The improvement of copepods intensive culture protocols as live feeds for aquaculture hatcheries

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Statement on the Contribution of Others

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ABSTRACT

To date, aquaculture hatcheries are mostly dependent on the production of rotifers (*Brachionus* spp.) and brine shrimp (*Artemia* spp.) as a source of live prey for the rearing of early marine larvae. However, these traditional live feeds have been repeatedly proven inappropriate for an expanding list of marine species, including numerous important commercial and ornamental species. As a result, hatchery production of many marine larvae is uneconomical or impossible using either rotifers or *Artemia*, which puts significant limitations on commercial larviculture. The identification of alternative live prey items that do not have the inadequacies of traditional live feeds is hence of critical importance to increase the variety and survival of species cultured commercially and to insure the long-term growth and economic performance of the aquaculture industry.

Marine copepods are the most abundant metazoans throughout the world's ocean and constitute the majority of plankton biomass in the epipelagic zone. Their importance as natural prey items, in addition to their ubiquitous distribution in marine systems, makes them valuable live feeds candidates for marine hatcheries, and their superiority over traditional live feeds is well established in the literature. Among copepods, families from the genus calanoid possess fully pelagic developmental stages and are considered to have the best potential as live prey.

Although copepod culture technology has gain a great deal of interest over the last 30 years, most intensive culture protocols are still in their experimental phases and only a few species of calanoids are routinely mass cultured as food for larviculture. The present study focused on improving our knowledge of intensive culture techniques for calanoid copepods, in order to help

realize their full potential as live feeds for commercial larviculture. A series of experiment was conducted with *Acartia sinjiensis* and *Bestiolina similis*, two calanoid copepods with excellent potential as live prey for marine larvae, and recommendations were made to optimize their intensive culture protocol.

This thesis consists of 8 chapters. Following a general introduction (chapter 1) and a general materials and methods section (chapter 2), the subsequent experimental chapters are comprised of two parts: Chapters 3, 4 and 5 focuses on improving culture techniques for *Acartia sinjiensis,* while Chapters 6 and 7 are focused on ameliorating culture methods for *Bestiolina similis*. The final chapter (Chapter 8) summarizes the main results from all of the data chapters and provides recommendations on potential application in aquaculture hatchery settings.

In Chapter 3, the influence of photoperiod on the productivity of the calanoid copepod *Acartia sinjiensis* was assessed. The effects of various photoperiod regimes (Light:Dark = 0:24; 6:18; 12:12; 18:6 and 24:0h) on important productivity-related parameters in cultures of *Acartia sinjiensis* were evaluated. Photoperiod significantly affected *A. sinjiensis* productivity overtime, and a clear trend of increasing daily egg production with longer illumination period was observed. Adult sex ratio was the only parameter that was not significantly influenced by photoperiod. Based on results from this chapter, a photoperiod of 18L:6D is recommended to improve *A. sinjiensis* intensive culture protocol.

In chapter 4, the effects of adult stocking density (125, 250, 500, 1000 and 2000 adults l^{-1}) over a wide range of parameters relating to *Acartia sinjiensis* productivity in culture were investigated in a series of laboratory experiments. *A. sinjiensis* adult cannibalism, population growth and 48h hatching success were all significantly affected by adult stocking density, while 96h hatching

success and daily female egg output were not. *A. sinjiensis* was found to readily tolerate being cultured in important stocking density when compared to other calanoid species. A stocking densities as high as 2000 adults l^{-1} is recommended for its intensive cultivation.

As cannibalism was determined to be a potential drawback in *Acartia sinjiensis* intensive cultivation in chapter 4, a detail assessment of cannibalism under intensive culture conditions was conducted in chapter 5. With respect to predators, cannibalism in adult males was negligible and significantly lower when compared to adult females cannibalism rates. With respect to prey, early nauplius stages (NI to NIII) were significantly more prone to cannibalism by adults than later stages (NIV to NVI). Cannibalism by adult females was significantly influenced by encounter rates between adults and prey, and was found to saturate at a prey concentration of 751 nauplii l^{-1} in the absence of microalgal food. Furthermore, factors such as adult starvation period and microalgae quantity were also found to significantly influence *A. sinjiensis* cannibalistic behaviour. Cannibalism can be significantly lessen under intensive culture conditions, providing some simple culture management techniques.

The Chapters 6 and 7 present data on investigations of both optimal quality and quantity of microalgal diets for the calanoid copepod *Bestiolina similis*. In chapter 6, an optimal microalgal diet for *B. similis* intensive cultivation was determined by conducting a range of laboratory experiments. The tri-algal diet T-Iso+Tet+Pav (at a 1:1:1 carbon ratio) was recommended to maximize *B. similis* productivity in culture, as it was responsible for the best egg production rate and the highest cumulative egg production during female lifespan, in addition to the shortest egg incubation time, the highest female longevity, the best 48h and 96h egg hatching rates, the highest naupliar survival and the best population increase over a 12 day culture period.

After having determined *B. similis* optimal food quality parameters in the previous chapter (T-iso+Tet+Pav), the effects of various food concentrations (1800, 1500, 1200, 900, 600, 300 and 150 µg C l^{-1}) of this microalgal diet were investigated in chapter 7. Microalgae concentration significantly influenced *B. similis* egg production and faecal pellet production rates. Other parameters significantly affected by food concentration include population growth, egg hatching success, naupliar and copepodite survival as well as development time, adult female lifespan and cumulative egg production. A microalgae concentration of 664 µg C l^{-1} was determined to saturate *B. similis* female cumulative egg production over their lifespan. Such a food ration should be implemented as part of *B. similis* culture protocol, as it will ensure that its productivity is not significantly limited by the quantity of microalgal food.

Publications

Peer-reviewed journal articles associated with this thesis

Camus, T., Zeng, C., 2010. Roles of microalgae on total egg production and incubation time, naupliar and copepodite survival, sex ratio and female lifespan of the copepod *Bestiolina similis.* Aquaculture Research, 41, 1717-1726

Camus, T., Zeng, C., McKinnon, A. D., 2009. Egg production, egg hatching success and population increase of the tropical paracalanid copepod, *Bestiolina similis* (Calanoida: Paracalanidae) fed different microalgal diets. Aquaculture, 297, 169–175

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CHAPTER 1

GENERAL INTRODUCTION

1.1 The world fisheries and the aquaculture industry

1.1.1 The capture fisheries

Historically, the oceans were considered limitless and thought to harbour enough living resources to feed an ever increasing human population (Tidwell and Allan, 2001). However, at the dawn of the new century, approximately 65% of the fish stocks assessed worldwide require either rebuilding or lower exploitation rates in order to reverse their decline trend or prevent their collapse (Worm et al., 2009). This is believed to be largely attributable to a combination of increased fishing capacity, poor fisheries governance and unsustainable management practices (Pauly et al., 2002; Costello et al., 2008). Over the last three decades, the rising human population has contributed to a doubling of the annual consumption of seafood worldwide, while the global catches from wild fisheries have levelled off around 80 million tons and fell short of the increasing demand (Neori et al., 2004; FAO, 2010). By 2050, the human population is expected to reach 9.1 billion, along with a projected 70% increase in food demand (FAO, 2009; Merino et al., 2012). With such a rapid increase in food demand against the backdrop of worldwide decline in wild fisheries stocks, any further increases in global seafood consumption will have to be met by aquaculture (Naylor et al., 2000; FAO, 2009; Awal et al., 2012). Indeed, aquaculture is the only means to compensate for the shortfall in ocean harvests and its ecological

threat is believed to be lower than that of continuing to supply the majority of seafood from the overexploitation of wild stocks (Tidwell and Allan, 2001).

1.1.2 Aquaculture: challenges and future prospects

Aquaculture is defined as the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants, and originated primarily from freshwater production systems in Asia (FAO, 2010). By the year 2000, aquaculture had become a global and robust industry, producing more than 220 different finfish and shellfish species worldwide (Naylor et al., 2000). Today, aquaculture is the fastest growing food-producing sector in the world, with an annual average growth rate of 8.8% since late 1970's, faster than the human population growth over the same period (Merino et al., 2012). This growth is forecast to continue in the decades to come, as aquaculture is developing, expanding and intensifying in almost all regions of the world, contributing significantly to global food security, nutritional well-being, poverty reduction and economic development (FAO 2009; Subasinghe et al., 2009).

The proportion of aquatic animal food destined for human consumption from aquaculture was approximately 48% in 2011 and is expected to surpass 50% by 2015 (FAO, 2010). Although there are indications that aquaculture might be able to fill the gap between the expected demand and supply by capture fisheries, the aquaculture industry itself is currently facing several significant challenges, particularly with respect to sustainability, which will determine its future development (Subasinghe et al., 2009; Bondad-Reantaso et al., 2012).

1.1.3 The importance of marine hatcheries

Marine aquaculture currently relies on two distinct methods of cultivation: (i) stocking wild-caught seedlings, and (ii) stocking hatchery-reared juveniles. Examples of the former include tuna (Scombridae) farming in South Australia or eel culture (Elopomorpha) in Europe and Japan (Naylor et al., 2000). As the seedlings are sourced from the wild, this approach does not ultimately relieve pressure from natural populations, and is rather a method to raise wild fish to marketable size in captivity (Naylor et al., 2000).

Marine hatchery production of seedlings is a multistep process that requires broodstock cultivation, maturation and spawning techniques, as well as subsequent rearing of newly hatched

larvae to a stage that is suitable for stocking in grow-out farms (Takeuchi, 2001). This approach reduces the utilization of natural resources and is therefore substantially more sustainable than relying on wild-caught seedlings, in addition to allow for a more reliable supply of seedlings, irrespective of season, and more control over the life cycle of the species of interest by manipulating culture conditions (Aiking, 2011).

1.2 Traditional live feeds in marine hatcheries

1.2.1 Current status

A major bottleneck in the larviculture of marine fishes is the transition from an endogenous (reliant on yolk) to an exogenous (reliant on external prey) feeding mode in early larval stage (Payne et al., 1998; Olivotto et al., 2010a) and high mortality are often experienced during this transitional period (Turingan et al., 2005). Most marine larvae faces bioenergetic constraints at first feeding because their high growth rates requires high weight-specific metabolism but their digestive system is still rudimentary with poorly developed digestive enzyme activity resulting in low assimilation efficiencies and high weight-specific metabolism (Conceicão et al., 2010; Takeuchi, 2001; Chesney, 2005). Such digestive systems are in most cases incapable of processing formulated diets, which themselves lack enzymes and/or enzyme activators (Person Le Ruyet et al., 1993) and consequently a vast majority of marine larvae requires live prey items during their early life stages (García-Ortega et al., 1998; Shields et al., 1999).

To date, marine hatcheries are largely dependent on the production of rotifers (*Brachionus* spp.) and brine shrimp (*Artemia* spp.) as live feeds for fish and crustacean larvae (Evjemo and Olsen, 1997; Hagiwara et al., 2001; Takeuchi, 2001; Lee et al., 2005; Hoff and Snell, 2008). Rotifers are relatively easy to culture, and can rapidly achieve high culture densities (Lubzens et al., 2001). *Artemia* can be obtained commercially in the form of dry cysts, and their nauplii exhibit a good tolerance to culture conditions and handling (Conceicão et al., 2010). More than 700t of *Artemia* cysts are currently sold annually worldwide (Hoff and Snell, 2008) but there is a growing concern over the continued supply of *Artemia* cysts in the future, as it may be insufficient to meet the demands of the aquaculture industry due to both increased demand and unreliable production (Lavens and Sorgeloos, 2000; Conceicão et al., 2010).

Most rotifer species live in freshwater, but some are also found in coastal marine habitats and salt lakes (Duggan et al., 2002; Yoshida et al., 2003; Hoff and Snell, 2008), while *Artemia* populations are restricted to inland high saline lakes and also possess very little oceanic distribution, with the notable exception of coastal salterns associated with commercial salt production (Browne and MacDonald, 1982; Van Stappen, 2002). As a result, *Artemia* are rarely part of the natural diet of marine species while rotifers are only found in the larvae of a minority of estuarine and coastal species. As they are rarely part of the larvae's natural diet, it is well known that both *Artemia* and rotifers lack some of the essential nutrients for the survival and development of most marine larvae and their nutritional limitation and inappropriateness for many species of interest are well documented in the literature (Doi et al., 1997;Støttrup, 2000; Olivotto et al., 2010a).

1.2.2 Biological limitations and nutritional deficiencies

For first feeding larvae with small mouth-gape, *Artemia* and rotifers are often too big to be ingested (Knuckey et al., 2005; O'Bryen and Lee, 2005; Gopakumar and Santhosi, 2009; Lindley et al., 2011). Moreover, live feeds are also known to elicit weak feeding responses in some fish larvae (Cassiano and Ohs, 2011). When they are successfully ingested by the larvae, the digestibility of *Artemia* and rotifers is still a cause for concern as it is believed to be insufficient to promote proper digestive system growth and development in most marine larvae. For example, motile, undigested rotifers were observed being defaecated from larval golden snapper *Lutjanus johnii* (Schipp et al., 1999) while partially digested *Artemia* were observed as far as the anus in halibut larvae (Luizi et al., 1999).

