatively affected the copepods' perception of food and feeding behaviour (Drillet et al., 2014; Jiang and Osborn, 2004), thereby decreasing their food intake. Despite the fact that cannibalism has been proven to affect other copepods in high density culture (Camus and Zeng, 2009; Miralto et al.,

1996), but it is unlikely to be a major contributor for *P. crassirostris* as cannibalistic behaviour is not normally observed for this species.

High stocking densities also significantly reduced *P. crassirostris* egg hatching success. The significantly lower egg hatching success at high stocking densities of 7000 and 9000 adults L^{-1} is most likely also related to deteriorated physiological and nutritional conditions under overcrowded conditions as mentioned above. Moreover, it is likely that high male mortality during the first 2 days (> 40%) under the two high density conditions further contributed to the low egg hatching success, particularly considering that *P. crassirostris* has a highly skewed sex ratio toward females (female:male ~88% to 12%). Although it is known that for copepods, a male can fertilise multiple females (Titelman et al., 2007), the scarcity of males and the stress caused by overcrowding are likely to reduce the fertilisation rates, resulting in increased unfertilised eggs (Kiørboe, 2007).

Adult copepod populations are generally female-biased, and paracalanid copepods in particular have been described as a female dominated population (Gusmao and McKinnon, 2009a; Kiørboe, 2006). Although it is still unclear how sex is determined in copepods, environmental factors, including temperature (Lee et al., 2003), food quality (Carotenuto et al., 2011) and quantity (Irigoien et al., 2000), have been reported to affect the sex ratio of copepods (Gusmao and McKinnon, 2009a). In the present study, high stocking density led to a major shift in the sex ratio from a highly female skewed population to close to an equal ratio at the high density of 9000 adults L^{-1}. While the mechanism underlying such a dramatic shift in sex ratio is unknown, it might represent a population self-regulation mechanism by which under overcrowding conditions, more males are produced to limit the reproductive capacity of the population to lessen conspecific competition that may jeopardise the health of the

population. Within some species, individuals adjust their sex ratios in response to the quality of the environment, producing more offspring of the gender likely to contribute most to their individual fitness (Sapir et al., 2008). Clearly, whether this was the case and how such a substantial shift in sex ratio was achieved warrant further research.

The mortality data provide key insights into population dynamics and offer clues for potential improvements in the management of culture. In addition to the fact that *P. crassirostris* males have a shorter lifespan than females because they are non-feeding, the present results show that *P. crassirostris* males generally had a higher mortality rate in crowding conditions. Sex-specific mortality based on the relative susceptibility to stress has been reported in other copepods in which male copepods also experienced higher mortality than females when under stress (Avery et al., 2008). Traditionally little attention has been paid to the potential significance of males in influencing the reproductive productivity of copepods; however, with the sex ratio highly skewed toward females and the high male mortality rates, males may play a more prominent role than expected in affecting the culture productivity of *P. crassirostris*.

The distribution of different developmental stages in the final populations after 15 days culture of different density treatments demonstrated that adults generally made up less than 50% of total population. For example, for the highest final population of 6429.4 ± 202.2 ind L^{-1} achieved by the 5000 adults L^{-1} treatment, adults only accounted for 30% of the final population. Because adults contribute directly to the reproductive capacity of the population, and premature individuals (nauplii and copepodites) are likely to restrain the adult population by competing for space and interfering with their feeding, techniques that can control the number of early developmental individuals might help enhance culture productivity. In fact, VanderLugt and Lenz (2008) reported

that the continuous harvesting and dilution of the culture helped maintain high culture densities for *Bestiolina similis,* another paracalanid copepod. Toledo et al. (2005) also suggested that segregation methods such as an egg collector could enhance the adult population. Because the primary portion of the copepod population required for the critical first feeding larvae are nauplii (Buttino et al., 2012; Payne and Rippingale, 2001a; Schipp et al., 1999), methods that can partially segregate/harvest newly hatched nauplii from the culture should help achieve high adult density and hence a high productive capacity of the culture population. Due to the smaller size of nauplii compared to other stages, this process could be performed relatively simply by gently circulating culture water through an outlet with a screen attached. The mesh size of the screen should allow only nauplii to pass and water pass through the screen could go through a second screen with smaller mesh size to retain nauplii before water's being circulated back to the culture tank. An added benefit of such management is that adult cannibalism on nauplii can also be reduced for cannibalistic species (Schipp et al., 1999). The current results of this chapter demonstrated that a maximum population growth could be achieved at an initial density of 1000 adults L^{-1}. However, the initial culture density depends on the purpose or application of cultured copepods.

In conclusion, the present study demonstrated that for *P. crassirostris*, an adult stocking density as high as 5000 L^{-1} has no obvious negative effects on various productivity-related biological parameters, and a mixed population with a sustainable culture density of approximately 7 ind. mL^{-1} can be achieved. In fact, the full potential of high density culture of *P. crassirostris* is more likely to be realised in larger scale culture than the current study in which 200 ml vessels were used. In fact, in our laboratory larger scale culture of *P. crassirostris* within 250 L tanks, densities between 12 to 17 ind. mL^{-1} have been routinely achieved (unpublished data). Clearly, such

densities are substantially higher than any other calanoid species documented so far (Støttrup and McEvoy, 2003), suggesting *P. crassirostris* is a highly promising species for rearing in hatcheries as larval prey.

4.5 Summary

The difficulty in culturing calanoid copepods, particularly at high culture density, hinders their use in aquaculture hatcheries. The aim of this chapter was to evaluate the effects of initial stocking density on a range of biological parameters affecting the culture productivity of the calanoid copepod *P. crassirostris*. Five initial stocking densities of 1000, 3000, 5000, 7000 and 9000 adults L^{-1} were evaluated. Among the treatment differences on both daily and total egg production, the egg hatching rate and mortality rate were all highly significant (p <0.001). Egg production was monitored over seven consecutive days, and the density of 5000 adults L^{-1} produced significantly (p <0.001) more eggs daily than did the other treatments. Egg hatching success was very high at 91.8 to 92.3% at the lower initial stocking density of ≤ 5000 adults L^{-1}; however, as density increased to ≥ 7000 adults L^{-1}, it dropped sharply to only 55 to 73%. Similarly, significantly higher adult mortality rates were recorded for densities ≥ 7000 adults L^{-1}. Moreover, population growth was evaluated for a 15-day culture period. It showed that the initial stocking density of 5000 adults L^{-1} produced the highest final population (6429.4 ± 202.2 individuals L^{-1}) at the end of the culture period. However, in term of percentage population growth over the initial population, it was the lowest density of 1000 adults L^{-1} that achieved the highest growth (319.4 ± 20.0%), while high density treatments of 7000 and 9000 adults L^{-1} suffered negative percentage growth. Interestingly, the sex ratio was also significantly affected by initial stocking densities as the male to female ratio increased significantly at a

density ≥ 7000 adults L^{-1}. Our results demonstrated that *P. crassirostris* is a highly promising species for culture as live prey for aquaculture hatcheries, and it can be stocked at a high initial density of 5000 adults L^{-1} without negatively impacting culture productivity.

CHAPTER 5

IMPROVEMENT IN THE REPRODUCTIVE PRODUCTIVITY OF THE TROPICAL CALANOID COPEPOD *PARVOCALANUS CRASSIROSTRIS* THROUGH SELECTIVE BREEDING[3]

Optimizing culture parameters to improve the reproductive capacity of copepods has limitations. Applying breeding strategies on copepods that had been used successfully in aquaculture for different animals are not yet exploited. Thus, this chapter investigates the practicality of selective breeding as an approach to enhance the reproductive capacity of *P. crassirostris*. Results from this chapter form a new field of research for the development of copepod culture techniques.

[3]Published as: Alajmi, F., Zeng, C., Jerry, D.R., 2014. Improvement in the reproductive productivity of the tropical calanoid copepod *Parvocalanus crassirostris* through selective breeding. Aquaculture. 420–421, 18-23.

5.1 Introduction

The difficulty in culturing calanoid copepods, particularly at high culture density, hinders their use in aquaculture hatcheries. Moreover, improving culture conditions has its limitations, and an established culture condition for a copepod might not be applicable to other species or even strains of the same species from different geographic locations. Selective breeding may be an additional approach for the improvement of copepod egg production. This approach may be very effective because the potential of selection to improve various commercially important aquaculture production traits has been extensively demonstrated in farmed fish, crustacean and molluscs (e.g. Argue et al., 2002; Dove and O'Connor, 2012; Gjedrem et al., 2012; Jerry et al., 2005), as well as for *Artemia*, in which selection for smaller cyst size and shorter hatching time led to a reduction in the size of newly hatched nauplii and the hatching time of cysts (Briski et al., 2008; Shirdhankar and Thomas, 2003; Van et al., 2012). To date, reports of the enhancement of copepod life history traits through selective breeding are scarce; the only such report in the literature is a single study in which the effect of selection on the fecundity of the cyclopoid copepod *Paracyclopina nana* was examined (Lee et al., 2012a). The study reported an increase in the fecundity of *P. nana*, but the increase was not statistically significant. Hence, the aim of the present study was to apply a selective breeding approach to examine if targeted selection could significantly enhance the reproductive output of the tropical paracalanid copepod *P. crassirostris*.

5.2 Methods

5.2.1 General procedures

P. crassirostris adult pairs were collected randomly from the stock cultures to form the base population (G_0) consisting of 50 pairs. Between 100 to 150 pre-matured females at the final copepodite stage V (CV) were individually isolated in petri dishes and observed until sexual maturation was reached. Maturation was confirmed by the moult of the CV copepodites (Lawson and Grice, 1973). Each mature adult female was then paired with an adult male. To ensure that the ages of selected females were similar, this step was undertaken simultaneously within 6 h of the time when the majority of females reached maturity. Each pair was then cultured in a 200 ml vessel submerged in a beaker. A 74 μm mesh was attached to the bottom to ensure that the adults would remain inside the vessel. This design permitted the eggs produced by the females to pass through the mesh and accumulate at the bottom of the beaker for counting. It also reduced the stress of daily water changes, which were performed simply by moving the vessel to a new beaker with fresh seawater and food every 24 h. Care was taken before moving the culture vessels to a new beaker through gently rinsing the sides of the culture vessel while partially submerged in the beaker to make sure all eggs/nauplii had passed through the bottom mesh. The eggs that accumulated at the bottom of the beakers were counted following daily water exchange with a Bogorov counting chamber and a dissecting stereo-microscope (Nikon SMZ645). The size of the eggs, first nauplii (NI) and adult males and females were measured to determine the effects of selection on the final “Select” and “Control” line generations. Size measurements were made using a Leica DM LB microscope and an Olympus DP25 camera. Photos were then analysed with the Olympus DP2-BSW software.

Throughout the experiment, *P. crassirostris* were fed the Tahitian strain of *Isochrysis* sp. and the diatom *C. muelleri* at 1000 µg C l^{-1} daily at a ratio of 1:1 (cell volume; Strathmann, 1967). Temperature, salinity and photoperiod were maintained at similar parameters to the stock culture conditions (see section 2.2.2).

5.2.2 Selection procedures

Family selection was adopted to develop a *P. crassirostris* strain with higher egg production capacity (Fig. 5.1). It has been previously reported that after reaching sexual maturity, daily egg production of calanoid copepods undergoes a pattern of steady increase for the first few days, subsequently peaking and plateauing for several days before dropping off significantly (Alajmi and Zeng, 2015). Based on these reports, a pilot experiment was conducted to determine the egg production pattern of *P. crassirostris*. We examined female (*n*= 50) egg production during their lifespan following similar procedures as in section *5.2.1*. The results consistently showed that egg production was highest at days 3 and 4 after sexual maturation, accounting for 47.8% of total egg production (Fig. 5.2). Thus, egg production on these days was used as the basis for selection decisions.

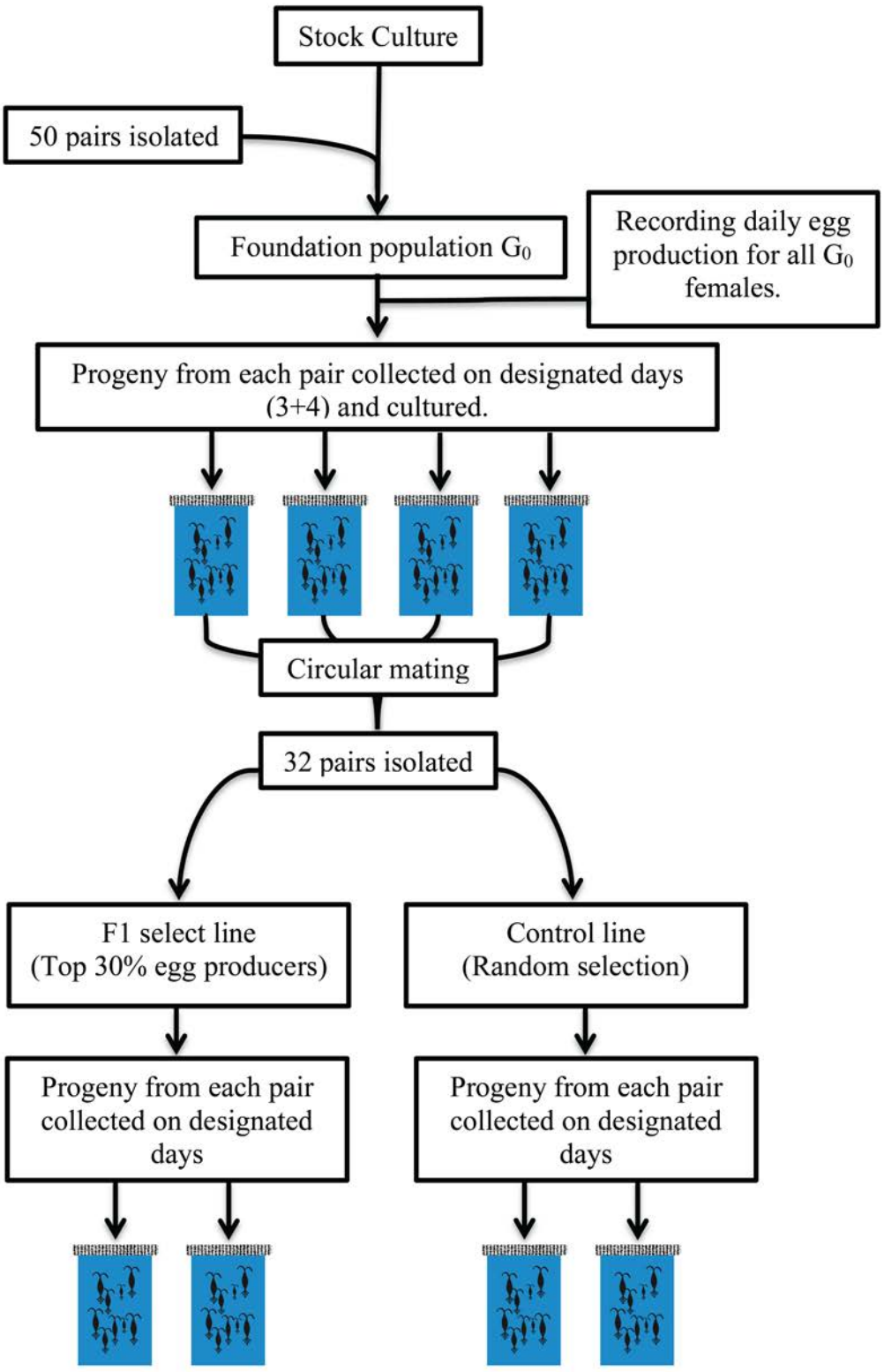

Figure 5.1: Schematic illustration of selection experiment.

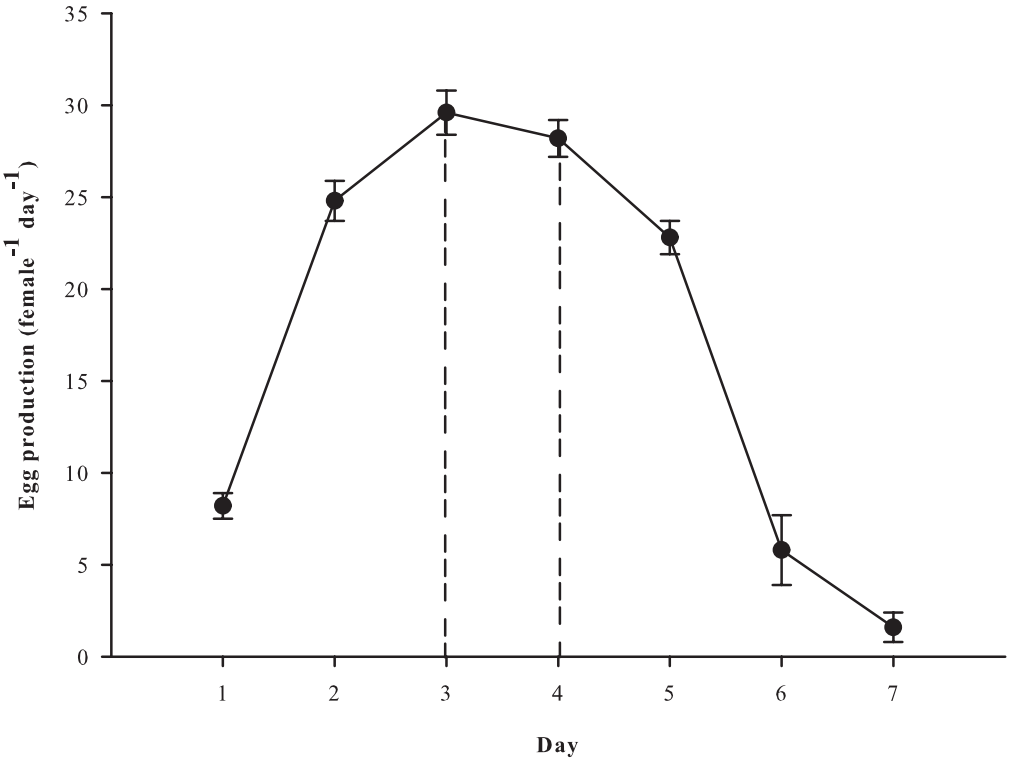

Figure 5.2: Daily egg production of *P. crassirostris* (eggs female^{-1}) prior to selection (*n = 50*; total egg production = 112 ± 1.2), dotted lines indicates days of highest egg production. Data are presented as mean ± standard error (Alajmi et al., 2014).

A foundation population (parents; G_0) comprising of 50 randomly selected pairs was formed, as described in section 5.2.1. Every day, eggs produced by each G_0 (parents) female were collected on a 25 μm mesh and counted to monitor total egg production, but only eggs produced on the two designated days (day 3+4) were put in a designated culture container for the hatching of nauplii. These nauplii were cultured to reach adulthood using the procedure described in section 5.2.1. Accumulating eggs from each G_0 pair on day 3+4 was done to ensure that a sufficient number of offspring were collected from each G_0 pair to form the next generation (progeny). The Select line (G_1) was formed by selecting as parents the offspring of the 30% G_0 females with the highest egg production on the designated days, further, an equal number of male individuals were selected from these top 30% females as well. The first Control line

was formed through randomly pairing male and female individuals from the offspring of G_0. Thirty-two pairs were formed for each line (Select and Control) by mating recently matured adults, however, to avoid inbreeding a circular mating design was implemented (Nomura and Yonezawa, 1996). This strategy involves the transfer of males from each group to the neighbouring group in a rotational manner for mating. Overall, five generations of selection were performed with the same selection criteria as for the Select line. For each subsequent generation, a Control line was maintained alongside as described above for G_1.

5.2.3 Calculation of response to selection and realized heritability

Response (R) to selection for egg production was determined by the difference in mean phenotype (egg production) between the progeny generation and the previous generation, whereas the selection differential (S) was calculated based on the difference in mean phenotype between the selected parents and the entire parent population. The selection differential was calculated by weighing each parent to the number of its offspring.

The realized heritability (h^2) was measured from the slope of the regression line of generation means plotted against the cumulative selection differential (Falconer, 1989). Genetic gain (G) accomplished over each two consecutive generations of selection was calculated according to the following equation:

$$G = \frac{Sn - Cn}{Cn}$$

where *S* is the mean phenotypic value of the Select line and *C* is the mean phenotypic value of the Control line; *n* represents the generation number.

The coefficient of inbreeding (f) was determined using the software PEDIGREE VIEWER, version 6.5b (Brian Kinghorn, University of New England, Armidale, Australia).

5.2.4 Statistical analysis

The data are presented as the mean ± standard error (SE). All data collected were tested for normality and homogeneity of variance prior to mean comparison procedures. Differences in mean egg production of each day and size (eggs, nauplii and adults) between Select and Control lines were analysed using a one-way ANOVA. All statistical analyses were conducted using Sigmaplot, version 11.

5.3 Results

Table 5.1 summarises the results from five generations of selection. Mean egg production prior to selection was 56.7 ± 0.6 eggs female^{-1} (day 3 + 4 pooled). As a consequence of selective pressure, the egg production of Select lines increased and was significantly greater than the Control lines across subsequent generations (p <0.001; Fig. 5.3). At G_5, egg production was 20.5% higher than that of the Control line. The G_5 Select and Control *P. crassirostris* females displayed a similar daily egg production pattern over their life span, and on any given day G_5 Select females daily egg production was significantly higher from the Control line (p <0.001; Fig. 5.4). It was also observed that select females continued to produce eggs on day 8, while all females from the Control line ceased egg production on day 7. The mean total egg production over female lifespan at G_5 was again significantly higher for the Select line when compared to that of the Control line at 155.3 ± 1.3 and 124.7 ± 1.5 eggs female^{-1}, respectively (p <0.001). Therefore, when measured by total egg production per female, G_5 females produced 24.5% more eggs when compared to the Control line.

Table 5.1: Mean egg production (day 3+4 pooled), cumulative genetic gain and response to selection (%) of selected line across five generations of *P. crassirostris* females.

Generation	Selected population	Selected parents	Control line	R (S_n-C_n)	G (%)	[a]Selection differential
G_0	56.7 ± 0.6	61.3 ± 0.4	-	-	-	6.6
G_1	57.9 ± 0.5[a]	61.2 ± 0.6	53.7 ± 0.9[b]	4.3	8	3.5
G_2	59.8 ± 0.5[a]	62.7 ± 0.4	52.8 ± 0.8[b]	7.1	13	4.2
G_3	58.1 ± 0.5[a]	60.8 ± 0.3	53.4 ± 0.8[b]	4.7	9	2.8
G_4	63.1 ± 0.6[a]	67.4 ± 0.5	54.4 ± 0.8[b]	8.7	16	4.1
G_5	63.5 ± 0.7[a]	68.1 ± 0.6	52.7 ± 0.9[b]	10.8	20	-

Mean values are expressed as ± SE. Values in the same row with different superscripts are significantly different ($P < 0.05$). [a]Selection differential was calculated by weighing each parent by the number of its offspring.