Marine larvae require dietary provision of essential fatty acids (EFA) for normal growth and survival, particularly highly unsaturated fatty acids (HUFAs) such as docosahexaenoic acid (22:6n-3; DHA) and eicosapentaenoic acid (20:5n-3; EPA) (Rainuzzo et al., 1997; Bell et al., 2003). The HUFA content of live prey is often considered to be the critical factor indicating their nutritional quality, and failure to provide required quantities of HUFAs is a primary cause for the unsuccessful larval rearing of some marine species (Sargent et al., 1999). In addition, HUFAs must be provided in appropriate ratios, as high amounts of dietary EPA in relation to DHA may create structural imbalances in the composition of phospholipids and negatively affect growth and quality in cultured larvae (Rainuzzo et al., 1997).

In addition to the biological limitations mentioned previously, *Artemia* and rotifers are known to possess very low HUFA content, particularly with respect to DHA, and only possess limited ability to convert short-chain poly unsaturated fatty acids (PUFA) into long chain DHA and EPA (Navarro et al., 1993; Liu and Xu, 2009). As a result, marine hatcheries often rely on enrichment protocols to increase their biochemical profile (Hamre et al., 2008) and several types of enrichment media have been developed since the 1980's, including microalgae, oil-based emulsions and microencapsulated preparations (Shields et al., 1999; Takeuchi, 2001). However, enrichments are often insufficient to achieve optimal EFA profile (Helland et al., 2003) as traditional live feeds sequester lipids predominantly in the form of triacyglycerols (energy-yielding fatty acids) rather than phospholipids (Shield et al., 1999; Sargent et al., 1999). Moreover, *Artemia* tend to convert DHA to EPA, with the incorporated DHA being used for energy, thus making difficult for enrichment preparations to provide a DHA/EPA ratio >1 (Navarro et al., 1995; Bell et al., 2003). Rotifer enrichment seems promising only with respect to a few selected micronutrients and vitamins (Hamre, 2008; Srivastava et al., 2011).

1.2.3 The need for alternative live feeds

Today, various finfish and crustacean species are cultured successfully with traditional live feeds but there is an increasing list of species which could not been reared successfully using rotifers and *Artemia*, even when previously enriched. The number of species reared today in marine hatcheries is hence limited and the lack of appropriate live prey is one of the most important limiting factors for commercial larviculture (Payne and Rippingale, 2001; Holt, 2003; Busch et al., 2010; Ajiboye et al., 2011). Research focusing on the identification of alternative feed sources that overcome the inadequacies of traditional live feeds is critical to increase the variety and survival of species that can be successfully cultivated, and ultimately enhance the growth, sustainability and economic performance of the aquaculture industry. Copepods appear to be the most valuable candidate for this role as they are the most important natural prey for a vast majority of marine larvae (Sampey et al., 2007), and their inclusion as live prey in larviculture may increase the number of fish species that can be successfully reared (Payne and Rippingale, 2001).

1.3 Copepods as alternative live prey for marine hatcheries

1.3.1 Copepods in the wild

Copepods are minute aquatic crustaceans that have successfully colonized all salinity regimes from freshwater to marine and hypersaline inland waters, as well as all temperature regimes from sub-zero polar waters to hot springs (Huys and Boxshall, 1991). More than 11,500 species have been identified so far (Humes 1994), with approximately one-third of these species being parasitic or associates. Copepod nauplii, i.e. the first 6 developmental stages in the copepod life cycle, are numerically the most abundant multicellular zooplankters in marine systems (Titelman and Kiørboe, 2003a), and the various developmental stages of copepods typically account for most of the zooplankton biomass in the epipelagic zone (Mauchline, 1998; Xu and Wang, 2001; Bunker and Hirst, 2004).

Pelagic copepods are generally competitively superior to other similarly sized zooplankton (Kiørboe, 2011) and are the most important consumers of primary production in the wild. Their secondary production supports most of the food webs of the open sea, directly affecting pelagic fish populations and the biological pump of carbon (Frost, 1972; Ohman and Hirche, 2001; Freese et al., 2012;). Numerous field studies have demonstrated that copepod nauplii are the most important prey for marine fish larvae, typically making up 50% or more of their stomach content (Hunter, 1981; Munk and Nielson, 1994; Chesney, 2005; Kleppel et al., 2005). Within the families of larval fish that specialized on copepod prey, there is a clear preference for calanoid copepods, particularly small species (Sampey et al., 2007). The importance of copepods as a natural prey for early fish larvae is also evident by various reports that their production drives recruitment in various marine species in the wild (Castonguay et al., 2008; Bi et al., 2011). The ubiquitous distribution of copepods in marine systems, in addition to their importance as natural prey items for marine larvae, has prompted substantial interests in culturing them as live prey for marine larviculture (Shansudin et al., 1997; O'Bryen and Lee, 2005). High survival, improved growth, normal pigmentation and digestive tract development, low frequencies of skeletal deformities and higher ability to tolerate stress are characteristics of marine fish larvae reared on calanoid copepods (Koven et al., 2001; van der Meeren et al., 2008; Lemus et al., 2010; Hansen, 2011). The efficiency of copepods as live feeds is largely attributable to their high

ingestability and digestibility when compared to traditional live feeds as well as their superior biochemical composition, which matches the nutritional requirements of most marine larvae.

1.3.2 Biological characteristics of copepods enhance larval ingestion and digestion

1.3.2.1 Size variation

Copepod nauplii are generally smaller than the smallest strain of rotifers or *Artemia* (Table 1.1), making them ideal prey for many marine larvae with small mouth-gapes at first feeding (Gopakumar and Santhosi, 2009). Moreover, copepods molt multiple times through their development and undergo dramatic morphological changes (i.e. 6 naupliar stages followed by 6 copepodite stages before becoming sexually mature adults), providing a broad spectrum of sizes offering options for the cultured larvae (O'Bryen and Lee, 2005; Gemmell and Buskey, 2011).

Table 1.1: Summary of the size range and main nutritional characteristics of copepods compared to the traditional live feeds rotifers and Artemia.

Prey categories	Size range	Motion Pattern	Main nutritional characteristics					Sources
			Digestive enzymes	Micronutrients and vitamins	HUFA	DHA/EPA ratio	Phospholipids	
Artemia	400-500µm (Instar I)	continuous (weak feeding responses)	Low	deficient, enrichment needed	very low enrichment needed	generally <1, even when previously enriched	mostly triacyglycerols enrichment needed	Browne and MacDonald, 1982; Luizi et al., 1999; Van Stappen, 2002; Bell et al., 2003; Liu and Xu, 2009;
Rotifer	~100-150µm (s type)	continuous (weak feeding responses)	Low	deficient, enrichment needed	low, enrichment needed	generally <1, even when previously enriched	mostly triacyglycerols enrichment needed	Navarro et al., 1993 Schipp et al., 1999; Duggan et al., 2002; Yoshida et al., 2003; Hoff and Snell, 2008;
Copepod	<80µm (early nauplii)	"stop and go" (strong feeding responses)	higher level compared to Artemia and rotifer	Copepods generally contain more vitamins, pigments and trace minerals than rotifers and Artemia	naturally high level	substantially >1 significantly higher levels of DHA and EPA when compared to enriched rotifers or Artemia	more phospholipids (>50%) than triacylglycerols in proportion when compared to Artemia and rotifers	Buskey et al., 1993; Luizi et al., 1999; Bell et al., 2003; van der Meeren et al., 2008; Conceicão et al., 2010; Koedijk et al., 2010;

1.3.2.2 Motion pattern

Hydro-mechanical disturbances associated with locomotory patterns of prey affect the selective feeding of larval fish, and prey that frequently jump from location to location are more attractive to larvae than those that spend prolonged periods motionless in the water column (Buskey et al.,

1993). The characteristic "stop and go" movement pattern of copepods is believed to trigger strong predatory responses in early larvae, resulting in substantially increased ingestion rates (Buskey et al., 1993) (Table 1.1).

1.3.2.3 Digestive enzymes

Digestive enzymes are the biochemical agents that catalyze hydrolysis of the dietary components and are an important link between ingestion and assimilation (Kreibich et al., 2011; Freese et al., 2012). Live prey, upon digestion, leach their enzymes into the digestive system of the larvae, thus increasing its digestive capability. The amount of digestive enzymes released in the larval digestive system is dependent upon the type and nutritional composition of the prey consumed (Person Le Ruyet et al., 1993; O'Brien-MacDonald et al., 2006; Kreibich et al., 2011; Freese et al., 2012). While *Artemia* and rotifers tend to pass through the digestive system of cultured larvae very fast, the retention time of copepods tends to be much higher, ensuring that they are better digested, and therefore offer more potential for nutrient uptake when compared to traditional live feeds (Støttrup et al., 1986; Luizi et al., 1999; Olivotto et al., 2010b). For example, larval Atlantic halibut (*Hippoglossus hippoglossus*) can digest copepods more efficiently than *Artemia*, and copepod-fed larvae had an adult-like digestive tract with functional stomach (high digestion efficiency) by the end of metamorphosis (Luizi et al., 1999).

1.3.3 Nutritional profile of copepods

1.3.3.1Essential fatty acids and free amino acids

There is considerable evidence that an important contribution to the superior performance of copepods when compared to traditional live feeds is their naturally high level of HUFAs (Shansudin et al., 1997; Liu and Xu, 2009; Conceicão et al., 2010). Copepods contain significantly higher levels of DHA and EPA when compared to enriched rotifers or *Artemia* (Toledo et al., 1999; Garcia et al., 2008; Koedijk et al., 2010; Lindley et al., 2011) and they also exhibit DHA/EPA ratios that are substantially >1 (Shields et al., 1999; Bell et al., 2003; Conceicão et al., 2010) (Table 1.1).

Phospholipids are referred to as the 'gold standard in larval lipid nutrition' and are antioxidants essential for the synthesis of highly unsaturated fatty acids in marine larvae (Sargent et al.,

1997). The ability of dietary phospholipids to enhance growth and development in larval fish is well established and copepod nauplii usually contain more than 50% phospholipid on average, with more phospholipids than triacylglycerols in proportion when compared to *Artemia* and rotifers (Bell et al., 2003; Liu and Xu, 2009).

An exogenous supply of free amino acids (FAA) is necessary to support growth and survival in first feeding larvae (Helland et al., 2003; Lindley et al., 2011). Copepods contain higher levels of free amino acids when compared to rotifers, and more than twice the amount per gram wet mass when compared to *Artemia* nauplii (Næss et al., 1995; Helland et al., 2003; van der Meeren et al., 2008).

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

1.3.3.2 Micronutrients: vitamins, minerals and pigments

Copepods generally contain more vitamins and trace minerals than traditional live feeds (Hamre et al., 2008) (Table 1.1). Copepods are rich in vitamin E and ascorbic acid, suggesting a high antioxidative capacity and making them particularly suitable for larvae with potential for high growth rates (van der Meeren et al., 2008). Furthermore, copepods are rich in pigment content, particularly astaxanthin, which may be an important source of retinoids for larval fish (van der Meeren et al., 2008). A copepod diet has been reported to promote correct pigmentation in Atlantic halibut larvae, with 55% of the copepod-fed larvae exhibiting correct pigmentation of the ocular and blind sides as compared to only 13% in those fed *Artemia* (Shields et al., 1999).

1.3.4 Utilization of copepods in larviculture

1.3.4.1 Copepod rearing techniques

In the past, copepods were mostly cultured extensively or semi-extensively in outdoor ponds (van der Meeren and Naas, 1997; Liao et al., 2001; Conceicão et al., 2010). Unreliable production resulting from environmental variations such as monsoonal rains, and the difficulty to rear sufficient copepods 'out of season', has prompted the urgent call for the development of intensive culture techniques. When compared to extensive or semi-extensive copepod production

methods, intensive culture protocols offer a more reliable, consistent and year-round production, along with better defined nutritional profiles and the exclusion of predators and competitors (Støttrup, 2000; Knuckey et al., 2005). As a result of increased R&D efforts over the past 30 years, intensive large scale cultivation of calanoid copepods has become gradually more reliable and increasing number of species have been cultured using intensive culture protocols (Ajiboye et al., 2011; Drillet et al., 2011). There is an increasing body of literature on the batch culture techniques of calanoid copepods (Lavens and Sorgeloos, 1996; Liu and Xu, 2009; Lemus et al., 2010; Cassiano and Ohs, 2011) and protocols for intensive cultivation have been reported for species such as *Acartia tonsa* (Støttrup et al., 1986; Ogle et al., 2002; Marcus and Wilcox, 2007), *Gladioferens imparipes* (Payne and Rippingale, 2001), *Calanus helgolandicus* (Carotenuto et al., 2012) and *Acartia* spp. (Schipp et al., 1999).

However, most of these intensive culture protocols are not considered cost-effective (Støttrup, 2000; Conceicão et al., 2010; Lemus et al., 2010), which is a substantial drawback for their utilization in commercial aquaculture. However, the question of cost-effectiveness is complex when dealing with the utilization of copepods in commercial settings (Schipp, 2006) and culturing copepods, even if it is more expensive than traditional life feeds, might be cost-effective in the case of important commercial or ornamental species for which copepods are essential at first feeding (see section 1.3.5).

1.3.4.2 Feeding methods

As cost-effective intensive culture protocols for calanoid copepods are not fully established yet, only a few commercial hatcheries have the capacity to provide copepods as sole diet to cultured larvae. Hence, copepods are often offer as a supplement to traditional live feeds.