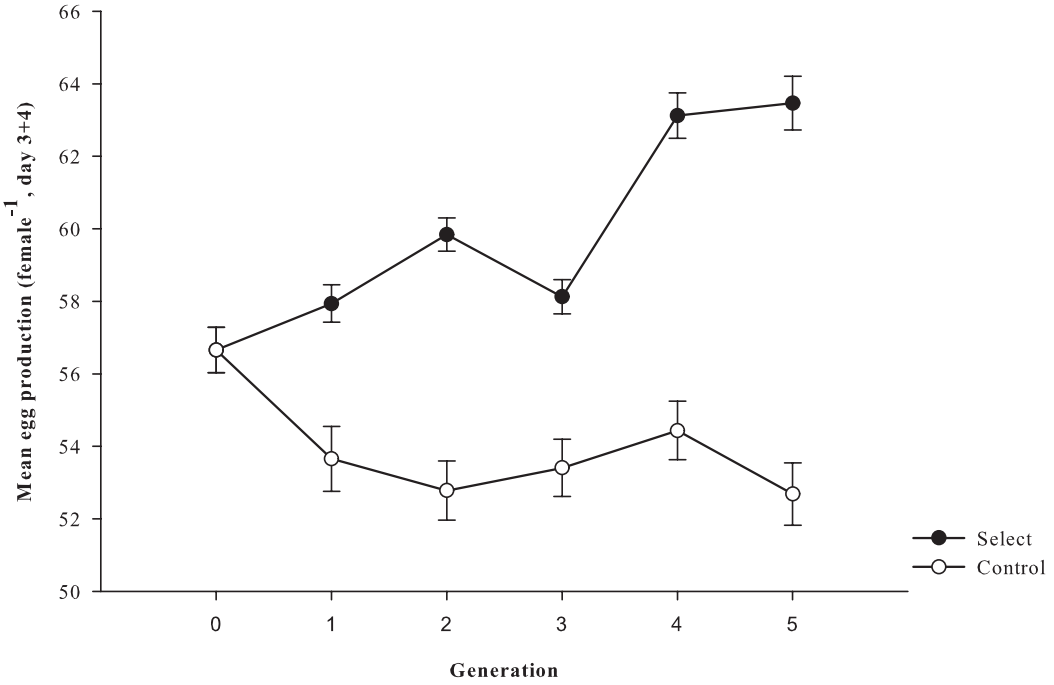

Figure 5.3: Mean egg production (eggs female^{-1}) of *P. crassirostris* of both the Select line and the Control line across 5 generations of selection for egg production capacity (day 3+4 pooled). Data are presented as mean ± standard error.

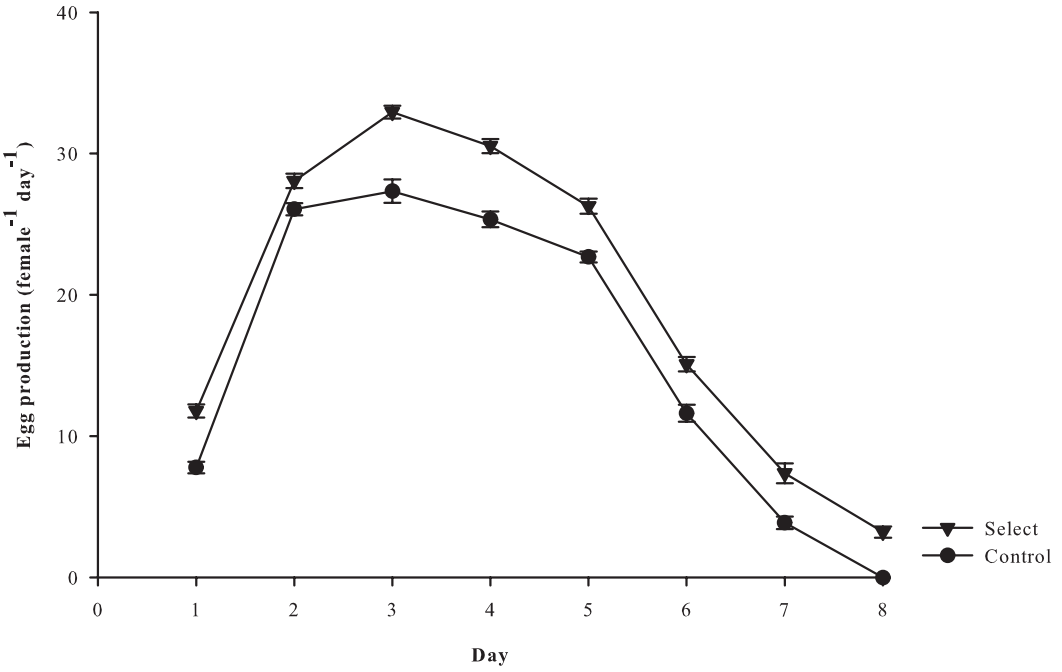

Figure 5.4: Responses to selection for daily egg production capacity (eggs female^{-1}) of *P. crassirostris* after 5 generations (Select line = generation 5). Data are presented as mean ± standard error.

Egg, nauplii and adult female sizes were also found to be significantly different between *P. crassirostris* G_5 Select and Control lines (p <0.001; Fig. 5.5), while no significant difference was detected among adult male sizes (p >0.05). The mean diameter of eggs from the Select line (G_5) was significantly larger at 71.5 ± 0.2 µm when compared to 59.7 ± 0.1 µm of the Control line. The size of G_5 nauplii was 86.3 ± 0.3 µm for the Select line, whereas Control line nauplii were 80.5 ± 0.5 µm. Finally, adult G_5 female size was 490.7 ± 0.9 µm, whereas females from the Control line were smaller at 443.0 ± 1.3 µm.

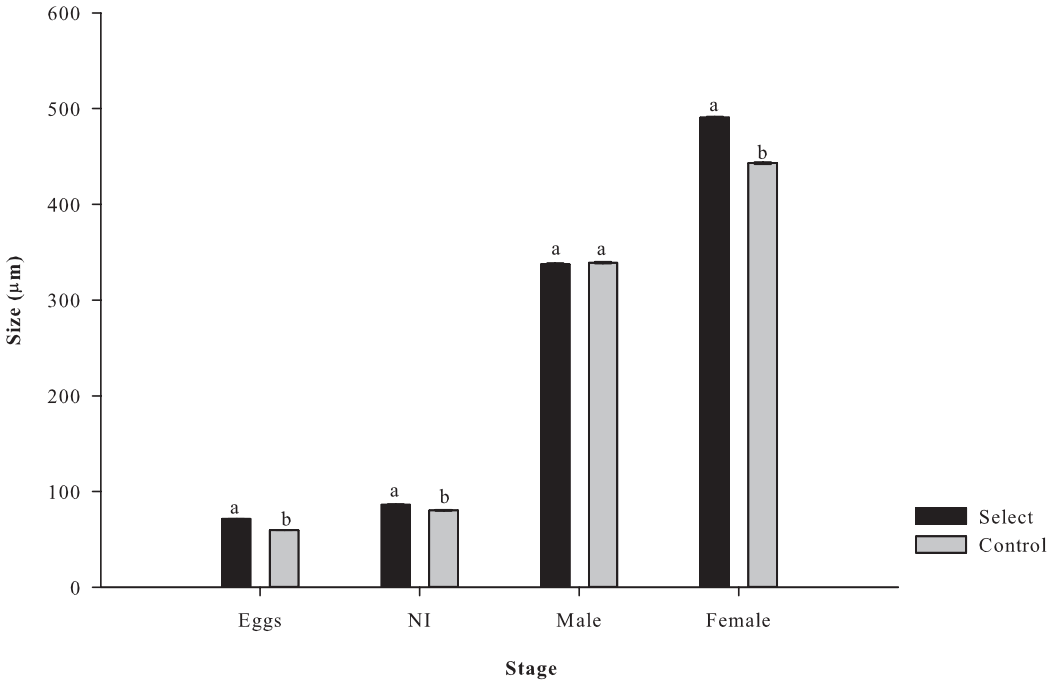

Figure 5.5: The effect of selection for egg production capacity (egg female^{-1}) of *P. crassirostris* after 5 generations (Select line = generation 5) on size. Data are presented as mean ± standard error. Different letters indicate significant differences within life stage of copepod ($p < 0.05$).

Overall, after five generations of selection, the percentage of pooled egg production for days 3 and 4 increased from 8% (G_1) to 20% (G_5). Based on the selection responses observed, the calculated heritability (h^2) for egg production in *P. crassirostris* is 0.38 ± 0.15 (Fig. 5.6), while the inbreeding coefficient calculated from the pedigree at G_5 was 12%.

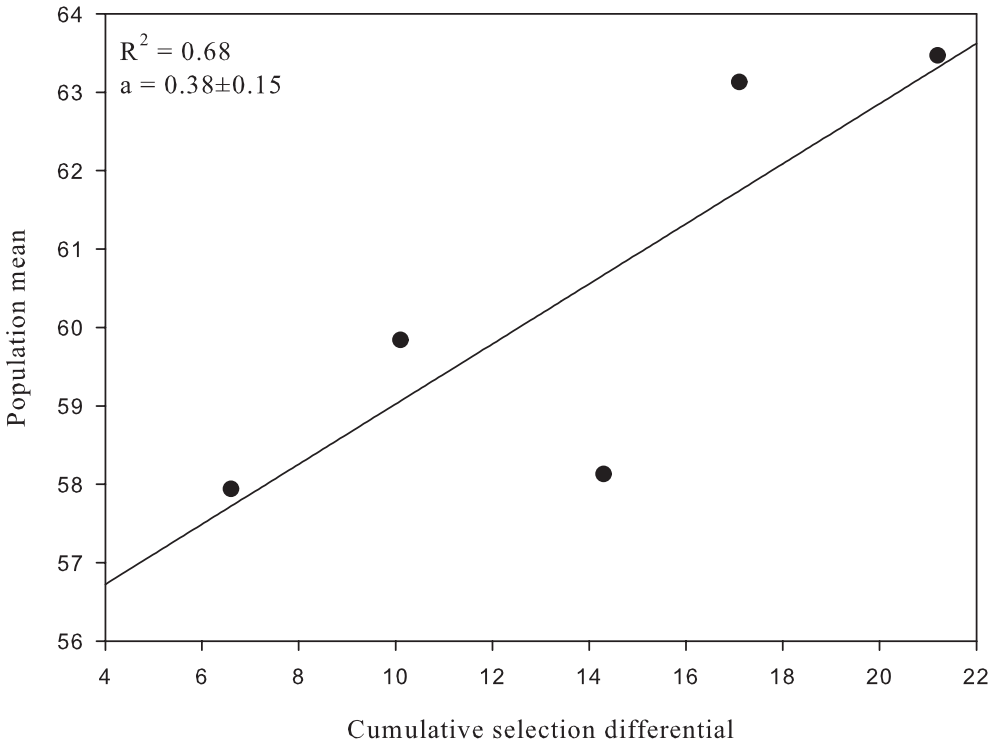

Figure 5.6: Regression between the population egg production means (day 3+4 pooled) across 5 generations and cumulative selection differential.

5.4 Discussion

Selective breeding has been shown to be a very effective approach for enhancing desirable traits in aquaculture species as well as for some live feed species (Argue et al., 2002; Gjedrem et al., 2012; Rezk et al., 2009; Shirdhankar and Thomas, 2003; Van et al., 2012). However, there had been no reports on any selection programs designated for copepods until recently when Lee et al. (2012a) examined the effect of selection on the fecundity of the cyclopoid copepod *P. nana*. Interestingly, in this study, Lee et al. (2012) observed no statistically significant sustained improvement. Conversely, in the present study, our approach resulted in a highly significant 24.5% increase in the reproductive productivity of *P. crassirostris* (in terms of overall egg production) after 5 generations of selection. This clearly demonstrates the potential of selection as an approach to increasing egg production in copepods.

The selective breeding program implemented in our experiment suited the biological features of the free spawning tropical calanoid copepod species. The relatively short generation time and adult lifespan compared to temperate copepod species demand a simple method for selection; thus, family selection was adopted. However, the high selection intensity limited the number of pairs selected, which impacted the rate of inbreeding with an increase of 12% at G_5. This high increase in inbreeding suggests that a larger number of pairs per generation are required to maintain a lower inbreeding rate in any commercial strains that are subsequently produced via selection (Gjedrem, 2005).

Under controlled environmental conditions and consistent selection of appropriately aged (mature) females, we were able to obtain a sustained positive selection response. Nevertheless, it should be noted that the initial transition from stock culture to experimental vessels resulted in a low, yet significant, decrease in egg production of the Control line from G_0 to G_1. This drop in egg production in the Control line is most likely linked to daily handling and stress caused by the environmental changes associated with transferring brood animals to substantially smaller culture vessels. This transfer is also likely to have affected egg production in the Select line but was masked by the larger genetic gain in egg production as a result of the first selection event. However, further generations (G_1 to G_5) of the Control line maintained consistent mean egg outputs between 52.7 to 54.4 eggs (day 3 and 4 pooled).

In a recent study, Lee et al. (2012a) evaluated the effects of selection and different strains on fecundity of the cyclopoid copepod *P. nana*. These authors stated that an “obvious improvement” in egg production capacity by 17.8% was obtained through selection; however, this increase was not statistically significant when

compared to the reproductive capacity of the foundation generation. High inbreeding due to the selection strategy implemented through their experiment may have contributed to the inconclusive results (Lee et al, 2011). Our results, therefore, represent the first conclusive evidence that selection is an effective strategy to increase egg production for copepods. This is evident by the response at G_5 where there is a 38.5% higher fecundity than the base population versus 24.5% with the same generation of the unselected Control line for *P. crassirostris*. Although such high response is not common for traits with moderate heritability such as fecundity, the number of eggs produced by calanoid copepods is substantially lower (i.e., < 50 per day) than most aquaculture species, which usually produce thousands to millions of eggs per spawning event (Gjedrem, 2005; Støttrup, 2006). Thus, even small increases in the egg production by copepods through selection would represent a large response. For species that produced a large number of eggs, the improvement in reproductive capacity is normally not a major concern relative to other traits (Gjedrem, 1985), whereas for copepods whose utilisation as a potential live feed for fish larvae is largely constrained by its low production level, improvement in fecundity is a major goal to overcome the disadvantage of low culture productivity (Drillet et al., 2011).

Whilst our selection goal was primarily egg production, positively correlated responses were also observed in other important copepod traits, including the size of eggs, nauplii and adult females. The size of eggs, nauplii and adult females of *P. crassirostris* were all significantly larger within the Select line compared to that of the Control line. The positive correlation between female size and egg production has been described previously in copepods and denotes that larger females (prosome size) have the capacity to produce more eggs (Campbell and Head, 2000; Smith, 1990). Therefore, although we were selecting for egg production, the positive genetic correlation with

female copepod size suggests that we were also indirectly selecting for larger females. Furthermore, the slight increase of 7% in first nauplii (NI) size might be the result of egg size increase.

Finally, the success in intensive culture of copepods rests on the selection of suitable species. The vast number of calanoid species (Mauchline, 1998), each adapted to various habitats, implies that there are vast possibilities for selection. Our study clearly illustrated that a simple selective breeding approach over a relatively short time frame can substantially enhance the reproductive productivity of *P. crassirostris.* These results are the first clear evidence of the effectiveness and high potential of selective breeding to significantly improve culture productivity of copepods for their use in aquaculture hatcheries.

5.5 Summary

The aim of this study was to evaluate the practicality of applying a simple selective breeding program to improve the reproductive capacity of the calanoid copepod *P. crassirostris.* Using a family selection program combined with a circular mating strategy, *P. crassirostris* was cultured for five generations. Females were selected for their high reproductive capacity, and a control chosen by random selection was maintained alongside. After five generations, the Select line exhibited a positive response for selection with a significant increase in total egg production of 24.5% over the female lifespan, with a calculated heritability (h^2) of 0.38. Selection also resulted in a significant increase in the size of female nauplii and eggs when compared to the Control line. Our results provide the first clear evidence of the effectiveness and high potential of selective breeding in enhancing the reproductive capacity of calanoid copepods. Furthermore, our results suggest that further investigations into improvement

of other life history traits can be implemented as a similar method for the improvement of culture productivity and other traits to generate suitable calanoid copepods for larval live food.

CHAPTER 6

DOMESTICATION AS A NOVEL OPPORTUNITY FOR IMPROVING CALANOID COPEPOD CULTIVATION

Copepods have been cultured successfully for some time; however, the implications of long-term cultivation or domestication on copepod life history traits have not yet been assessed. Therefore, this chapter evaluates the effects of domestication after multiple generations of controlled cultivation to determine if the domesticated and wild populations of *P. crassirostris* are phenotypically or physiologically different.

[4]Published as: Alajmi F, Zeng C, Jerry DR. Domestication as a Novel Approach for Improving the Cultivation of Calanoid Copepods: A Case Study with *Parvocalanus crassirostris*. PLoS ONE. 2015;10(7):e0133269.

6.1 Introduction

Previous research has demonstrated many difficulties in mass culturing calanoid copepods due to their low tolerance to changes in water quality (Payne and Rippingale, 2001a) and low culture productivity compared to other taxonomic groups (Støttrup, 2006). Recently, several calanoid species have been identified as great candidates for aquaculture, including the calanoids *Acartia tonsa*, *Acartia sinjiensis* and *P. crassirostris*, which have been cultured over multiple generations in laboratories and aquaculture facilities (Alajmi and Zeng, 2014; Camus and Zeng, 2009; Jepsen et al., 2007; Kline and Laidley, 2015). Despite the increased use of domesticated copepods, the implications of long-term cultivation or domestication on copepod life history traits has not been quantified to date.

Generally, domestication is characterized by genetic changes in behaviour, morphology, or physiology caused by cultivation under artificial conditions (Doyle, 1983). The benefits of domestication have been demonstrated for many aquaculture species, with improved productivity and tolerance to culture conditions resulting simply from culturing a species for several generations without direct selection (Coman et al., 2013; Zuberi et al., 2011). However, domestication could also result in loss of fitness, particularly if pedigrees are not adequately kept and inbreeding occurs. Furthermore, the effects of domestication are likely to accumulate over generations (Araki et al., 2007).

In comparison, copepod generation times are extremely short compared to most other cultured aquatic species (i.e., weeks). These multiple generations within a short time could result in either positive or negative phenotypic responses due to changes in environmental conditions. For example, inconsistent behavior and reproductive capacity was found between laboratory-cultured and wild *Acartia tonsa*, with more

eggs produced by laboratory-cultured copepods (Tiselius et al., 1995). Furthermore, significant differences in fatty acid profiles have been reported between captive and wild copepods (Parrish et al., 2012; van der Meeren et al., 2008). These changes in vital culture characteristics warrant further investigation into the effects of domestication that might have occurred over multiple generations.

The calanoid copepod *P. crassirostris* has been isolated from the wild and cultivated as a closed population at James Cook University, Queensland, for over 2 years. The short life cycle of *P. crassirostris* (≈18 days) theoretically means that more than 80 generations have passed since the James Cook University domesticated strain was collected from the wild. Because there was no intentional selection on the base population for any trait, this population offers a unique opportunity to evaluate the effects of domestication on a range of biological traits in comparison to wild copepod populations. To date, there is no information on the potential effects of domestication of copepods on traits relevant to culture productivity and utilization in aquaculture (i.e., reproductive capacity). Therefore, the aim of this experiment was to investigate the effects of domestication after multiple generations of controlled cultivation to determine if the domesticated and wild populations of *P. crassirostris* are phenotypically or physiologically different.

6.2 Materials and methods

6.2.1 Wild caught copepods

To ensure the same genetic stock was sampled as the domesticated strain, wild *P. crassirostris* were collected from the original location of the earlier collection in September 2013. Pure copepod cultures were maintained in 20-L polycarbonate

carboys filled with 1 µm filtered seawater (36±1) with gentle aeration. The culture temperature was kept at 26 ±1°C, and a photoperiod of 16:8 h light/dark cycle was maintained. The propensity of the culture salinity to increase due to evaporation was adjusted by adding dechlorinated distilled water if needed. Experiments did not commence until wild *P. crassirostris* completed at least one generation of breeding post-collection to ensure that the differences between the two stocks were not simply due to a lack of acclimatization to the experimental environment and/or stress.

6.2.2 Life history traits

The size of developmental stages, fecundity and lifespan of adult females were evaluated to determine the response to domestication. One hundred randomly collected individuals of each stage (eggs to adults) from each population were measured. Size measurements were made using a Leica DM LB microscope and an Olympus DP25 camera. Photos were analysed with Olympus DP2-BSW software. Fecundity and lifespan were evaluated using a design adapted from Alajmi et al. (2014). Pre-matured females were collected from each population at the final copepodite stage V (CV) and then individually isolated in petri dishes to be observed until sexual maturity was reached. Maturation was confirmed by the moult of the CV copepodites (Lawson and Grice, 1973). Each mature adult female was paired with an adult male (replicates, n = 10). Each pair was then cultured in a 200-mL vessel submerged in a beaker. A 74-µm mesh was attached to the bottom to ensure that the adults would remain inside the vessel while eggs would pass through and accumulate at the bottom of the beaker. The eggs that accumulated at the bottom of the beakers were counted following daily water exchanges with a Bogorov counting chamber and a dissecting stereo-microscope (Nikon SMZ645). Similarly, the lifespan of adult females was evaluated. The 200-mL

culture vessels were stocked with 10 pre-matured adults (n = 10) from each population and monitored every day for mortality. Dead copepods were removed daily. The experiment stopped when all copepods died.

6.2.3 Culture density

Three stocking density treatments of 1, 3 and 5 adults mL^{-1} were evaluated (n = 10) following the methods described in (Alajmi and Zeng, 2014). Stage V copepodites (CV) were randomly collected from stock cultures of the wild and domesticated populations and then cultured in 200-mL vessels at designated densities. The experiment started 24 h after stocking CV individuals to allow time for their development into adults (Alajmi and Zeng, 2015). Mean daily egg production was evaluated over 7 days of culture, while the mean daily mortality rate of adults was calculated over 5 days of culture using the following formula:

$$\text{Daily mortality \%} = \left[\frac{\text{No. of dead copepods found on a particular day}}{\text{Total surviving adults of the previous day}}\right] \text{x100}$$

6.2.4 Ingestion rate and faecal pellet production

To evaluate the ingestion rate, 30 recently matured females (n = 10) were cultured in 75-mL containers filled with fresh seawater (36±1) and T-Iso at 1000 µg C l^{-1}. Culture containers were rotated for 24 h on a plankton wheel at 1 rpm to maintain a constant suspension. Five control replicates without copepods were additionally maintained to estimate the cell growth coefficient k. After 24 h, all individuals were removed from the containers and mortality was calculated. Initial (C_0) and final (C_t) algae concentrations were calculated and the ingestion rate (I) was evaluated according to Marin et al. (1986) as follows:

$$F = \frac{V\left(\frac{\ln C_0 - \ln C_t}{t}\right) + k}{N}$$

$I = F \times C_0$

$k = \left(\frac{\ln C_0 - \ln C_t}{t}\right)$

F = clearance rate (µg C l^{-1} $female^{-1}$ h^{-1}); V = container volume (mL); N= number of individuals; C_0 = initial algae concentration (µg C l^{-1}); C_t = final algae concentration (µg C l^{-1}); t = time (h); k = cell growth coefficient (h^{-1}).

Faecal pellet production was measured using similar methods described for the collection of eggs (described in section 6.2.2). Recently matured females from wild and domesticated populations were individually cultured for the duration of their lifespan, and their daily faecal pellet production was counted.

6.2.5 Fatty acid analysis

To obtain an adequate sample for fatty acid analysis, *P. crassirostris* were cultured in 250-L tanks fed the designated diet treatments under similar conditions described for main stock cultures (described in section 2.2.2). *P. crassirostris* were harvested after 7 days of culture by filtering the content of the culture tanks through a 150-µm mesh followed by a 25-µm mesh. This process ensured the collection of second generation eggs, nauplii and early copepodites (I-III; $\overline{x}$ = 4.5x10^5). The selected size fraction represented the dominant component of the cultured population. The collected copepod samples (n = 3) were then flushed three times in 10‰ saltwater to remove salt and transferred to 2-mL vials for freeze drying.