When provided in supplement to traditional life feeds, copepods are the preferred food items of fish larvae and usually dominate their gut contents (Payne et al., 2001; Olivotto, 2010b). Nauplii were the dominant prey item in the stomach contents of first feeding larvae even when they are provided at a density as low as 0.5 ml^{-1}, demonstrating that fish larvae are very capable of selective feeding on copepod nauplii, even if present in low numbers (Schipp, 2006). Supplemental copepods can potentially ameliorate the nutritional deficiencies of traditional live feeds but are also known to improve the overall digestion rates of live prey by the cultured

larvae. For example, the inclusion of supplemental copepods to a rotifer dominated diet resulted in improved growth and condition of spotted seatrout (*Cynoscion nebulosus)* larvae and the digestion of the copepods facilitated the assimilation of rotifer (Lemus et al., 2010).

Encouraging results were also obtained with emerging feeding technologies, such as the use of cold preserved copepods in the larviculture of the clownfish *Amphiprion clarkii*, with a positive effect on its growth and survival reported (Olivotto et al., 2010b). Similarly, the utilization of frozen copepods to feed seahorses was also successful (Woods, 2003).

1.3.5 Cultured larvae benefit from dietary copepods

Copepods are not only the most promising live feed candidates for a variety of economically important fish species that cannot be reared using traditional live feeds, but they also possess excellent track records in improving survival and development in species that can be cultured with traditional live feeds (Lee et al., 2005). Copepods are utilized in all regions of the world as live feeds for important commercial and ornamental species (Table 1.2).

Table 1.2: Literature review of the utilization of calanoid copepods in the larviculture of commercial and ornamental fish species around the world.

Species	Common name	Location	Effect of dietary copepods	Source
Clupea harengus	larval herring	Denmark/Norway	The enzyme trypsin was retained in the cultured larvae and copepod prey had an average carbon assimilation of 90%.	Pedersen & Hjelmeland, 1988
Pagrus major	red seabream	Japan	Success in culturing the red seabream using calanoid copepods.	Ohno, 1992
Coryphaena hippurus	mahimahi	Hawaii, USA	Second stage survival (9-20 days) was significantly higher when larvae were fed copepods. Larval requirements in EPA and DHA were met by feeding copepod.	Kraul, 1993
Stigmatopora argus	young pipefish	Perth, Australia	Copepods significantly improve survival and growth	Payne et al., 1998
Hippocampus subelongatus	west Australian seahorse	Perth, Australia	Early growth and survival significantly greater when fed copepod nauplii. Copepod nauplii were well digested by juvenile seahorses whereas Artemia were not.	Payne & Rippingale, 2000
Glaucosoma hebraicum	west Australian dhufish	Perth, Australia	Growth and survival were significantly greater in larvae fed with supplemental copepods.	Payne et al., 2001
Scophthalmus maximus *Sparus aurata*	flatfish spp.	Scotland, U.K.	Copepods are best live prey for first-feeding and are nutritionally beneficial due to high presence of HUFA, EPA and DHA, predominantly in the form of phospholipids.	Bell et al., 2003
Hippocampus abdominalis	seahorse sp.	Wellington, New Zealand	Successfully weaned onto frozen copepods.	Woods, 2003
Lates calcarifer	larval barramundi	Tamil Nadu, India	Significant influence of copepods on weight gain and survival compared to a rotifer diet.	Rajkumar & Kumaraguru vasagam, 2006
Centropyge loricula *Cephalopholis argus*	flame angelfish peacock hind	Darwin, Australia Hawaii, USA	Requires copepod nauplii to successfully negotiate past first feeding. Do not survive well, if at all, if copepods are not used.	Schipp, 2006
Lates calcarifer *Seriola rivoliana* *Caranx ignobilis* *Coryphaena hippurus*	barramundi almaco jack giant trevally common dolphinfish	Darwin, Australia Hawaii, USA	Can be reared well on rotifers or *Artemia* but when copepods are used as a supplement, their growth is significantly enhanced.	Schipp, 2006
Amphiprion clarkii	yellowtail clownfish	Ancona, Italy	Larvae fed copepod nauplii showed higher survival and growth compared to those fed a standard rotifer/*Artemia* diet as well as a significant increase of insulin-like growth factors I and II.	Olivotto et al., 2008
Paralichthys olivaceus	japanese Flounder	Qingdao, China	Significantly higher growth rate and survival along with superior content in DHA, EPA and Arachidonic Acid (20:4n-6; ARA) when fed copepods instead of *Artemia.*	Liu & Xu, 2009

Dascyllus aruanus	damselfishes spp.	Tamil Nadu, India	Total mortality observed when fed with rotifers but successfully grown using copepods.	Gopakumar & Santhosi, 2009
Chrysiptera cyanea	sapphire devil damselfish	Tamil Nadu, India	Rotifer alone and rotifer plus supplemental copepods provided total mortality in cultured larvae. Only a pure copepod diet worked and provided the best survival.	Gopakumar et al., 2009
Trachinotus carolinus	larval Florida pompano	Florida, USA	Significantly higher survival and growth.	Cassiano & Ohs, 2011
Elacatinus figaro	barber goby	Florianópolis, Brazil	Feeding supplemental copepod nauplii enhanced growth compared to a ciliate or an enriched rotifer diet.	Côrtes & Tsuzuki, 2012

Advantages of copepods as live feeds are especially evident in some high valued food fish species, such as tropical groupers (Serranide spp.), snappers (Lutjanide spp.) and for temperate species such as the Atlantic cod (*Gadus morhua)* and Atlantic halibut (*Hippoglossus hippoglossus*).

1.3.5.1 Grouper and snapper

Larvae of high valued tropical reef fish are typically very demanding with respect to live feeds and often do not accept traditional live feeds at first feeding. When provided with copepod nauplii as well as rotifers during the first 0-2 days following the commencement of exogenous feeding, gut contents of larval red snapper (*Lutjanus argentimaculatus)* consisted solely of copepod nauplii (Doi et al., 1997), showing a strong prey preference for copepod nauplii. Furthermore, the transformation from larvae to juvenile did not occur in this specie when fed only with enriched rotifers (Rabalais et al., 1980), while larvae were successfully reared to healthy juveniles when copepods were provided as supplemental live prey (Leu et al., 2003). Similarly, the early larval survival of crimson snapper (*Pristipomoides filamentosus*) is strongly linked to feeding on copepod nauplii (Schipp, 2006) and faster larval growth was achieved in the pink snapper (*Pagrus auratus*) when copepod nauplii were provided (Payne et al., 2001). Larvae of the spotted seatrout (*Cynoscion nebulosus*) fed with copepods exhibited superior growth rates and were generally in better general condition than larvae fed with rotifers only (Lemus, 2010). Finally, Toledo et al. (1999) reported an improvement in feeding incidence, gut content, growth and survival of early larval grouper *Epinephelus coioides* when they were fed copepod nauplii.

1.3.5.2 Atlantic cod (Gadus morhua) and Atlantic halibut (*Hippoglossus hippoglossus*)

Atlantic cod larvae fed copepods rather than enriched rotifers grow considerably faster, and increased their mean weight 2000–fold during the first 50 days of exogenous feeding (Finn et al., 2002; Koedijk et al., 2010). Larvae reared on copepods also showed a higher survival rate and a lower frequency of deformities than larvae fed traditional live feeds (Hamre et al., 2008; Busch et al., 2010; Koedijk et al., 2010; Hansen, 2011). Copepods performed better than enriched *Artemia* for Atlantic halibut larvae (*Hippoglossus hippoglossus),* resulting in higher survival, pigmentation and retinal morphology. It was reported that 40% of copepod-fed larvae exhibited perfect metamorphosis attributes, whereas only 3.5% of those fed *Artemia* fell into this category (Shields et al., 1999). Halibut larvae can digest copepods more efficiently than *Artemia*, and copepod-fed larvae had an adult-like digestive tract with functional stomach by the end of metamorphosis (Luizi et al., 1999).

1.4 Limitations of copepod utilization

The many advantages of copepods over traditional live feeds, individually as well as interactively, improve the opportunity for new larvae to be cultured commercially and enhance their developmental success (Koedijk, 2010). Yet, despite the many biological and nutritional advantages that copepod possess over traditional live feeds, in addition to their excellent track record as a live prey in the literature, their utilization in marine hatcheries remains overall sporadic (Marcus et al., 2004).

This under-utilization is mainly attributable to their relative low productivity in intensive culture (O'Bryen and Lee, 2005), which could in turn be partially attributed to the lack of research on this field, especially when compared to the attention received by rotifers and *Artemia*. By the year 2004, it was estimated that fewer than 4% of marine planktonic calanoid species (i.e. approximately 70 species) have had their fecundity measured (Bunker and Hirst, 2004) while intensive culture protocols were established only for a few species (see section 1.3.4.1). In addition to this lack of research, copepods possess different bio-ecological characteristics when compared to rotifers and *Artemia.* Calanoid copepods do not rely on parthenogenesis for reproduction, which constitute a handicap for productivity when compared to traditional live feeds..

In order to gain more knowledge about calanoid optimal cultivation parameters and help realize their full potential as live feeds for marine hatcheries, more research is urgently needed to establish reliable, cost-effective intensive culture systems. Given the little research attention that copepods have received from an aquaculture point of view, the recent advances in their cultivation methods mentioned previously are encouraging, and it is not unreasonable to expect that with the increasing R&D efforts in this area there will be major development or even breakthroughs in the near future.

1.5 About this thesis

1.5.1 The selection of candidate copepod species

Among the families of larval fish that specialized on copepod prey, a vast majority shown a clear preference for calanoid copepods, and more specifically for small calanoid species (Sampey et al., 2007). Calanoids are hence considered to be the most promising order of copepod for production and use as live prey items for marine hatcheries (Doi et al., 1997, Støttrup, 2000). Specifically, pelagic calanoid species from coastal waters, with a high tolerance to wide ranges of environmental conditions are preferred as live prey candidates (Støttrup, 2003; Conceicão et al., 2010).

Two calanoid copepod species, *Acartia sinjiensis* and *Bestiolina similis,* were selected as the targeted species for the study for this PhD project. Both species are euryhaline with a tropical and sub-tropical distribution and can tolerate fluctuations in temperature and turbidity. They also have a substantially short generation time (7-10 days) compared to temperate species and have been proven to be promising candidate for commercial larviculture (McKinnon et al., 2003). Following are brief introductions to these two species.

1.5.1.1 Acartia sinjiensis (Mori, 1940)

Acartia sinjiensis (Calanoida: Acartiidae) is a small copepod (adult <800 µm; early nauplii <100 µm) common in coastal and estuarine waters of the Indo Pacific (McKinnon et al., 2003). As a natural prey for larvae of tropical fishes and crustaceans, this species is amenable for aquaculture hatcheries, as the nauplii are small enough to be efficiently ingested by most first feeding larvae

(Knuckey et al., 2005). Previous research has assessed the effects of diets (McKinnon et al., 2003; Knuckey et al., 2005; Milione and Zeng, 2007), temperature and salinity (Milione and Zeng, 2008) on *A. sinjiensis* productivity. However, many other important factors remains unknown and need to be further examined, such as photoperiod, stocking density and cannibalism.

1.5.1.2 Bestiolina similis (Sewell, 1914)

Bestiolina similis (Calanoida: Paracalanidae) is a small (adult <600 μm; early nauplii <100 μm) pelagic species that is often a major constituent of inshore tropical Australian copepod assemblages (McKinnon and Duggan, 2001; McKinnon et al., 2003; Boxshall and Halsey, 2004). It is known to be a favorite prey item for several families of tropical fish (Sampey et al., 2007). Paracalanid copepods are widely distributed in both tropical and temperate waters and frequently dominate the copepod communities of surface waters (McKinnon and Duggan, 2001; Boxshall and Halsey, 2004). The small size and natural abundance of paracalanids, particularly the fact that they are a favorite prey for a vast majority of marine larvae make them excellent live feed candidates for marine hatcheries. McKinnon et al. (2003) suggested that *B. similis* was the best paracalanid candidate for larval fish diets copepods on the basis of its size, susceptibility to predation, growth rate and nutritional composition.

The aim of this study was to improve intensive culture techniques for *Acartia sinjiensis* and *Bestiolina similis,* two calanoid copepods with excellent potential as live prey for marine hatcheries.

1.5.2 Outlines of thesis chapters

This thesis consists of 8 chapters. Some introductory material and some materials and methods will be repeated in different chapters as each chapter is presented in publication format. However, a general materials and methods chapter was included to limit such repetitiveness.

Following the present General Introduction chapter (chapter 1) and a General Materials and Methods chapter (chapter 2), the subsequent experimental chapters are comprised of two parts: Chapters 3, 4 and 5 are focused on improving culture techniques for *A. sinjiensis,* while chapters 6 and 7 are focused on *B. similis*. The final chapter (chapter 8) summarizes the main results and

provide major recommendations for applications of the results in aquaculture settings in order to improve intensive culture protocols of these two calanoid copepods.

Although photoperiod is a major environmental parameter that can be easily manipulated with minimum costs in aquaculture hatcheries, only very limited research were conducted to investigate its effects on copepod productivity (Chinnery and Williams, 2003; Peck and Holste, 2006), with most publications mainly focused on its roles in inducing diapauses egg production (Hairston and Kearns, 1995; Ohman et al., 1998; Chinnery and Williams, 2003; Avery, 2005; Marcus, 2005). In chapter 3, the influence of photoperiod on the productivity of *Acartia sinjiensis* was assessed. The developmental and reproductive response of *A. sinjiensis* was quantified in a series of laboratory experiments using six different photoperiod regimes ranging between 0 and 24 hours of illumination per day. The best photoperiod regime was determined and recommendation made regarding optimal light regime for *A. sinjiensis* intensive production systems.