Fatty acid methyl esters (FAMEs) were prepared by transesterification using 14% BF, (boron trifluoride/methanol, w/w*)*, following the method of Morrison and Smith (1964). FAMEs were quantified by an Agilent 6890 gas chromatograph (Agilent

Technologies, Santa Clara, CA) coupled to a flame ionization detector, with an Omegawax 320 fused silica capillary column (30 m x 0.32 mm; Supelco, Bellefonte, PA). The injector and detector temperature was maintained at 260 °C. The column temperature was initially set to 60 °C, then increased at a rate of 50 °C min^{-1} to 170 °C, followed by an increase at a rate of 2 °C min^{-1} to 180 °C, and was then held for 2 min. The temperature was further increased at the same rate of 2 °C min^{-1} to 230 °C and held for 1 min before another increase at a rate of 1 °C min^{-1} to the final temperature of 240 °C, until all FAMEs had been eluted. The total time was 46.2 min. The carrier gas was helium with a flow velocity of 25 cm s^{-1}. Peaks were identified by comparing retention times with known standards (Nu-Chek-Prep Inc, Elysian, MN). Fatty acid contents were expressed as a percentage of a particular fatty acid to the total fatty acids (%).

6.2.6 Statistical analysis

Data are presented as the mean ± standard error (SE), except for fatty acid composition data (mean ± standard deviation). All data collected were tested for normality and homogeneity of variance prior to mean comparison procedures, and data expressed in percentages were arcsin-transformed prior to analysis. Size measurements, ingestion rates and fatty acid composition data were compared using one-way ANOVA, while egg and faecal pellet production data were analysed with repeated-measures ANOVA. When significant differences (p <0.05) were found, Tukey's multiple comparison test was performed to determine specific differences among treatments. All statistical analyses were conducted using Sigmaplot, version 11.

6.3 Results

6.3.1 Life history traits

Table 6.1 summarizes the size measurements of domesticated and wild developmental stages. Generally, size over all developmental stages was significantly different between strains (p <0.001), with domesticated individuals being significantly larger than the wild strain.

Table 6.1: Mean length (µm) of developmental stages of domesticated and wild *P. crassirostris*.

	Strain	
Stage	**Domesticated**	**Wild**
Egg	71.0 ± 0.4[a]	66.9 ± 0.4[b]
Nauplii I	80. ± 0.6[a]	76.8 ± 0.5[b]
Nauplii II	87.4 ± 0.4[a]	83.5 ± 0.5[b]
Nauplii III	112.1 ± 0.4[a]	106.3 ± 0.4[b]
Nauplii IV	134.8 ± 0.8[a]	126.1 ± 0.9[b]
Nauplii V	155.6 ± 0.8[a]	150.0 ± 0.9[b]
Nauplii VI	172.7 ± 0.7[a]	165.9 ± 0.8[b]
Copepodite I	195.4 ± 0.6[a]	185.1 ± 0.6[b]
Copepodite II	257.1 ± 1.1[a]	245.0 ± 1.2[b]
Copepodite III	299.7 ± 1.7[a]	290.2 ± 1.7[b]
Copepodite IV	338.9 ± 1.1[a]	332.0 ± 1.2[b]
Copepodite V	389.8 ± 1.1[a]	381.2 ± 1.3[b]
Adult male	384.1 ± 0.8[a]	345.0 ± 0.9[b]
Adult female	444.7 ± 2.9[a]	432.6 ± 2.5[b]

Lengths of copepodites and adults were measured as the prosome length only. Data are presented as mean±S.E (n = 100). Superscript letters indicate significant differences (p < 0.05).

Within each strain, *P. crassirostris* adult females displayed a similar daily egg production pattern over their lifespan. On any given day, the domesticated female's daily egg production was significantly higher than the wild strain (p <0.001; Fig. 6.1a).

The domesticated females continued to produce eggs on day 8, whereas all females from the wild strain ceased egg production on day 7. The mean total egg production over a female's lifespan for the domesticated strain was significantly higher compared with the wild strain at 112.3 ± 1.8 vs. 64.6 ± 3.3 eggs female^{-1}, respectively (p <0.001; Fig. 6.1b). Thus, when compared to the wild strain, the domesticated copepods produced 73.8% more eggs per female.

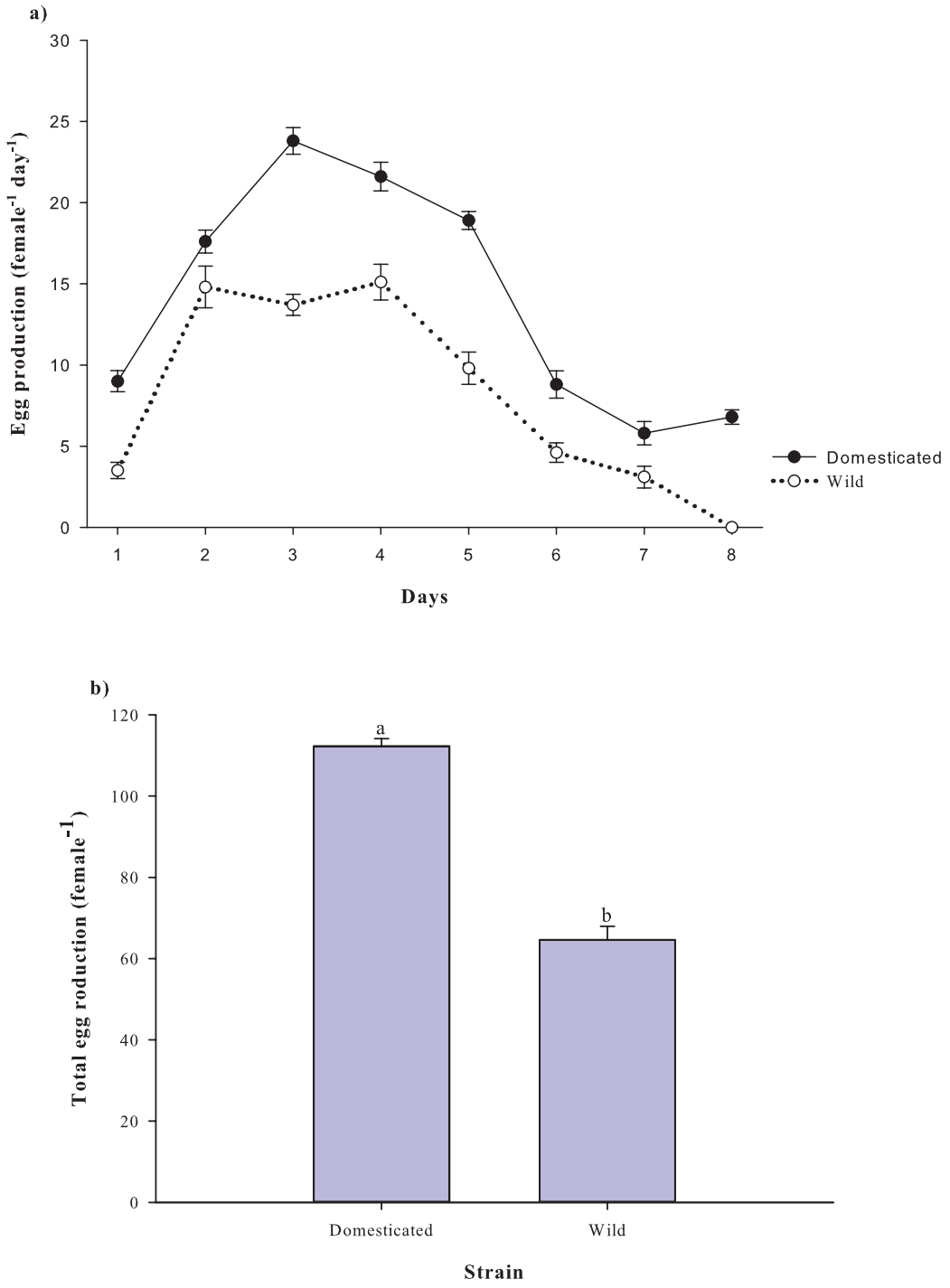

Figure 6.1: Egg production of *P. crassirostris* (female^{-1}) strains (n = 10). a) Mean daily egg production female^{-1} over 8 days; b) mean total egg production female^{-1} over 8 days. Data are presented as the mean ± standard error. The different letters on the tops of the bars indicate significant differences (p <0.001).

The mean female lifespans of the *P. crassirostris* strains were significantly different (p <0.001; Fig. 6.2). Domesticated females had a longer lifespan (8.8 ± 0.1 days), while wild females lived a day less (7.5 ± 0.1 days).

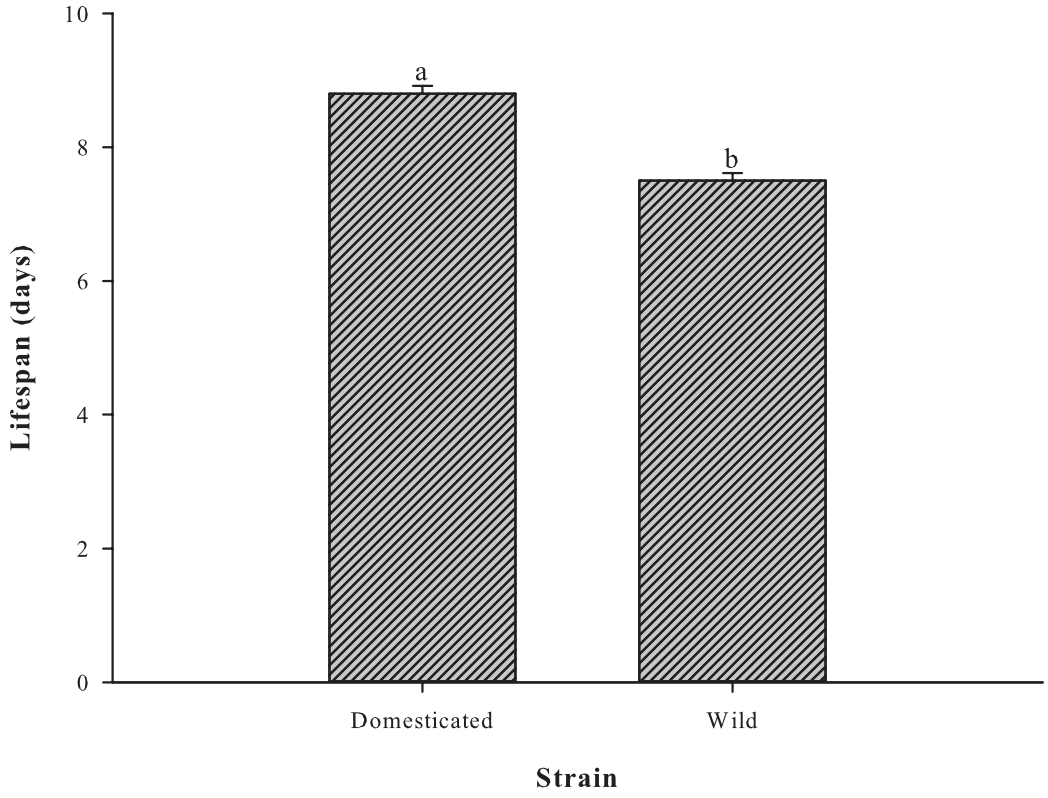

Figure 6.2: Adult female lifespan of *P. crassirostris* strains (n = 10). The different letters on the tops of the bars indicate significant differences (p <0.001).

6.3.2 Culture density

Figure 6.3 shows the mean daily egg production of *P. crassirostris* over 7 days at different initial stocking densities. The differences in mean daily egg production were highly significant between strains at different density treatments (p <0.001). Two very different patterns were demonstrated between the strains; the first pattern was characterized by a low mean egg production on the first day but increased sharply on

day 2, with a peak production between days 2 and 5. A steep decrease followed on days 6 and 7, although egg production was still found on day 7. This pattern was shown by the domesticated strain at stocking densities of 1, 3 and 5 adults mL^{-1}. The second pattern was characterized by steady and substantially lower mean egg production from days 2 to 5, followed by a significant drop in mean egg production on day 6 and the termination of all egg production after day 6. This pattern was shown by the wild strain at stocking densities of 1, 3 and 5 adults mL^{-1}.