Despite their many perceived advantages as larval prey, the inability to culture calanoid copepods at a sustainable high density is a major bottleneck that hinders their utilization in aquaculture hatcheries. Yet, only a few studies have investigated the effects of stocking density on copepod productivity and aimed at their application in aquaculture (Medina and Barata, 2004; Jepsen et al., 2007; Peck and Holste, 2006). In Chapter 4, the influence of stocking density on productivity of *A. sinjiensis* was investigated at stocking densities ranging between 10-2000 adult l^{-1}. The ideal stocking density was determined for this species, and recommendations are made regarding suitable culture protocols.

Cannibalism can potentially become a problem in intensive culture settings, especially at high stocking density due to the increased encounter rates of individuals. In Chapter 5, cannibalistic behaviour in the copepod *Acartia sinjiensis* was investigated. Though this is not the first study to describe cannibalism in the genus *Acartia* (see Lonsdale et al. 1996), it was the first to focus on cannibalism from the perspective of maintenance of copepod cultures. Results from this chapter demonstrate that cannibalism is more pronounced under conditions where the adult copepods are subject to some degree of food limitation, and is significantly influenced by the density of naupliar prey in culture. In the light of the results presented in this chapter, recommendation are

made to manage the impact of cannibalism under intensive culture conditions and increase productivity overtime.

Microalgae quality and quantity are key biotic factors affecting copepod culture productivity. As such, the Chapter 6 and 7 present data on investigations on both quality and quantity of microalgal diets on culture productivity of *Bestiolina similis*. Selecting appropriate microalgal diet is crucial to determine appropriate intensive culture protocols for calanoid copepods. Through a series of laboratory experiments, Chapter 6 examines the effects of 10 different microalgal diets (mono or mixed microalgal diets) on thus far unstudied parameters related to *B. similis* productivity in culture. Recommendations were made regarding optimal microalgal diet for *B. similis.*

The influence of food concentration is now recognized as a dominant factor in determining copepod metabolism. While an optimal microalgal diet for *B. similis* intensive cultivation was determined in the previous chapter, chapter 7 investigated the influence of various concentrations (between 150-1800 μg C l^{-1}) of this optimal microalgal diet on several productivity-related parameters of *B. similis.* Optimal microalgal concentration for maximizing *B. similis* productivity in culture was determined in this chapter, and recommendation to implement a minimal microalgal diet for optimal productivity were made.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 *Acartia sinjiensis* stock culture

Acartia sinjiensis were collected from zooplankton tows performed in October 2006 at the mouth of the Ross River, Townsville, Queensland, Australia. After collection, samples were brought back to the Marine and Aquaculture Research Facility Unit (MARFU), James Cook University, within an hour. Upon arrival at the laboratory, *A. sinjiensis* were isolated from the rest of the zooplankton. The culture was gradually scaled up and inoculated into several 20 L plastic carboys filled with 1μm filtered seawater and gentle aeration as the stock cultures. Light intensity was about 700 lux, as measured by a light meter (MC-88, TPS), on a light:dark cycle of 12h:12h. All carboys were gently aerated and culture temperature was kept at 30±1°C in a temperature controlled room.

A. sinjiensis stock cultures were fed daily with 40,000 cells/ml of the Tahitian strain of *Isochrysis* sp. (T-Iso), and 3,125 cells/ml *Tetraselmis chuii* (Tet), as recommended by a previous diet experiment (Milione and Zeng, 2007). Depending on water quality condition, between 30 to 50% of culture water was exchanged every 2 days using a siphon with a 25 μm mesh attached to the end to prevent the removal of any *A. sinjiensis*. Every 10 to 15 days, carboys were drained

through a 150 µm sieve to remove detritus. The 150 µm sieve retained adult and copepodites but eggs and nauplii were mostly lost. The carboys were then cleaned and sterilized with chlorine before the cultures were restarted.

2.2 *Bestiolina similis* stock culture

Bestiolina similis were obtained on December 2008 from a plankton tow performed at the mouth of the Ross River in Townsville, Northern Queensland, Australia. Immediately after collection, plankton samples were brought back to the Marine and Aquaculture Research Facility Unit (MARFU), James Cook University, within an hour. *B. similis* were isolated from the rest of the zooplankton and gradually scaled up to four 20 L carboys filled with 1µm filtered seawater and gentle aeration. Salinity was 30±1‰ and the culture temperature was maintained at 26±1°C. Light intensity was about 700 lux, measured by a light meter (MC-88, TPS) and the photoperiod was set as light:dark cycle of 12h:12h.

B. similis were fed daily with a mixture of 3 microalgae: the Tahitian strain of *Isochrysis* sp. (T-Iso), *Tetraselmis chuii* (Tet) and *Pavlova* 50 (Pav) at a carbon ration of 1500 µgC.L^{-1}, with an equal ratio of biomass (i.e. T-Iso: Tet: Pav =1:1:1). About 20% of the culture water was exchanged every 2 days using a siphon with a 22 µm mesh attached to the end to prevent removal of copepods. *B. similis* stock cultures were entirely drained through a 150 µm sieve every 10 to 15 days to remove the buildup of detritus. The 150 µm sieve retained adults and copepodites, but eggs and nauplii were mostly lost. The carboys were then cleaned and sterilized with chlorine before the cultures were restarted.

2.3 Microalgae Culture

Microalgae species relatively easy to obtain and commonly used in marine hatcheries throughout the world were selected. The Tahitian strain of *Isochrysis* specie ('T-iso', class Prymnesiophyceae; CS-177), *Pavlova 50* ('Pav', class Prymnesiophyceae; CS-50), *Tetraselmis chuii* ('Tet', class Prasinophyceae; CS-26) and *Chaetoceros muelleri* ('Chaet'. class Bacillariophyceae; CS-176), were inoculated from starter cultures supplied by the

Commonwealth Scientific and Industrial Research Organization (CSIRO) Microalgal Supply Service, Hobart, Tasmania, Australia. Nutrients used for the culture of all species were f/2 concentration (Guillard and Ryther, 1962) with the addition of silicates for the diatom *Chaetoceros muelleri*.

All microalgae were cultured indoors using 20 L polycarbonate carboys and were maintained in a temperature controlled room (25±1°C) using 1 µm-filtered and UV irradiated seawater at 30 ppt with vigorous aeration (0.2 µm filtered air). The photoperiod was set at light: dark cycle =12h:12 h with a light intensity of approximately 5000 Lux as measured by a MC- 88 light meter. Concentrations of algal culture were assessed using a haemocytometer and a microscope. The microalgal cultures were fed to copepods during the exponential growth phase.

CHAPTER 3

EFFECTS OF PHOTOPERIOD ON EGG PRODUCTION AND HATCHING SUCCESS, NAUPLIAR AND COPEPODITE DEVELOPMENT, ADULT SEX RATIO AND LIFE EXPECTANCY OF THE TROPICAL CALANOID COPEPOD *ACARTIA SINJIENSIS* [1]

3.1 Introduction

Copepods are primary consumers in the oceans and are perhaps the most numerous metazoans on earth (Ohman and Hirche, 2001). Copepods, especially their nauplii, are a natural prey items of fish larvae (Chen et al., 2006; Chesney, 2005), typically making up fifty percent or more of their stomach contents (Støttrup, 2000). This makes copepods attractive candidates for possible use as live feeds for larviculture in commercial hatcheries (Shansudin et al., 1997; Knuckey et al., 2005).

Compared to brine shrimp *Artemia* spp. and rotifers *Brachionus* spp., the two most commonly used live feeds in hatcheries, copepods offer a numbers of advantages. Firstly, copepod nauplii

[1] Chapter 3 is adapted from Camus T, Zeng C (2008) Effects of photoperiod on egg production and hatching success, naupliar and copepodite development, adult sex ratio and life expectancy of the tropical calanoid copepod *Acartia sinjiensis.* Aquaculture 280: 220-226

are generally smaller than the smallest rotifer strain, allowing first feeding larvae to effectively ingest them (Chesney, 2005). Copepod nauplii are in fact an essential prey items for first feeding larvae of a variety of fish, including tropical snappers (*Lutjanide* spp.), groupers (*Serranide* spp.), the seahorse *Hippocampus subelongatus* and the dhufish *Glaucosom hebraium* (see Chapter 1, Table 1.2). Moreover, their numerous naupliar and copepodite stages provide a broad range of prey sizes for larvae of different developmental stages (Chen et al. 2006). Secondly, the nutritional profiles of copepods are far superior to rotifers and *Artemia* and generally match the requirements of fish larvae (Drillet et al. 2006; Evjemo et al., 2003). Improved survival, enhanced pigmentation and increased resistance to stress are often observed in larvae fed on copepods (Støttrup, 2000; Knuckey et al., 2005). Finally, the swimming pattern of copepods, especially calanoid species, is believed to stimulate stronger foraging responses in some fish larvae, resulting in improved ingestion (Støttrup, 2000).

Despite their promising characteristics, copepod utilization in aquaculture hatcheries to date remains sporadic (Marcus et al., 2004). This is mainly due to their relative low productivity in intensive culture, which could be partially attributed to lack of research on this field (Drillet et al., 2006a). The productivity of copepod culture is affected by a range of factors, including diet, stocking density and environmental conditions for culture such as temperature, salinity and photoperiod (Ambler, 1986; Castro-Longoria, 2003; Kleppel et al., 1998; Koski and Kuosa, 1999; Leandro et al., 2006; Rodriguez et al., 1995). Photoperiod is one of the most significant cues for seasonality in nature (Hairston and Kearns, 1995) and could therefore be a key factor controlling female copepod reproductive status and population dynamics. Meanwhile, photoperiod is an environmental parameter that can be easily manipulated with minimum costs in aquaculture hatcheries (Chinnery and Williams, 2003).

The productivity of a copepod culture is firstly linked to the rate of eggs produced by the females. Due to obvious importance of egg production rate in determining copepod productivity, the effects of various environmental factors on egg production have been well documented (Calbet and Alcaraz, 1996; Castellani and Altunbas, 2006, Hirst and McKinnon, 2001; Kang and Poulet, 2000; Poulet et al., 1995; Shin et al., 2003; Yebra et al., 2005). However, aside from egg production, other biological parameters such as subsequent egg hatching success (Jepsen et al., 2007), nauplii and copepodite development rates (McKinnon et al., 2003), sex ratio and life

expectancy of the adults could also significantly affect copepod productivity in culture (Knuckey et al., 2005). Hence, a systematic evaluation of the effects of photoperiod on all these parameters is likely to provide comprehensive information helping to improve the productivity of copepod culture.

Acartia sinjiensis is a planktonic copepod indigenous to tropical North Queensland, Australia, which often occurs in large numbers in the coastal and estuarine waters (Uye, 1980; McKinnon et al., 2003). This species is amenable for aquaculture hatcheries, as their nauplii are small enough to be efficiently ingested by first feeding larvae of tropical reef fish with small mouth-gapes (Knuckey et al., 2005). Although previous research have assessed the effects of diets (McKinnon et al., 2003; Knuckey et al., 2005; Milione and Zeng, 2007), temperature and salinity (Milione and Zeng, 2008) on *A. sinjiensis* productivity, no information is available so far as to optimum photoperiod condition for their culture. In order to maximize the productivity of intensive culture of this species for use as live feed in aquaculture, experiments were conducted to investigate the effects of photoperiod on egg production and hatching success, naupliar and copepodite development rate, adult sex ratio and life expectancy of *A. sinjiensis*.

3.2 Materials and methods

3.2.1 General procedure

Five photoperiod conditions of Light : Dark = 0h:24h; 6h:18h; 12h:12h; 18h:6h; 24h:0h were set up for all following experiments. The light intensity reaching experimental vessels were kept constant at 1250 Lux for each photoperiod treatment and water temperature was maintained at 30±1°C throughout all experiments, unless stated otherwise. Observations and counting of *A. sinjiensis* eggs, nauplii, copepodites and adults were made using a Sedgewick-Rafter counter and a Leica CME optical microscope model TN-PSE30 (x 40 magnification).

3.2.2 Eight days egg production experiment

Egg production of *A. sinjiensis* was monitored on a daily basis over 8 consecutive days under the 5 photoperiod conditions mentioned above. This enabled the investigation of potential variations in egg production over time.

At the beginning of the experiment, three 1L beakers of *A. sinjiensis* "mixed population" cultures, consisting of all developmental stages, were established under each photoperiod condition. This "mixed population" was harvested directly from the stock cultures, well mixed before approximately same amount (volume of water) of *A. sinjiensis* was randomly introduced into each of the 1L beakers. Gentle aeration was provided to each beaker and every day 40 to 60% culture water was exchanged as well as fresh microalgae added as described in Chapter 2 for *A. sinjiensis* stock culture.