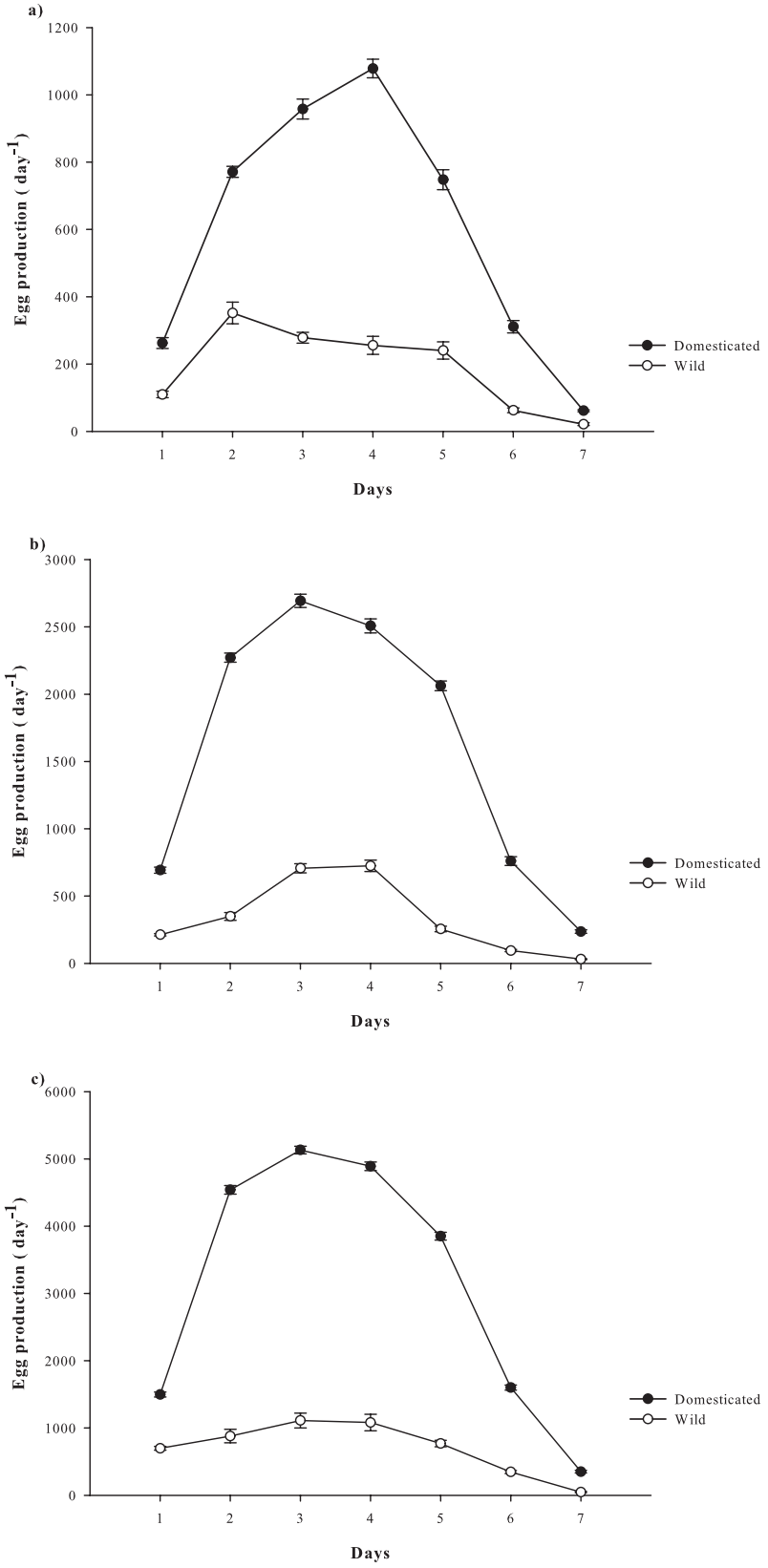

Figure 6.3: Mean daily egg production of domesticated and wild *P. crassirostris* strains cultured at initial densities of (a) 1, (b) 2 and (c) 3 mL^{-1} (n = 10). Data are presented as mean ± standard error.

Mean total egg production over 7 days was also significantly different across different stocking densities (p >0.001; Fig. 6.4). The domesticated females at all stocking densities produced significantly more total eggs than the wild strain (p <0.001). The domesticated strain produced 4189.8 ± 61.2, 11224.0 ± 71.7 and 21860.6 ± 103.6 eggs at 1, 3 and 5 adults mL^{-1}, respectively, while the wild strain produced 1319.5 ± 54.3, 2374.5 ± 80.9 and 4933.8 ± 269.5 eggs at 1, 3 and 5 adults mL^{-1}, respectively.

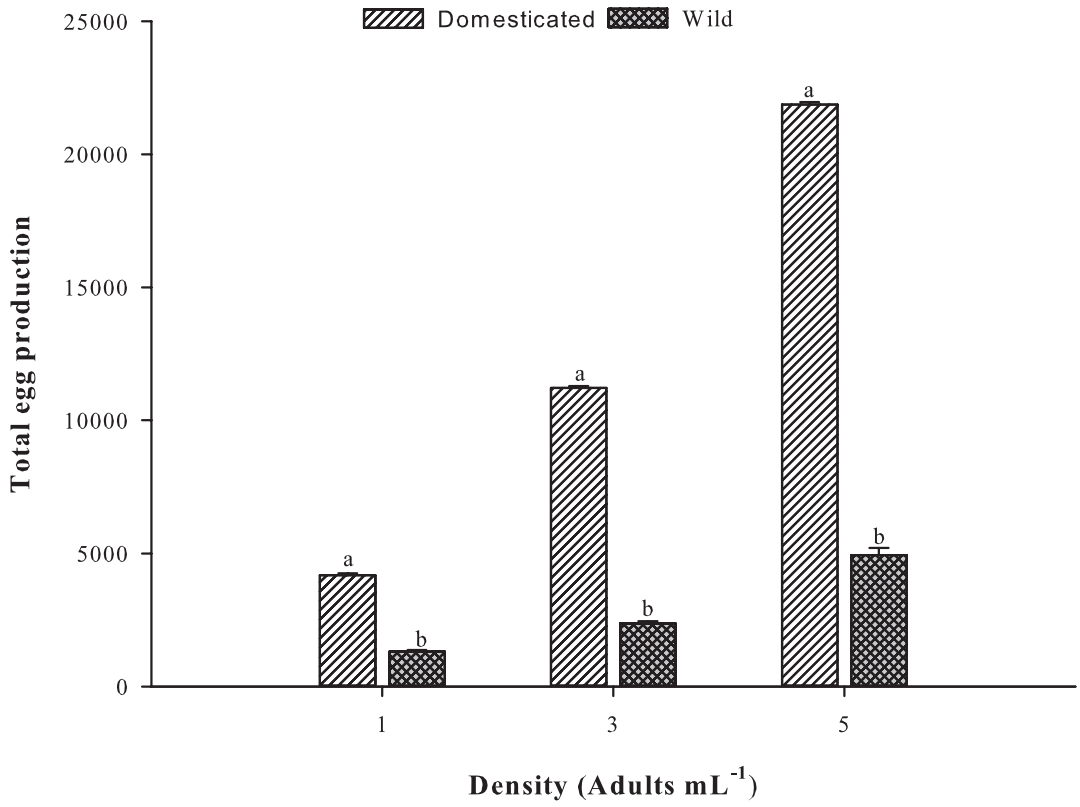

Figure 6.4: Mean total egg production of *P. crassirostris* strains under different initial adult stocking densities (n = 10). The different letters on the tops of the bars indicate significant differences (p <0.001).

The mean daily mortality rates were significantly different between the two strains (p <0.001; Fig. 6.5). While the mean daily mortality rate of the domesticated strain remained very low for the 1, 3 and 5 adults mL^{-1} treatments (5.6 ± 0.3, 5.6 ± 0.1 and 5.5 ± 0.3%, respectively) and no significant differences were detected between all densities (p >0.05), the wild strain had a significantly higher mean daily mortality rate across all densities (p <0.001). The wild strain's mean daily mortality rates were 22.9 ± 1.6, 29.8 ± 1.2 and 31.3 ± 1.3% at 1, 3 and 5 adults mL^{-1}, respectively, with no differences between treatments (p >0.05).

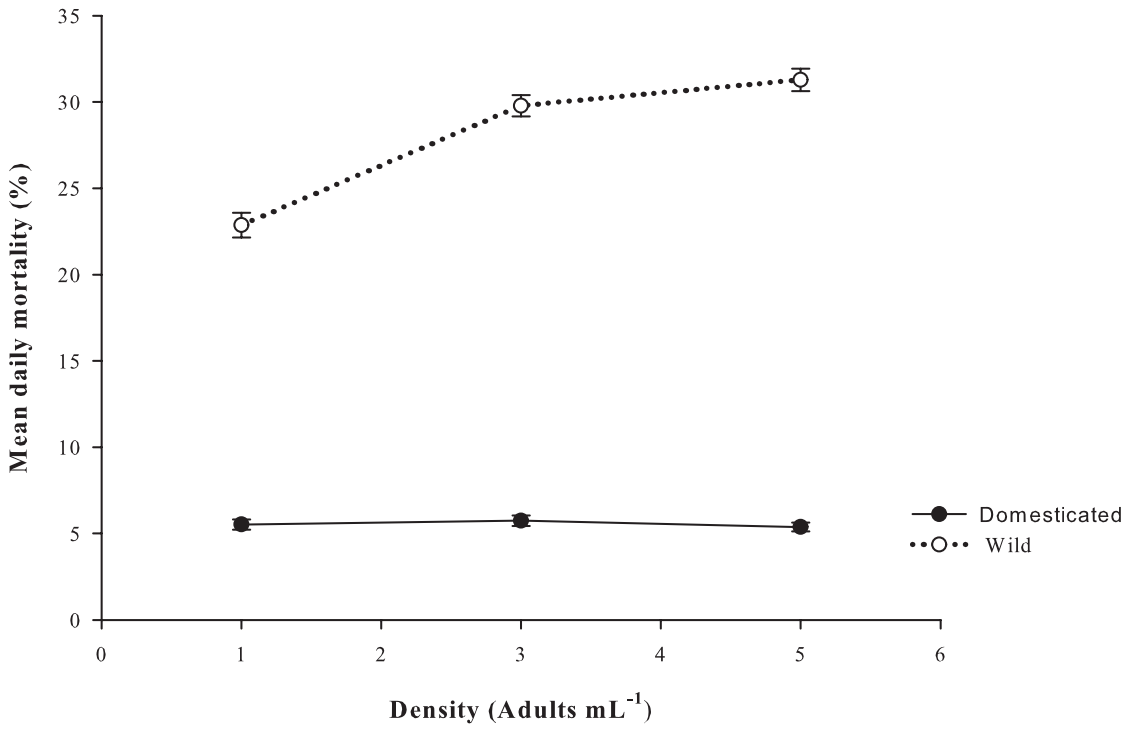

Figure 6.5: Mean daily mortality rate (%) of *P. crassirostris* strains under different initial adult stocking densities (n = 10). Data are presented as the mean ± standard error.

6.3.3 Ingestion rate and faecal pellet production

Ingestion rates obtained at an average food concentration of 1000 µg C l^{-1} were significantly different between the two strains (p <0.001; Fig. 6.6). The domesticated strain had a significantly higher ingestion rate at 888.4 ± 9.9 ng C $female^{-1}$ h^{-1} compared to 775.3 ± 11.2 ng C $female^{-1}$ h^{-1} for the wild strain (p <0.001). The domesticated strain grazed more efficiently compared to the wild strain, as depicted by a higher clearance rate of 0.9 ± 0.0 mL $female^{-1}$ h^{-1} compared to 0.8 ± 0.0 mL $female^{-1}$ h^{-1} (p <0.001).