At the same time of the day for the subsequent eight days, five healthy adult females of similar sizes were randomly isolated from 'mixed population' cultures and distributed individually into 300 ml plastic cylinders to monitor their 24 h egg production under each photoperiod condition. Each of these cylinders, with bottoms being removed and replaced with attached 100 µm sieve, was suspended in a 500 ml beaker filled with 1 µm-filtered and UV irradiated 30 ppt seawater. Such a design allowed the eggs to fall through the mesh but did not allow the adults to pass, preventing egg cannibalism from occurring. A same quantity of fresh algae (ca. 40,000 cells/ml T-iso and 3,250 cells/ml *Tetraselmis chuii)* was provided daily to each of the 500 ml beakers.

Every 24 hours, eggs resting on the bottoms of each 500 ml beaker were collected using a 25 µm sieve and fixed with 10% formalin. The total number of eggs was counted for each replicate to determine 24 h egg production under different photoperiods and the means of the five replicates were calculated. After egg collection, all females used were discarded while all 300 ml cylinders and 500 ml beakers were cleaned and then filled with new filtered seawater and fresh algae. Five new females were then randomly selected from the 'mixed population' cultures again and placed individually into 300 ml cylinders to replace the discarded females and to start a new 24 h egg production experiment. Daily introduction of new female *A. sinjiensis* ensured that experimental females remain viable and that the observed egg production rates were not affected by lack of remating (Ianora et al., 1996).

3.2.3 Egg hatching success experiment

Eggs collected from stock cultures pre-conditioned to the various experimental photoperiod regimes (> 48 hours) were collected, rinsed and placed in freshwater for 2.5 min to kill all post-egg-stages *A. sinjiensis*. This procedure is known not to affect viability of unhatched eggs

(Knuckey et al., 2005). The eggs were then returned to seawater and samples consisting of 53 to 79 eggs (exact number counted under a microscope) were randomly distributed into twenty five 50 ml sealed plastic flasks. The five containers, each treated as a replicate, were then placed under a given photoperiod condition for incubation. The incubation temperature was maintained at 29±1°C for all photoperiod regimes.

After 48 hours of incubation, containers were retrieved and their content fixed with 10% formalin. The number of unhatched eggs in each replicate was then counted and the Egg Hatching Success (EHS) was subsequently calculated as:

$$EHS\ (\%) = \frac{[(\text{No. of eggs introduced initially} - \text{No. of unhatched eggs}) * 100]}{\text{No. of eggs introduced initially}}$$

3.2.4 Naupliar and copepodite development rate, adult sex ratio experiment

The development of *A. sinjiensis* was followed from hatching to the adult stage under 5 different photoperiods (three replicates per treatment). Eggs were gently siphoned from the stock cultures and placed in freshwater to kill all post-egg-stage copepods. Approximately 1,000 eggs (±50) were randomly distributed to each replicates, consisting of 1 l beakers filled with 30 ppt filtered seawater.

Food was added daily but adjusted according to *A. sinjiensis* development and always provided in excess. To maintain an optimal food particle size, the diet offered to nauplii consisted of only *T-iso* (mean equivalent spherical diameter, ESD=4.5 μm). But from copepodite onward, a mixture of *T-iso* and *Tetraselmis chuii* (ESD=10 μm) was provided in equal biomass (Knuckey et al. 2005; Milione and Zeng, 2007). Microalgae concentration was assessed daily and adjusted by adding fresh algae when necessary.

Every 24 h, samples were taken from each beaker by siphoning between 25 to 30 ml of culture water onto a 40 μm sieve to obtain at least 30 individuals for determining their developmental stages. Prior to sampling, cultures were mixed thoroughly and siphoning was done by a quick and strong suction to ensure all developmental stages were equally sampled (McKinnon et al., 2003). Developmental stage of sampled copepods was identified to nauplii, copepodites and adults, respectively and any adults found were sexed to obtain sex ratio data.

The mean development time from eggs to copepodite/adult under different photoperiod conditions were computed using the following formula:

$$MDT(adults/copepodites/nauplii) = \frac{\sum_{n}^{n+1} N(development\ stage)_n * n}{\sum N(development\ stage)}$$

Where N is the number of nauplii, copepodites or adults found on a given day n.

3.2.5 Adult life expectancy experiment

The initial procedure for adult life expectancy experiment was the same as for the naupliar and copepodite development experiment (Section 3.2.3). Eggs obtained from *A. sinjiensis* stock cultures were randomly distributed into 1 l beakers for incubation and growth under 5 different photoperiod regimes until adults appeared (3 replicates per treatment). As the adults appearing on the first day were mostly males, they were discarded. Only on the following days, newly appeared adults with normal sex ratio were isolated from the culture and transferred to separate 200 ml beakers under same photoperiod condition (between 15 and 23 adults per replicate). All replicates were fed daily as described previously for stock culture (Chapter 2, Section 2.1), while their daily mortality monitored and recorded. The day these adults were introduced to the 200 ml beakers was considered as day 0 of their adult phase. Experiment was continued until all animals in a replicate died and the mean life expectancy of adult *A. sinjiensis* in a given replicate was obtained by averaging all individual adult lifespan in the replicate.

3.2.6 Data collection and analysis

All data met the parametric test assumption (i.e. normally distributed, homogeneity of variance, independent and randomness of the data). Egg hatching success and adult life expectancy data were analyzed using one way ANOVA while mean 24 h egg production data were analyzed with 2-factor blocked ANOVA. The development data were analyzed with two-way factorial ANOVA. If any significant differences were detected ($p<0.05$), Tukey multiple comparison test was performed to determine specific differences between treatments at a significant level of 0.05. The sex ratio data was log transformed and pooled across all replicates before being analyzed for significant difference between treatments, using the Chi square test. All statistical analyses were conducted using Statistica, version 7. Data are presented as mean ± standard error (SE).

3.3 Results

3.3.1 Egg production experiment

3.3.1.1 Egg production over 8 consecutive days

Under different photoperiod regimes, the daily egg production rates of *A. sinjiensis* over 8 consecutive days are showed in Figure 3.1. Daily fluctuations in egg production were observed under all photoperiod conditions, but a clear trend of increasing egg production with longer light phases was apparent (Fig. 3.1). Under constant light (24L:0D), *A. sinjiensis* was most productive among all treatment from the first 4 days, but the daily egg production started to drop and was below that of the 18L:6D treatment from day 5 onward. For the constant darkness treatment (0L:24D), acclimatization to the un-natural condition was evident as egg output increased steadily every day, from 5.6±0.68 eggs/female/day on day 1 to 10.8±2.71 eggs/female/day on day 8, an increase of near two folds (Fig. 3.1).

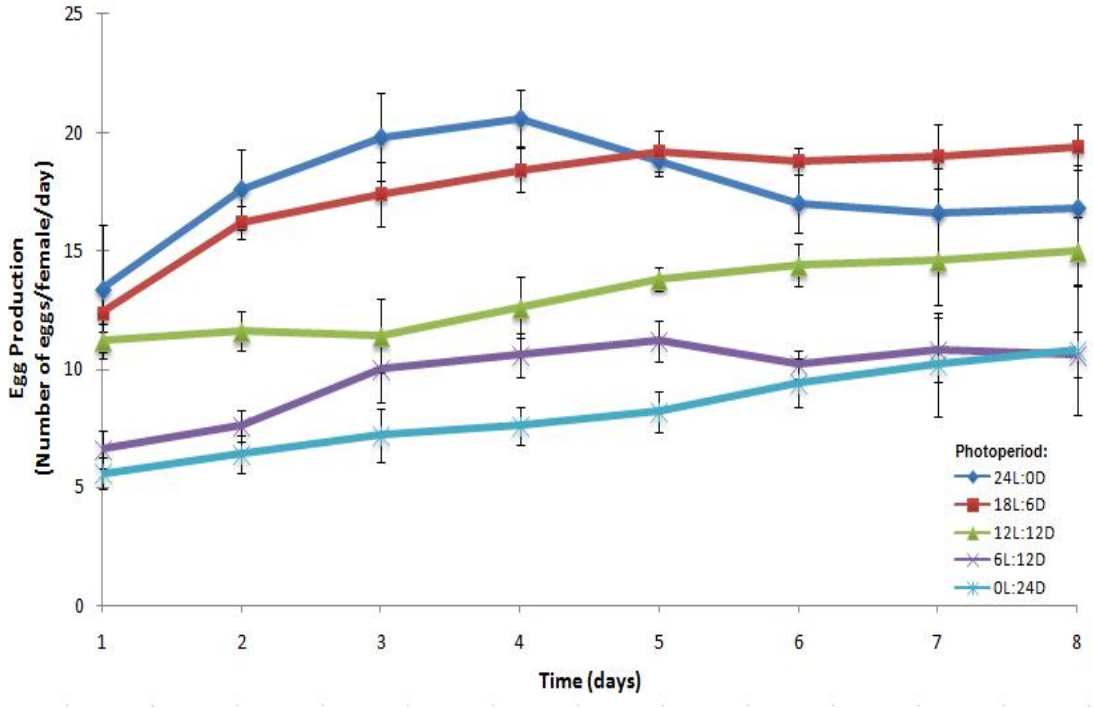


Figure 3.1: Daily egg production of *A. sinjiensis* females under different photoperiods for 8 consecutive days (5 replicates per treatment). Data are presented as mean ± standard errors.

3.3.1.2 Mean 24 h egg production

When daily egg productions were averaged over the 8 day period, the mean 24 h egg productions (#eggs/female/day) under different photoperiods were found to be significantly different ($p<0.001$) (Fig. 3.2). The mean 24 h egg production increased steadily with increasing light period; the highest egg production rate of 17.6 eggs/females/day was recorded at both 24L:0D and 18L:6D (Fig. 3.2). In contrast, the lowest egg production (8.2±1.2 eggs/female/day) was found under constant darkness (0L:24D) although it did not differ significantly from that of 6L:18D treatment (9.7 ± 1.1 eggs/female/day). Mean number of eggs produced daily at 12L:12D (13.1±1.1 eggs/female/day) was significantly higher than those of both 0L:24D and 6L:18D treatments, but significantly lower than that of the 24L:0D and 18L:6D treatments (Fig 3.2).

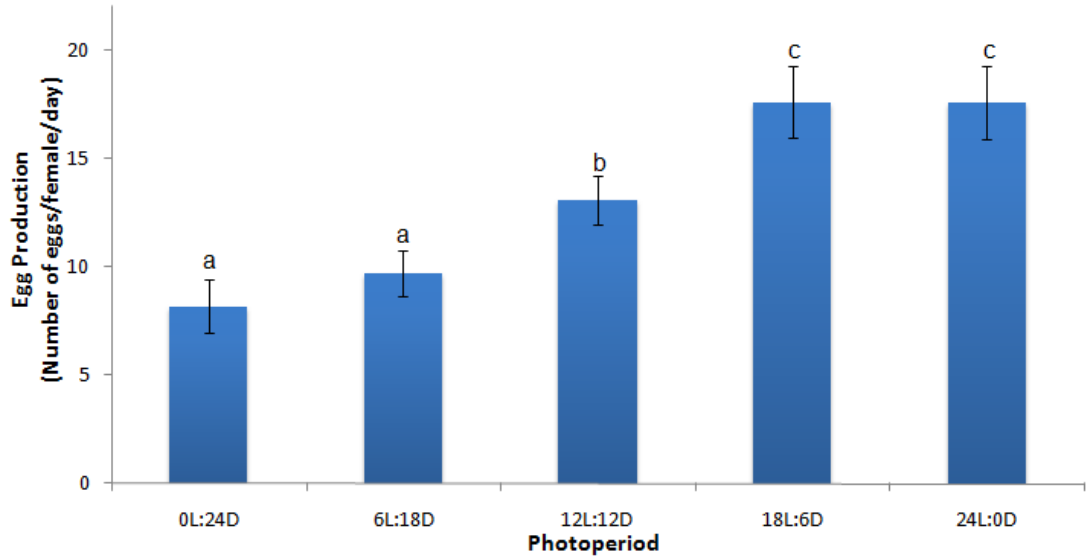


Figure 3.2: Mean 24 h egg production of *A. sinjiensis* females over 8 days under different photoperiod regimes. Data is presented as mean ± standard errors. Different letters on the top of bars indicate significant differences ($p<0.05$).

3.3.2 Egg hatching success

Photoperiod also had a significant influence ($p<0.005$) on *A. sinjiensis* egg hatching success. Improved 48 h hatching success corresponded to longer periods of illumination as hatching

success increased steadily from 72.9 ± 2.6% at constant darkness (0L:24D) to 87.2 ± 1.4% at constant light (24L:0D) (Fig. 3.3).

Hatching rate under constant light (24L:0D) was significantly higher than those of both 0L:24D and the 6L:18D treatments. Meanwhile, hatching rate at 18L:6D was also significantly higher than that of the 0L:24D treatment ($p<0.05$). No significant differences were found among the rest of the treatments ($p>0.05$).