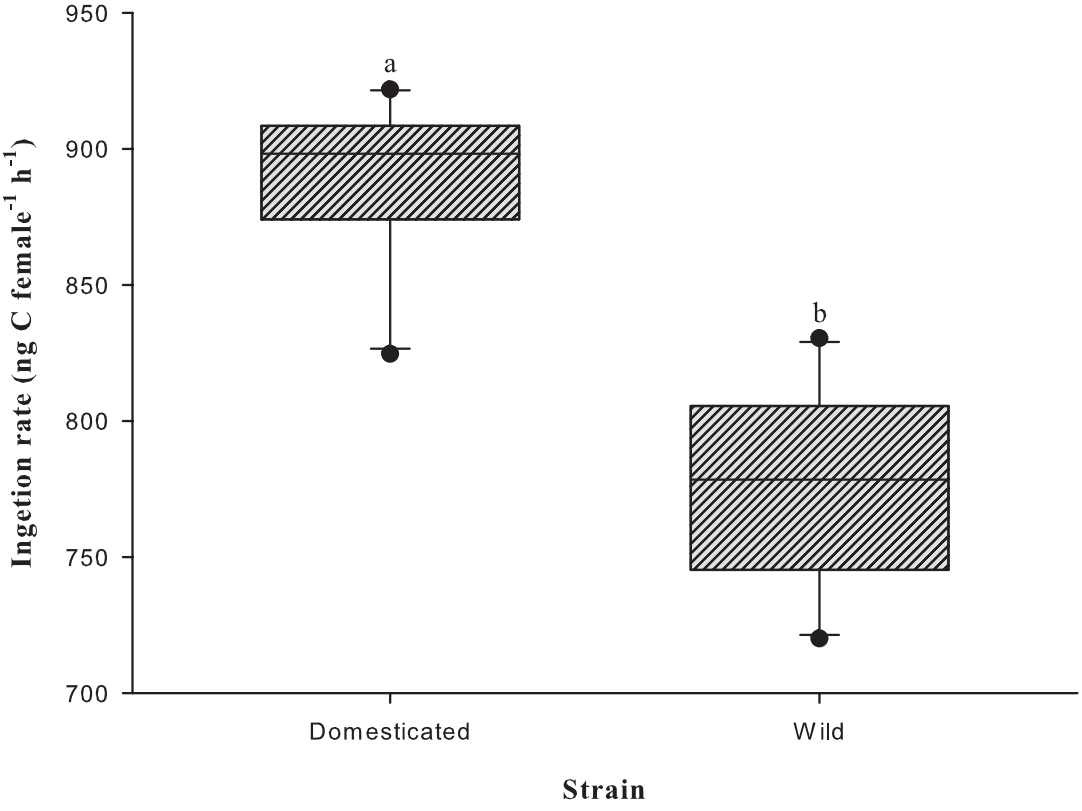

Figure 6.6: Mean ingestion rate (ng C $female^{-1}$ h^{-1}) of *P. crassirostris* strains (n = 10) at an average food concentration of 1000 µg C l^{-1}. The different letters on the tops of the boxes indicate significant differences (p <0.01).

Mean daily faecal pellet production (female^{-1} day^{-1}) was not significantly different between strains (p >0.05; Fig. 6.7a), at 30.1 ± 1.2 and 31.7 ± 1.7 female^{-1} day^{-1} for the domesticated and wild strain, respectively. Furthermore, the difference in the total faecal pellet production (female^{-1}) between strains was not statistically significant (p >0.05; Fig. 6.7b). The mean total faecal pellets produced were 241.1 ± 10.2 and 253.5 ± 14.2 female^{-1} for the domesticated and wild strains.

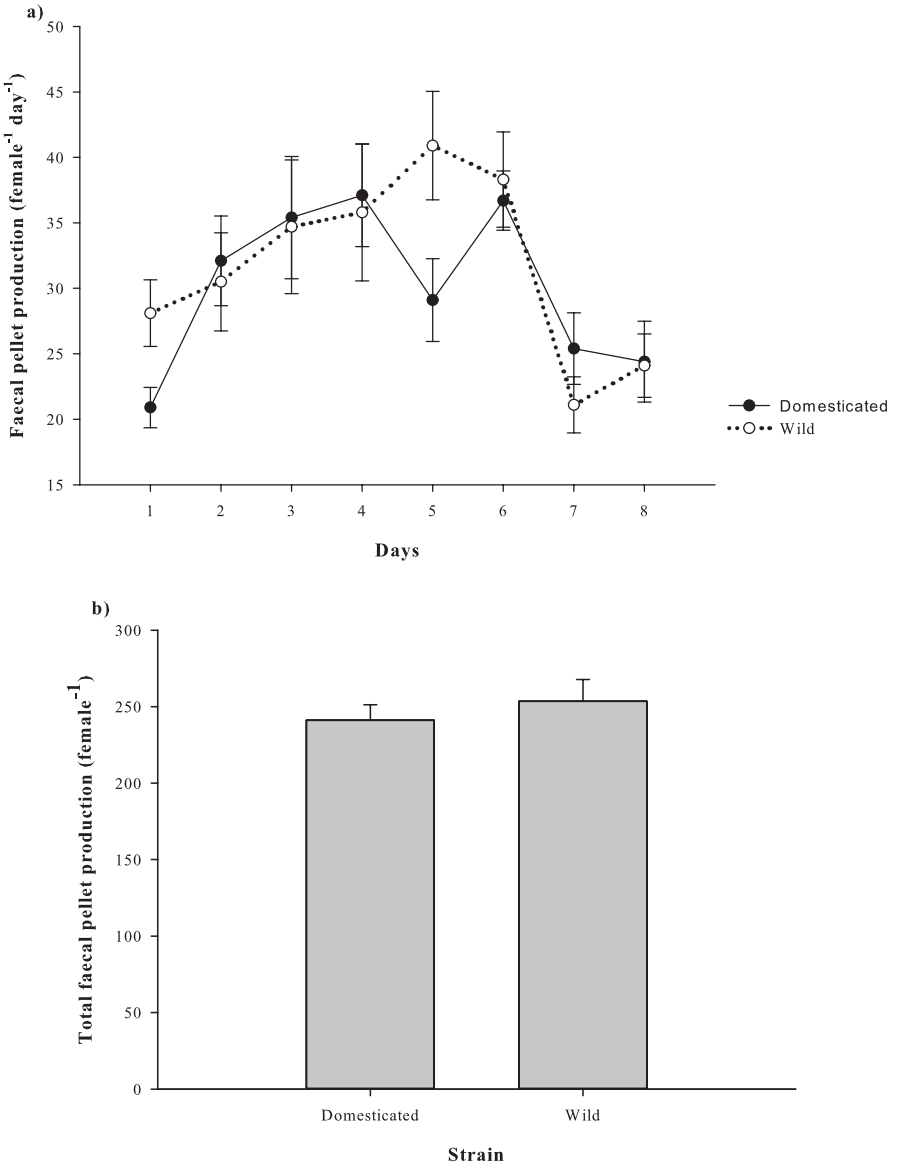

Figure 6.7: Faecal pellet production (female^{-1} day^{-1}) of *P. crassirostris* strains (n = 10). a) Mean daily faecal pellet production female^{-1} over 8 days; b) mean total faecal pellet production female^{-1} over 8 days. Data are presented as the mean ± standard error.

6.3.4 Fatty acid analysis

Table 6.2 summarizes the fatty acid composition of *P. crassirostris* strains. The domesticated copepods had significantly higher levels of C18:2ω6 (4.7±0.1%) and C18:3ω3 (3.3±0.1%) than the wild copepods (3.2±0.0 and 2.3±0.1%, respectively), while C20:4ω6 levels were not significantly different between strains. Domesticated copepods had significantly higher levels of EPA and DHA (5.4±0.1 and 22.3±0.3%, respectively) than the wild copepods (2.7±0.1 and 6.1±0.1%, respectively). LC-PUFA levels were significantly higher in the domesticated copepods (36.2±0.4%) compared to wild copepods (16.1±0.1%).

Table 6.2: Fatty acid composition of domesticated and wild cultured strains of *P. crassirostris* fed *Isochrysis* sp. (mean % of total fatty acids ± SD).

	Strain		
Fatty acids	**Domesticated (*n*=3)**	**Wild (*n*=3)**	**ANOVA (*p* value)**
C14:0	8.8±0.1	6.7±0.1	.000
C15:0	0.6±0.1	0.7±0.0	.212
C16:0	16.5±0.1	20.1±0.1	.000
C18:0	5.2±0.1	7.8±0.1	.000
C22:0	0.4±0.0	0.3±0.0	.001
C23:0	0.5±0.0	1.1±0.0	.000
C24:0	0.4±0.0	0.7±0.0	.000
C14:1ω7	1.0±0.0	0.5±0.0	.000
C16:1ω7	4.5±0.1	4.2±0.1	.025
C16:1ω5	0.5±0.0	0.4±0.0	.011
C18:1ω9	7.1±0.1	11.3±0.1	.000
C18:1ω7	3.5±0.1	3.1±0.1	.007
C20:1ω9	0.1±0.0	0.3±0.0	.001
C20:1ω7	0.2±0.0	0.6±0.0	.000
C16:2ω4	0.7±0.0	4.4±0.0	.000
C16:3ω4	1.2±0.0	0.6±0.0	.011
C18:2ω6 (LA)	4.7±0.1	5.4±0.1	.000
C18:3ω6	0.2±0.0	1.2±0.0	.000
C18:3ω3 (LNA)	3.3±0.1	1.2±0.0	.000
C18:3ω4	6.6±0.2	1.5±0.0	.000
C18:4ω3	0.3±0.0	0.8±0.0	.000
C20:2ω6	0.2±0.0	0.6±0.0	.000
C20:3ω6	0.7±0.0	4.2±0.1	.000
C20:4ω6 (ARA)	0.5±0.1	0.7±0.0	.417
C20:3ω3	0.4±0.0	0.3±0.0	.007
C20:4ω3	1.0±0.0	0.2±0.0	.000
C20:5ω3 (EPA)	5.4±0.1	2.7±0.1	.000
C22:2ω6	0.5±0.0	2.6±0.1	.000
C22:5ω3	2.1±0.1	9.6±0.1	.000
C22:6ω3 (DHA)	22.3±0.3	6.1±0.1	.000
ΣSFA	32.4±0.3	37.3±0.2	.000
ΣMUFA	2.2±0.0	20.4±0.2	.000
ΣPUFA	50.2±0.5	42.2±0.2	.000
ΣLC-PUFA	36.3±0.4	16.1±0.1	.000
DHA/EPA	4.1±0.1	2.3±2.2	.000

6.4 Discussion

The present study is the first to investigate the effects of domestication on the calanoid copepod *P. crassirostris* after 2 years of continuous culture. We observed a significant improvement in the reproductive capacity of the domesticated copepods compared to the wild copepods, with almost a 2-fold increase in egg production. In addition, the domesticated strain had a higher tolerance for different culture environments than wild copepods, as demonstrated by the low mortality rate and unaffected egg production at different culture densities.

Domestication often leads to improved adaptability to culture environments of cultured species and the enhancement of traits related to culture productivity (Liao and Huang, 2000). The current results coincide with the previous statement, as reproductive capacity of the domesticated strain exceeded that of the wild strain. Tiselius et al. (1995) reported similar results when comparing laboratory-cultured *Acartia tonsa* to wild-caught specimens, which produced only half as many eggs. However, the study compared adult females of various ages, which could have affected those results (Parrish and Wilson, 1978). A positive correlation between female size and egg production has been described previously in copepods and indicates that larger females (prosome size) have the capacity to produce more eggs (Campbell and Head, 2000; Smith, 1990). However, the increase in female prosome length of the domesticated strain (present study) compared to the wild strain was only 2.7%, which could not have solely accounted for the increase in egg production. Moreover, during a selective breeding program designed to increase the reproductive capacity of *P. crassirostris*, (Alajmi et al., 2014) found that selected females had a 10% increase in prosome length with an increase in egg production of 24.5%. This report suggests that the increase in prosome length played a small role in the current experiment. The heightened egg

production by the domesticated strain in the present study is likely due to improvements in oogenesis, which could be related to adaptations to the controlled culture environment (i.e., food satiated environment) (Niehoff, 2007). Interestingly, domesticated males were found to be larger than males of the wild strain. This was not observed in the previous chapter (selective breeding), which indicates that change in males' size took place after many generations (>5 generations). However, the possibility that size in domesticated males had reached its biological limit as no more possible increase could be achieved (Gjedrem, 2005), thus, explaining the observations during the selective breeding experiment in males size.