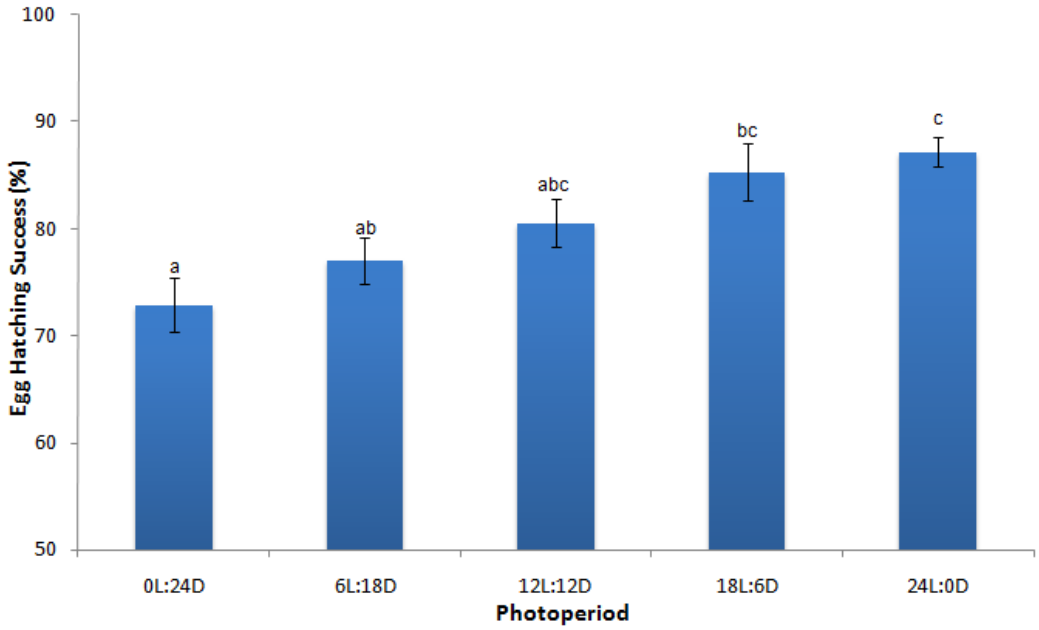


Figure 3.3: 48 h egg hatching success of *A. sinjiensis* under five different photoperiod conditions (5 replicates per treatment). Data are presented as mean ± standard errors. Different letters on the top of bars indicate significant differences ($p<0.05$).

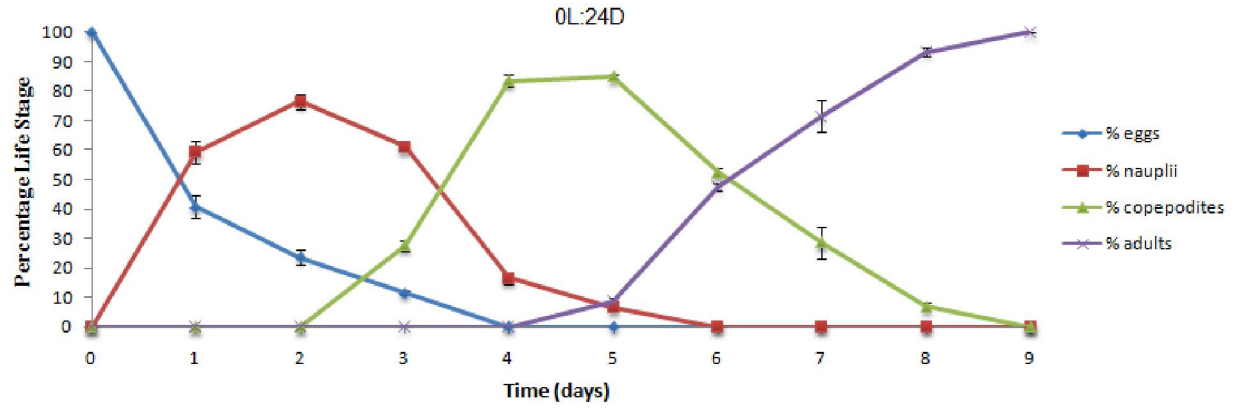
3.3.3 Naupliar and copepodite development rates and adult sex ratio

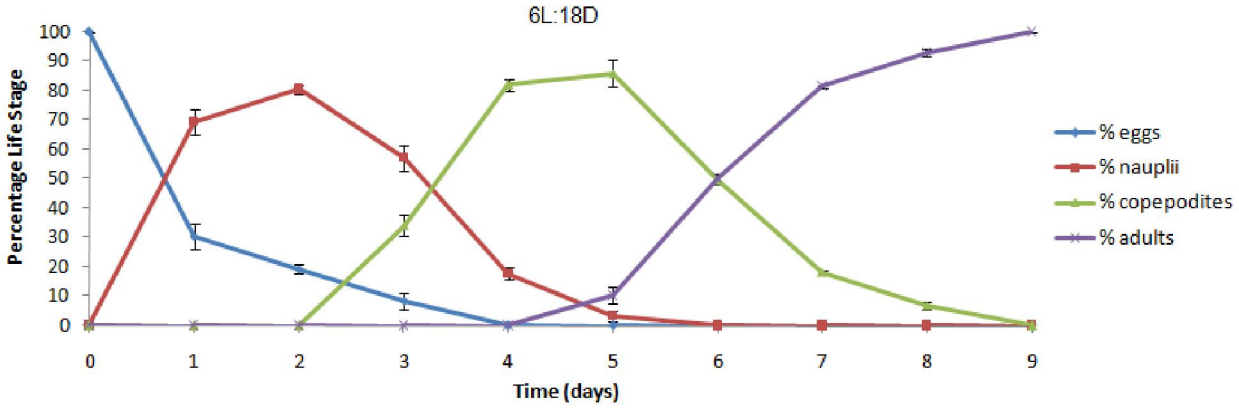
3.3.3.1 Naupliar and copepodite development rates

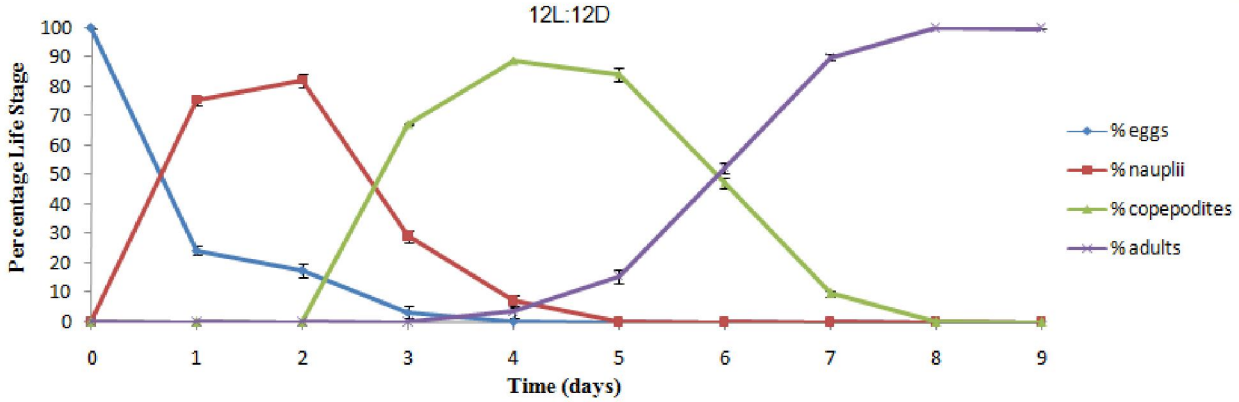
For easier comparison and better visual presentation, the daily presence of various *A. sinjiensis* developmental stages (i.e. eggs, nauplii, copepodites and adults) was converted into percentage of the population. Figure 3.4 shows the progress of *A. sinjiensis* development from the eggs to adult under various photoperiod regimes. A trend of accelerated development with increasing

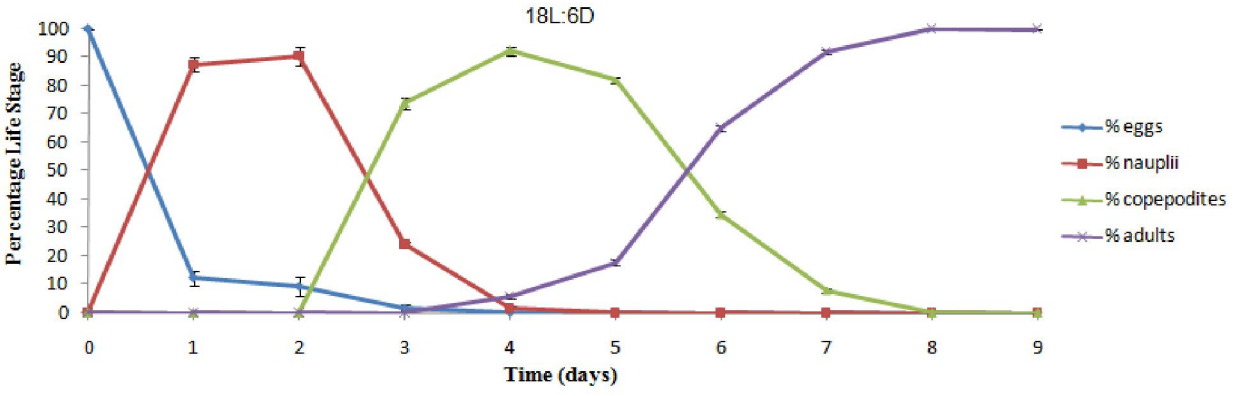
light duration was shown. For example, on day 5 of the experiment, the proportion of *A. sinjiensis* reaching the adult stage was 8.5%, 10.3%, 15.7%, 17.8% and 28.2% for photoperiod regimes of 0L:24D; 6L:18D;12L:12D; 18L:6D and 24L:0D, respectively. Furthermore, it took 9 days for the whole population to reach 100% adults under 0L:24D and 6L:18D photoperiod conditions whereas only 8 days were required under photoperiods with light phases of 12 hours or longer (Fig. 3.4).

It is worth noting that in contrary to the developmental rate, the duration of the naupliar stage was inversely related to hours of illumination. For example, naupliar stages accounted for more than 50% of the total population for 3 days under photoperiod of 0L:24D and 6L:18D, this was reduced to only 2 days for the photoperiod regimes with 12 to 24 h of illumination (Fig. 3.4).


0L:24D
Percentage Life Stage
Time (days)
% eggs
% nauplii
% copepodites
% adults


6L:18D
Percentage Life Stage
Time (days)
% eggs
% nauplii
% copepodites
% adults


12L:12D
Percentage Life Stage
Time (days)
% eggs
% nauplii
% copepodites
% adults


18L:6D
Percentage Life Stage
Time (days)
% eggs
% nauplii
% copepodites
% adults

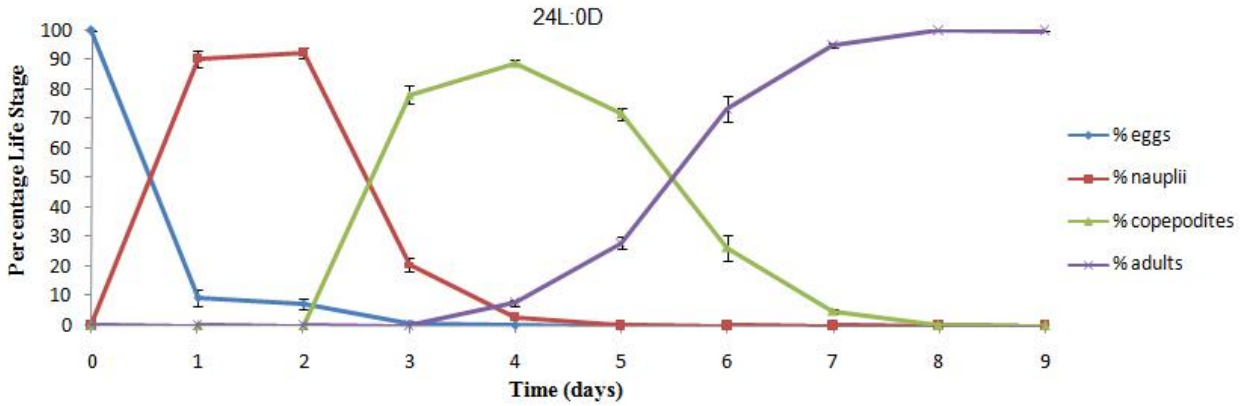


Figure 3.4: Percentage presence (%) of various developmental stages (eggs, nauplii, copepodites and adults) of *A. sinjiensis* under different photoperiod regimes (3 replicates per treatment). Data is presented as mean ± standard errors.

The influence of photoperiod on *A. sinjiensis* development was further evident when average development time from eggs to copepodites and adults were compared (Table 3.1). Significant differences in development time required from eggs to copepodites and from eggs to adults were detected ($p<0.005$). Average development time from eggs to copepodites ranged from 3.22 ± 0.12 days at 24L:0D to 3.99 ± 0.1 days at 0L:24D. Mean development time from eggs to adults was the shortest under constant light (6.00 ± 0.33 days), followed by 18L:6D (6.24 ± 0.24 days), both were significantly shorter than those of 0L:24D (6.84 ± 0.08 days) and 6L:18D (6.77 ± 0.07 days) treatments ($p<0.05$).

Table 3.1: Mean development time from eggs to copepodites and adults of *Acartia sinjiensis* under different photoperiod conditions.

Photoperiod	Mean Development Time from Eggs to Copepodites (Days)	Mean Development Time from Eggs to Adults (Days)
0L:24D	3.99 ± 0.10 ^{a}	6.84 ± 0.08 ^{B}
6L:18D	3.87 ± 0.05 ^{ab}	6.77 ± 0.07 ^{B}
12L:12D	3.40 ± 0.06 ^{abc}	6.57 ± 0.16 ^{AB}
18L:6D	3.36 ± 0.19 ^{bc}	6.24 ± 0.24 ^{A}
24L:0D	3.22 ± 0.12 ^{c}	6.00 ± 0.33 ^{A}

3.3.3.2 Adult sex ratio

No significant difference in sex ratio was found among the photoperiod treatments ($p>0.05$). For all photoperiod regimes tested, higher female to male ratio were observed across all treatments with female to male ratios ranging from 61.8%:38.2% to 73.7%:26.3% (Table 3.2).