Culture density is an essential parameter that is often described as the "bottleneck" in a cost-effective implementation of copepods in aquaculture (Alajmi and Zeng, 2014). The present culture density results are very promising, considering the obvious variation between both strains. The domesticated copepods sustained a higher culture density than the wild copepods. After a period of acclimatization, the wild *P. crassirostris* strain had a lower tolerance to the culture environment compared to the domesticated strain. This finding indicates that calanoid copepods require multiple generations of culture to reach their full potential to adjust to different culture environments. Hence, future studies on copepod culture density should reference the history of stock cultures.

Although no significant differences were found between strains when comparing faecal pellet production, the ingestion rate of the domesticated strain was higher than the wild strain. The higher ingestion rate by the domesticated copepods was achieved by adaptation to the offered food, while in nature, seasonality and food concentration is suggested to play a major role in copepod ingestion rate (Huskin et al., 2000). The change from diverse food particles with various sizes to a monospecific diet

could also explain the less efficient ingestion rate of the wild strain (Mayzaud et al., 1998).

Fatty acids are essential to the health and wellbeing of fish larvae (Sargent et al., 1999). The quantity and quality of essential fatty acids are vital for the development of fish larvae (Hamre et al., 2013). Copepods, as the natural prey of most fish larvae, provide a valuable source of essential fatty acids (Conceição et al., 2010). However, the fatty acid profile of cultured copepods can vary significantly (Parrish et al., 2012; van der Meeren et al., 2008). Our results showed an apparent difference between the domesticated and wild strain's fatty acid profiles. Although the wild strain was acclimatized to the same diet for one generation, the levels of essential fatty acids were lower than the domesticated strain. The lower levels of LC-PUFAs found in the wild strain fatty acid profile could be a reflection of their assimilation capacity (Mayzaud et al., 1998). Nonetheless, the diet shift and stress caused by a new environment requires energy, which will rapidly change the fatty acid profile (Kreibich et al., 2008). The enhanced nutritional value of domesticated copepods is a good investment, as the improvement in the fatty acid profile of domesticated copepods gives merit to long-term cultivation programs.

In conclusion, this study found that reproductive capacity and tolerance to the culture environment of the domesticated calanoid *P. crassirostris* has improved significantly. This improvement presents a new domain in the development of copepod culture techniques, as domestication could be a step towards the development of new copepod strains that require many generations to reach a balanced equilibrium under culture conditions. Developing copepod strains through strain selection and selective breeding could be coupled with domestication to produce a copepod strain that can be utilized by aquaculture hatcheries in the future.

6.5 Summary

The aim of this chapter was to determine if the domesticated and wild populations of *P. crassirostris* are phenotypically or physiologically different. Wild-caught *P. crassirostris* were compared to a long-held domesticated strain (>2 years) for size of developmental stage, fecundity and lifespan of adult females, culture density, ingestion rates, faecal pellet production and fatty acid profiles. The domesticated strain was significantly different from the wild strain in size (eggs, nauplii, copepodites and adults were larger in the domesticated strain), egg production (112.3 ± 1.8 eggs female^{-1} vs. 64.6 ± 3.3 eggs female^{-1}) and adult female lifespan (8.8 ± 0.1 days vs., 7.5 ± 0.1 days). At 1, 3 and 5 adults mL^{-1}, the domesticated strain performed significantly better than the wild strain in egg production (4189.8 ± 61.2, 11224.0 ± 71.7 and 21860.6 ± 103.6 eggs vs. 1319.5 ± 54.3, 2374.5 ± 80.9 and 4933.8 ± 269.5 eggs, respectively) and mean daily mortality rate (5.6% across all densities vs. 22.9 ± 1.6, 29.8 ± 1.2 and 31.3 ± 1.3%, respectively). The domesticated strain had significantly higher ingestion rates than the wild stain (888.4 ± 9.9 ng C l^{-1} and 775.3 ± 11.2 ng C l^{-1}, respectively), while faecal pellet production was not significantly different between strains. Fatty acid profiles indicated higher levels (as % of total fatty acid) of long-chain polyunsaturated fatty acids in the domesticated strain (36.2±0.4%) than the wild strain (16.1±0.1%). Overall, this chapter found that the reproductive capacity and tolerance to the culture environment of the calanoid *P. crassirostris* have improved significantly due to domestication.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

The present research demonstrated the great potential of the calanoid copepod *P. crassirostris*. The tropical calanoid species is globally found (Bradley et al., 2013; McKinnon and Klumpp, 1997a; Rong et al., 2002), and compared to other calanoid species, *P. crassirostris* is relatively easy to culture and can be cultured at high densities. This species nauplii were reported to be essential in larval first feeding of the flame angelfish *Centropyge loriculus*, bluefin trevally *Caranx melampygus*, and red snapper *Lutjanus campechanus* (Shields et al., 2005).

The findings of the current research represent a significant advance in the development of tropical calanoid copepods culture techniques for aquaculture. Particularly, through novel techniques of domestication and/or selective breeding that were very promising as demonstrated for the first time in this study and could lead to multiple new opportunities in the future. Moreover, *P. crassirostris* had been continuously cultured over the past 3 years for the present study, demonstrating *P. crassirostris* high potential of being implemented as a widely used live food for commercial aquaculture hatcheries in the near future.

7.1 Major findings of this thesis

7.1.1 Diet requirement and alternative diets

Chapter 2 and 3 focused on diet requirements of *P. crassirostris*. Results of chapter 2 showed that among 9 different algal diets examined, the reproductive capacity of *P. crassirostris* was maximized when a combination of the prymnesiophyte *Isochrysis* sp. and the diatom *Chaetoceros muelleri* (1:1) were used. However, a monoalgal diet of *Isochrysis* sp. was also sufficient to maintain a good reproductive yield, which represents a major advantage for aquaculture hatcheries since this could substantially reduce the costs for maintaining multiple algae species.

Despite live *Isochrysis* sp. shown to be an excellent diet for *P. crassirostris,* a very low reproductive performance was recorded when the copepod was fed a commercial *Isochrysis* sp. paste. A series of experiments were hence designed to identify the causes leading to that failure. These hypotheses were: I) Poor copepod culture productivity was largely attributed to low food availability caused by settlement of dead algae cells of pastes (Milione and Zeng, 2007); II) Copepod low reproductive capacity and survival were due to inefficient feeding caused by fouling algae cells of pastes to their body, particularly, to their swimming and mouth appendages (Puello-Cruz et al., 2009); and III) copepod inferior performance was a result of the low digestibility and nutritional value of dead algae cells (Milione and Zeng, 2007). The results rejected the first hypothesis, while demonstrating that the fouling of copepod body parts, particularly the feeding and swimming appendages, by algal cells from the paste led to low feeding efficiency, significantly reducing egg production and survival. Therefore, fouling was the main reason behind the unsuccessful use of the commercial algae paste for copepod cultivation. Meanwhile, low digestibility and nutrition value of dead cells from the paste were likely to contribute to that failure. Importantly, the use

of cold stored *Isochrysis* sp. yielded comparable results to the live algae, which was very promising, indicating that algae paste products could still be viable alternatives to live algae for copepods cultivation as long as the production procedure taking into consideration the special feeding requirements of copepods.

7.1.2 Culture density

The sustainable culture density of calanoid copepods is generally below 2 ind. mL^{-1} (chapter 4). The results of the density experiment from chapter 4 revealed that *P. crassirostris* is a very promising species due to the high sustainable density of >5 ind. mL^{-1} achieved. Larger calanoids such as *Acartia tonsa* and *Acartia sinjiensis* were found to be more productive at densities below 2 ind. mL^{-1}, as a negative correlation between species size and density could be extrapolated from the literature (Camus and Zeng, 2009; Medina and Barata, 2004). Cannibalism and hydrodynamic disturbance are believed to be the main factors regulating population density for calanoids (Drillet et al., 2014; Jiang and Osborn, 2004). For *P. crassirostris*, cannibalism was not observed even at high stocking densities (chapter 4) while hydrodynamic disturbance was likely not to be an issue when compared to larger calanoids (e.g. *Acartia* sp.) due to *P. crassirostris* small size (Jiang and Osborn, 2004), which may explain why *P. crassirostris* can be cultured at much higher density than other calanoids.

In fact, while the sustainable culture density in the laboratory was 7 ind. mL^{-1} in 20 L carboys, in larger scale cultures of 250 L tanks densities between 12 and 17 ind. mL^{-1} have been regularly recorded. This demonstrates the high potentials of large-scale culture, in which advance techniques, such as recirculating culture systems and automatic harvesting, could be implemented to enhance culture management (Buttino et al., 2012; Carotenuto et al., 2012; Payne and Rippingale, 2001a; Toledo et al., 2005; VanderLugt and Lenz, 2008).

7.1.3 Selective breeding and domestication

Although optimizing culture conditions can play a significant role in enhancing culture productivity of copepods, there is a limitation on what that might achieve. Therefore, improvements through genetic manipulations, which have been shown to be highly successful in many cultured species (Argue et al., 2002; Dove and O'Connor, 2012; Gjedrem et al., 2012; Jerry et al., 2005), could be the next step for copepod culture improvement, although to date research in this area appears to be largely void.

Selective breeding is a common technique utilized to improve culture productivity of many aquatic species (Argue et al., 2002; Dove and O'Connor, 2012; Gjedrem et al., 2012; Jerry et al., 2005). However, the investigation shown in chapter 5 represented the first evidence of significant improvement in fecundity of a calanoid copepod through selective breeding. The significant increase in the reproductive capacity of *P. crassirostris* over only 5 generations of selecting is encouraging and suggests that the technique could also be explored on other calanoid species targeted for aquaculture use. Additionally, the simple selection method adopted in chapter 5 could easily be adapted to develop other desirable traits for cultured copepods. Aside from targeted selective breeding programs, the effects of continuous cultivation over multiple generations or domestication also often leads to enhanced genetic traits for cultivation. In chapter 6, *P. crassirostris* that had been reared for over 2 years (>100 generation) was compared to wild-caught *P. crassirostris* over a range of biological parameters. The results showed that acclimatization to culture environment had led to the significant improvement of egg production and tolerance to high culture density. Such finding is again, a first for copepods and represents a valuable opportunity for the aquaculture industry. Suggesting that maintaining a domesticated stock culture of

copepods in laboratories or hatcheries is worthwhile and could represent a new approach for improving their culture productivity.

7.2 Future direction

The knowledge base for candidate copepods to be used in aquaculture is still small compared to rotifers and *Artemia*, the traditional hatchery feeds. The area of study is well young, yet significant progresses have been made over recent decades. The diversity of calanoid species found in marine environments is very large (Mauchline, 1998), and represents an opportunistic pool for candidate selection. Such a huge pool of candidate species holds great potentials but more research efforts are obviously needed.

As demonstrated by this study, in addition to traditional research focusing on generating general culture protocols and optimizing specific culture conditions for each candidate species, increased research on exploring new domains such as selective breeding and domestication, might prove effective in enhancing copepod culture productivity, eventually leading to more common use of copepods in aquaculture.

Finally, advances in the development of culture techniques for copepods from laboratory studies require results being tested in large-scale cultures to confirm their feasibility. Commercial up-scaling is the ultimate goal for such research and undoubtedly, the results generated in laboratory studies will need modification and adaptation to suit commercial conditions. Meanwhile, other technical advances, such as culture automation and harvesting with the aid of computers, also needs to be explored and developed. These advances could improve culture efficiency and reduce the costs of copepod cultivation, which will improve copepod utilization in aquaculture hatcheries.

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