3.3.4 Adult life expectancy

Photoperiod had a significant effect on life expectancy of adult *A. sinjiensis* ($p<0.005$). The lifespan of adult *A. sinjiensis* was the longest at 12L:12D (10.9±0.1 days) and the shortest at 24L:0D (9.4±0.4 days). Life expectancy under constant light was significantly shorter than all other photoperiod regimes tested (Table 3.2). However, no significant difference was found among other photoperiod treatments (Table 3.2).

Table 3.2: Sex ratio and life expectancy of adult Acartia sinjiensis cultured under five different photoperiod regimes.

Photoperiod	Percentage of Females (%)	Adult Life Expectancy (days)
0L:24D	73.7 ± 1.7 [a]	10.4 ± 0.2 [B]
6L:18D	67.6 ± 2.1 [a]	10.7 ± 0.1 [B]
12L:12D	68.1 ± 1.3 [a]	10.9 ±0.1 [B]
18L:6D	64.4 ± 1.0 [a]	10.5 ±0.1 [B]
24L:0D	61.8 ± 2.5 [a]	9.4 ± 0.4 [A]

3.4 Discussion

Although photoperiod is a major environmental parameter that can be easily manipulated with minimum costs in aquaculture hatcheries (Chinnery and Williams, 2003), effects of photoperiod on copepod productivity have not been well examined (Peck and Holste, 2006). Previous research on photoperiod has been mainly focused on its role in inducing diapause egg production

(Avery, 2005; Chinnery and Williams, 2003; Hairston and Kearns, 1995; Marcus, 2005; Ohman et al., 1998). Cost effective copepod culture for aquaculture hatcheries relies on maximizing productivity (Holste and Peck, 2006), the present study examined the effects of photoperiod on a range of productivity related parameters for the tropical copepod *Acartia sinjiensis*, a species with substantial potential as live feed for tropical aquaculture.

Results from the 8 day egg production experiment demonstrated that photoperiod significantly impacted *A. sinjiensis* egg production. Significantly higher daily egg productions were found for photoperiod treatments with light phase of 18 hours or longer. As a tropical species, *A. sinjiensis* is probably accustomed to longer light period, as tropical regions generally have long daily light phase year around. Among the very few studies published on the effect of photoperiod on copepods egg production, Peck and Holste (2006) reported that *A. tonsa* egg output was unaffected by photoperiod. This probably reflects species-specific responses to photoperiod, but can also due to photoperiod regimes tested in their experiments ranged only from 8L:16D to 20L:4D. In their study, Peck and Holste (2006) also described that the ratio of eggs produced during darkness to that during light (D/L) increased with increasing photoperiod duration, which is in agreement with the slight decrease in egg production observed in the current experiment at 24L:0D when compared to 18L:6D.

Past studies on copepod egg production normally examined egg output in 24 h or 48 h (Rodriguez et al., 1995; Hirst and McKinnon, 2001; Lincoln et al., 2001; Onoue et al., 2004; Sedlacek and Marcus, 2005). In the present study, *A. sinjiensis* daily egg production was monitored for 8 consecutive days under various photoperiod regimes in order to assess any potential fluctuations in egg production over time. A clear adaptation of *A. sinjiensis* to extreme constant darkness condition was shown as females egg production increased steadily over 8 day period and nearly doubled by the end of the experiment. In contrast, although daily egg production under constant light was the highest for the first 4 days, it decreased substantially from day 5 onward and was lower than that of 18L:6D treatment for the final 3 days. A possible explanation for this is that under constant illumination, copepods were probably active 24 h around, which required higher metabolic rate to sustain. Such a high metabolic rate plus high daily egg production for the first 4 days probably had rapidly depleted their energy reserves, leading to the decrease in egg production observed in the following days.

Data from the 48 hours hatching success trial showed that photoperiod also significantly affected the hatching process of *A. sinjiensis*. The hatching rate increased steadily with increased illumination time although no significant difference was detected between the two highest hatching rates at 24L:0D and 18L:6D. Hatching rates found in current experiment are comparable to those of *A. tonsa*, the only other calanoid species studied in relation to photoperiod (Peck and Holste, 2006).

The development of *A. sinjiensis* was similarly affected by photoperiod and accelerated development was found under photoperiods with longer illumination periods. The mean development time from egg to adult under photoperiod regimes of 24L:0D and 18L:6D were significantly shorter than those of the 0L:24D and 6L:18D treatment. This suggests that longer illumination could be used to increase generation turnover time, and therefore productivity in *A. sinjiensis* culture. However, on the other hand, due to the fact that copepod nauplii are often a crucial feed for first-feeding larvae, knowledge on whether the duration of naupliar stage are affected by photoperiod is also useful. The current study showed that the duration of nauplii was inversely related to photoperiod illumination time. This suggests that when required, a hatchery manager could potentially alter photoperiod regime to slow down the naupliar development and keep them available for longer period.

Sex in calanoid copepods is probably determined genetically during meiosis through an X-Y chromosome mechanism (Gilbert and Williamson, 1983) although it is also known to be influenced by temperature (Voordouw and Anholt, 2002). The proportions of male and female adults *A. sinjiensis* observed in this experiment were not significantly affected by photoperiod, and were always skewed in favor of females. This is the first report on sex ratio of *A. sinjiensis,* and the result is in accordance with the majority of previous reports from both laboratory and field studies, which usually showed a sex ratios skewed in favor of females (Fleminger, 1985; Medina and Barata, 2004).

Despite its significant implications for culture productivity, the life expectancy of adult *A. sinjiensis* has never been investigated before. Obviously, the productivity of *A. sinjiensis* is linked not only to daily egg production rates, but also to the duration for which a female can contribute to the total egg output in the culture. Significantly shorter adult life-span was found under the constant light when compared to the rest of the photoperiod treatments. Such finding

appears in accordance to results from the egg production experiment, in which *A. sinjiensis* under constant illumination started to decrease daily egg production after a few days. The constant illumination likely kept *A. sinjiensis* active 24 h, therefore requiring high metabolic rates to maintain, which could result in shorter life expectancy.

In summary, current experiments revealed that except for the adult sex ratio, photoperiod exerted significantly effects on all other productivity related parameters examined. In general, *A. sinjiensis* cultured under photoperiods of 18L and 24L illumination periods have the highest daily mean egg production rate and 48h egg hatching success, as well as the fastest naupliar and copepodite development rates. However, due to the fact that 24L constant light resulted in significant shorter adult life expectancy and there was also clear signs of decreasing egg production overtime under this particular photoperiod regime, it is therefore suggested that a photoperiod of 18L:6D should be adopted for *A. sinjiensis* culture to maximize its productivity. This study confirm the notion put forward by Peck and Holste (2006) that short dark periods seems necessary to insure efficient productivity. As a result, it could be of great interest to study alternating short-term pulses of darkness and light as a method to maximize egg production in intensive cultures of calanoid copepods. Finally, it is worth noting that all current experiments were conducted at laboratory scale, therefore the results might not be fully reproducible in large-scale production in aquaculture, however, it clearly served the purpose of identifying the optimal photoperiod for *A. sinjiensis* cultivation.

CHAPTER 4

THE EFFECTS OF STOCKING DENSITY ON EGG PRODUCTION AND HATCHING SUCCESS, CANNIBALISM RATE, SEX RATIO AND POPULATION GROWTH OF THE TROPICAL CALANOID COPEPOD *ACARTIA SINJIENSIS* [2]

4.1 Introduction

Copepods are primary consumers and perhaps the most common metazoans in the ocean (Feinberg and Dam, 1998). Copepods, especially their nauplii, are natural prey items for larvae of most fish (Kahan, 1992; McKinnon et al., 2003; Chen et al., 2006), typically making up fifty percent or more of their stomach contents (Støttrup, 2000). This makes them attractive candidates for mass culture as live feeds for marine larvae culture in aquaculture hatcheries (Shansudin, et al., 1997; McKinnon et al, 2003; O'Bryen and Lee, 2005).

To date, marine hatcheries are largely dependent on the production of rotifers (*Brachionus* spp.) and brine shrimp (*Artemia* spp.) as live feeds for fish and crustacean larvae (Støttrup et al., 1986; Jepsen et al., 2007). However, both rotifers and *Artemia* have been shown to possess sub-optimal nutritional profiles and are often too big for first feeding fish larvae with small mouth gapes

[2] Chapter 4 is adapted from Camus T, Zeng C (2009) The effects of stocking density on egg production and hatching success, cannibalism rate, sex ratio and population growth of the tropical calanoid copepod *Acartia sinjiensis*. Aquaculture 287, 145-151

(Payne and Rippingale, 2001; Knuckey et al., 2005; O'Bryen and Lee, 2005). In contrast, copepods have been proven to promote healthy development and improved survival for a variety of cultured fish larvae (Drillet et al., 2006; VanderLugt and Lenz, 2008), including tropical snappers (*Lutjanide* spp.) and groupers (*Serranide* spp.) (Schipp et al., 1999; McKinnon et al., 2003; Peck and Holste, 2006). The inclusion of copepods in larval diets has also been linked to improved pigmentation and increased resistance to stress (Næss et al., 1995; Knuckey et al., 2000).

Despite obvious advantages of copepods as live feeds for marine larvae, their utilization in hatcheries remains limited (VanderLugt and Lenz, 2008; O'Bryen and Lee, 2005). This can largely be attributed to the fact that methods/systems capable of producing copepods in large quantity and in a reliable, economically efficient way, have not yet been established (Kahan, 1992; Støttrup, 2003). The lack of research and knowledge in this field has contributed to the slow progress in copepod utilization in hatcheries.

Calanoid copepods are generally believed to have low tolerance to poor water quality (Payne and Rippingale, 2001); their cultivation at high densities is therefore considered difficult due to density-related stress factors (Jepsen et al, 2007). Nevertheless, only a very limited number of calanoid species have been thoroughly investigated for their potential as live feed, with attention rarely being directed to relevant biological endpoints, such as species-specific carrying capacity and density dependence (Sibly et al., 2000). As numerous and diverse as copepods are, it is likely that copepod species with good potential for high density culture are yet to be discovered (O'Bryen and Lee, 2005).

Past studies investigating effects of copepod stocking density on their culture productivity have mainly assessed egg production and egg hatching success and used them as major indicators (Medina and Barata, 2004; Peck and Holste, 2006; Jepsen et al., 2007). However, other biological parameters, such as adult sex ratio (Voordouw et al., 2005), cannibalism rate and population growth over time (Milione and Zeng, 2007, 2008) represent other important criteria. The combined knowledge of all these parameters are likely to provide more comprehensive information and better insights into how density may affect copepod productivity in culture.

Acartia sinjiensis is a calanoid copepod indigenous to the coastal and estuarine waters of tropical North Queensland, Australia (McKinnon et al., 2003). As a natural prey for larvae of tropical fishes and crustaceans, it is an amenable species for aquaculture hatcheries (Milione and Zeng, 2008). As a part of a series of laboratory studies aimed at optimizing culture conditions of *A. sinjiensis* (Knuckey et al., 2005; Milione and Zeng, 2007; Milione and Zeng, 2008), the present study evaluated the effects of stocking density on various productivity-related parameters, including egg production, egg hatching success, adult cannibalism upon nauplii, sex ratio and population growth of *A. sinjiensis*.

4.2 Materials and methods

4.2.1 General procedure

All experiments were conducted at MARFU, James Cook University. Throughout all experiments, water temperature was maintained at 30±1°C and salinity at 30 ±1‰ while light regime was set at 18L:6D. Such conditions were determined by previous experiments as optimal for the culture of *A. sinjiensis* (Milione and Zeng, 2008; Chapter 3 from current thesis). Observations and counting of eggs, nauplii, copepodites and adults were made using a Sedgewick-Rafter counter and a Leica CME optical microscope (model TN-PSE30).

Two previous studies reported that *A. sinjiensis* can reach a culture density up to 2,000 individual per liter (i.e. 2 ind./ml) (McKinnon et al., 2003; Milione and Zeng, 2007). This density was therefore selected as the highest stocking density of the following density experiments and five densities of 125, 250, 500, 1000 and 2000 ind./l were set up for all subsequent experiments.

Voordouw et al. (2005) reported that algal concentration could affect both sex ratio and size of adult calanoid copepods. To ensure that food did not become a limiting factor, algal concentration was carefully maintained in excess across all replicates throughout all experiments. To achieve this, a pilot trial was conducted to determine a suitable daily ration of algae that should be provided. This was done by setting up *A. sinjiensis* cultures at the highest stocking density to be tested (2000 adults/l) and fed them with T-iso and *T. Chuii* at various concentrations (3 replicates per treatment). Algal counts were then performed 24 hours later to

determine remaining algal densities. The result showed that after 24 h of grazing, the daily feeding ration of 80.000 cells/l T-iso + 6250 cells/l *Tetraselmis Chuii* resulted in remaining algal concentration in culture vessels close to the optimal density for *A. sinjiensis* culture (i.e. 40.000 cells/ml T-iso plus 3125 cells/ml *T. Chuii*, Milione and Zeng, 2007). This feeding ration was therefore chosen as the standard feeding ration across all density treatments for subsequent experiments.

4.2.2 Egg production experiment

The effects of initial stocking density on daily egg production rate of *A. sinjiensis* were investigated over 12 consecutive days. Healthy, actively swimming adults (CVI) and final stage copepodites (CV) were isolated from stock cultures and randomly distributed in 200 ml culture vessels at stocking densities of 125, 250, 500, 1000 and 2000 ind./l respectively. Five replicates were set up for each treatment. Previous study has shown that at 30°C, it took less than 24 h for *A. sinjiensis* CV copepodites to develop into adults (CVI) (see Chapter 3). The experiment was therefore started 24 h after *A. sinjiensis* were stocked into individual culture vessel. This not only ensured that all *A. sinjiensis* in the experimental vessels were adults when the experiment commenced, but also allowed 24 h acclimatization to the experimental condition for the copepods.

Following the method described by Medina and Barata (2004), 24 h egg production was monitored and recorded for 12 consecutive days for each replicate. This was done by gently filtering the content of each replicate firstly through a 100 μm mesh sieves (to retain adults but allow egg and nauplii to pass through, the use of smaller 100 μm mesh was to ensure all adults were retained) and then a 25-μm mesh sieves (to retain eggs and nauplii). The adults retained on 100 μm mesh were promptly returned to their respective culture vessels filled with fresh algae and seawater (1 μm-filtered, UV irradiated), eggs and recently hatched nauplii separated out on 25-μm mesh were resuspended in 20 ml seawater and fixed with 10% formalin for later counting. This procedure ensured no new recruitments were added to the initial stocked adult population.

The initial sex ratio of *A. sinjiensis* population used for the egg production experiment was determined prior to the experiment by randomly siphoning 50 adults for sexing under a

microscope. The sex ratios from 5 replicates were averaged and the mean was used for the calculation of the mean egg output per female on day 1 using the following formula:

$$\text{Mean egg production per female} = \frac{\text{Total no.of eggs produced daily in a replicate}}{(\text{Total no.of adults} * \text{female \% ratio of the population})}$$

On the final day of the experiment, all surviving *A. sinjiensis* were counted for each replicate to allow the calculation of adult survival rate over the 12 day period. The surviving adults were also sexed for the computation of 24 h egg production per female on the final day of the experiment.

4.2.3 Egg hatching success experiments

To assess hatching rates of eggs produced by *A. sinjiensis* reared under different stocking densities, *A. sinjiensis* cultured at five stocking densities of 125, 250, 500, 1000 and 2000 ind./l (5 replicates per treatment) were set up as described in Section 4.2.1. All cultures were maintained similar to the egg production experiment for 3 days, i.e. egg and nauplii removed from culture and fresh food and water added daily. On day 4, eggs produced over the last 24 h were collected from each replicate. They were then briefly submerged in fresh water (approx. 2.5 min.) to kill any post-egg-stages of *A. sinjiensis* (Knuckey et al., 2005) before being put back into seawater. Similar numbers of eggs (around 100) from each replicate were then counted and placed in 50 ml sealed plastic flasks filled with filtered seawater (1 µm) for incubation at 30± 1°C and under a 18L:6D photoperiod.

Hatching success experiments were run twice with eggs incubated for 48 and 96 h respectively. After incubation time, all hatching containers were emptied and their content fixed with 10% formalin. Unhatched eggs in each replicate were counted under a microscope and the Egg Hatching Success (EHS) was subsequently calculated as:

$$\text{EHS (\%)} = \frac{[(\text{No. of eggs introduced initially} - \text{No. of unhatched eggs}) * 100]}{\text{No. of eggs introduced initially}}$$

4.2.4 Cannibalism experiment

The effect of adult stocking density on cannibalism rate toward newly hatched nauplii was assessed in a separated experiment. Eggs of *A. sinjiensis* were siphoned out from stock cultures, rinsed in fresh water for 2.5 minute to kill all post-egg-stages and incubated at 30± 1°C for 24 h

in filtered seawater (1 µm). Newly hatched nauplii (hatched within 24 h) were then isolated from unhatched eggs by attracting them to a light source utilizing their positive phototoxic behaviour. The nauplii collected were subsequently counted and an identical number of 60 nauplii (i.e. 300 nauplii/l) were randomly introduced into each of twenty-five 200 ml replicate vessels (5 replicates per treatment)

Using a 250 µm mesh sieve, the predators consisting of late copepodites (CV) and adults, were pre-isolated from stock culture and suspended in Petri dishes with fresh microalgae. After nauplii had been distributed into each replicate vessel, the pre-isolated adults and copepodites were then counted and added to each replicate vessel to form 5 densities of 125, 250, 500, 1000 and 2000/l, respectively. Fresh T-iso and *Tetraselmis chuii* were added to all replicates *ad labium,* as in Section 4.2.1.

To avoid possible confounding effects of nauplii hatched out from eggs produced by adult females introduced as predators, a pilot experiment was conducted to determine a suitable experimental duration for the cannibalism experiment. The pilot experiment assessed the hatching rates of *A. sinjiensis* eggs (laid within 24 h) after 4, 8, 12 and 16h incubation periods respectively. The result showed that only 0.5% of the eggs had hatched after 8 hours of incubation, and hence an experimental duration of 8 h for the cannibalism experiment was determined. At the end of the 8 hours experiment, content of each replicate was collected on 40 µm mesh sieve and fixed with 10% formalin. The remaining nauplii were then counted using a dissecting microscope. As it has been shown that under similar culture conditions, survival of *A. sinjiensis* nauplii were normally very high (cumulative survival of 5 naupliar stages >95%, Authors' unpublished data), the difference between initial and final number of the nauplii in each replicate was assumed due to cannibalism.

4.2.5 Sex ratio and population growth experiment

With five different initial stocking densities (125, 250, 500, 1000 and 2000 adults/l, 5 replicates per treatment), population growth of *A. sinjiensis* over 12 days was examined. The initial set up of the cultures was similar as described in the section 4.2.1. During the 12 day experimental period, 20% of the culture water was exchanged daily for each replicate by gently siphoning. The siphon has a 25 µm mesh attached at the end to prevent the removal of any eggs or post-egg-

stages of *A. sinjiensis* from the culture. Algae were added daily at a pre-determined ration (4.2.1) before the addition of fresh seawater. As under such culture condition, average adult life expectancy of *A. sinjiensis* is known to be about 10.5 days (Chapter 3), the experiment was hence run for 12 days to ensure that the final populations mainly consisted of new generations and that the sex ratio of adults stocked initially had limited effects on the final sex ratio. After 12 days, contents from each replicate were emptied onto a 25 μm mesh and all eggs, nauplii, copepodites and adults retained on the mesh were fixed with 10% formalin for later counting. Adults were also sexed to obtain the sex ratio of the final population.

4.2.6 Data collection and analysis

For the convenience of presentation and easy comparison between results, data that were a function of culture volume (i.e. total daily egg production and cumulative egg production), were converted to 1 l of culture water. All data were verified to have met parametric test assumption (e.g. normally distributed, homogeneity of variance, independent and randomness of the data). Egg hatching success, nauplii cannibalism and percentage increases in final populations were analyzed using one way ANOVA while mean egg production data were analyzed with 2-factor blocked ANOVA. If any significant differences were detected ($p<0.05$), Tukey's multiple comparison test was performed to determine specific differences among treatments.

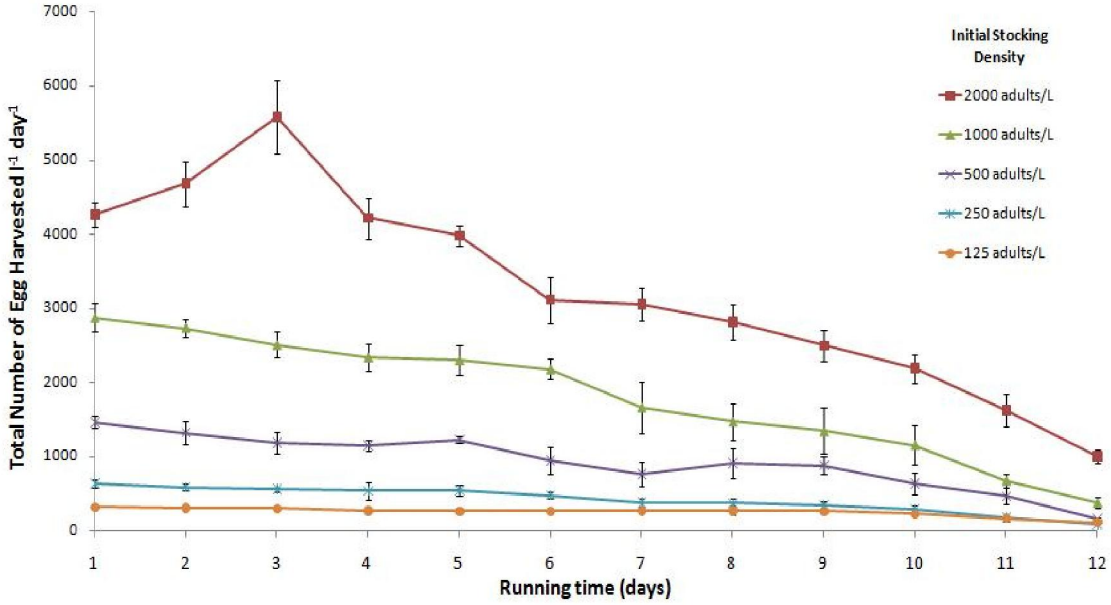
The sex ratio data was log transformed and pooled across all replicates before being analyzed for significant differences among treatments using the Chi square test. All statistical analyses were conducted using Statistica, version 7. Data are presented as mean ± standard error (SE).

4.3 Results

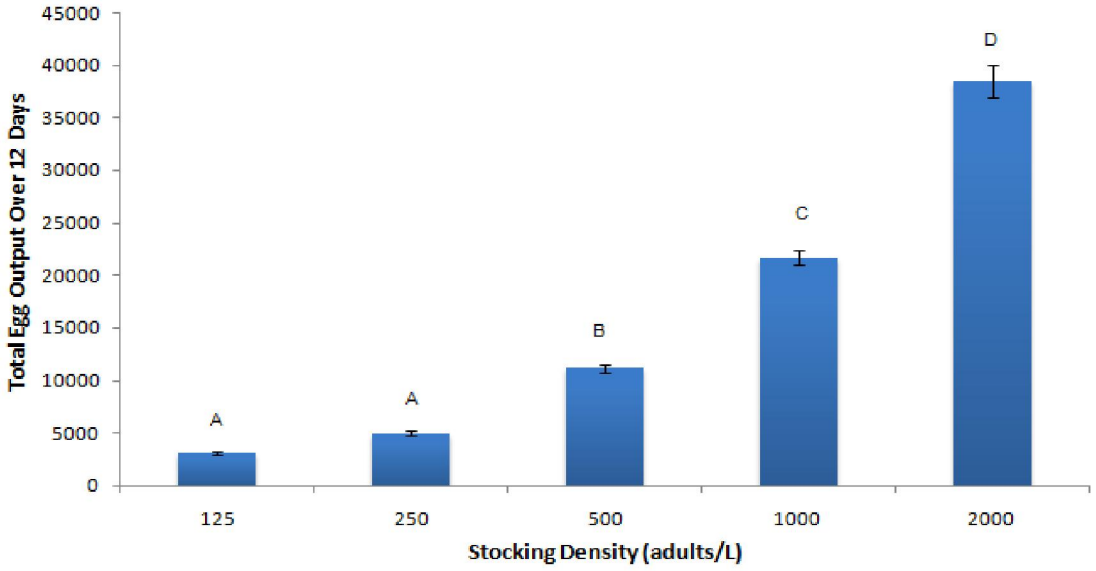
4.3.1 Egg production experiment

Over 12 consecutive days, a general trend of gradual decrease in total daily egg production was observed for all densities tested (Fig 4.1a). The only exception was that at the highest density treatment (2000 adults/l): daily egg production increase for the first 2 days, but was followed by a steep decrease during the subsequent 3 days (Fig. 4.1a). As expected, the accumulated total egg production over the 12 days at various stocking densities was significantly different, with higher

stocking densities generally producing significantly more eggs (Fig. 4.1b) ($p<0.001$). However, an exception was found at the two lowest density treatments where no significant differences in cumulated egg production were found (Fig. 4.1b) ($p>0.05$).



a)



b)

Figure 4.1: Effects of initial adult stocking density (n=5) on egg production of *Acartia sinjiensis*. Data are presented as mean ± SE. **a)** daily total egg production over 12 consecutive days; **b)** accumulated total egg output over the 12 days. Different letters on the tops of bars indicate significant differences ($p<0.05$).

To avoid excessive handling of the animals, mortality of *A. sinjiensis* was not checked every day over the 12 day experimental period. However, at the end of the experiment, final number of surviving *A. sinjiensis* in each replicate was counted, enabling the calculation of survival over the 12 day period for each treatment (Fig. 4.2). The results revealed that stocking density significantly impacted the cumulative survival of adult *A. sinjiensis* ($p<0.05$) over the 12 days. The highest survival was found at the lowest density of 125 adults/l, which was significantly higher than that of 250, 500 and 1000 adults/l treatment (Fig. 4.2). However, no significant differences was found among all other treatments, including those of the 125 and the 2,000 adults/l treatments (Fig. 4.2).

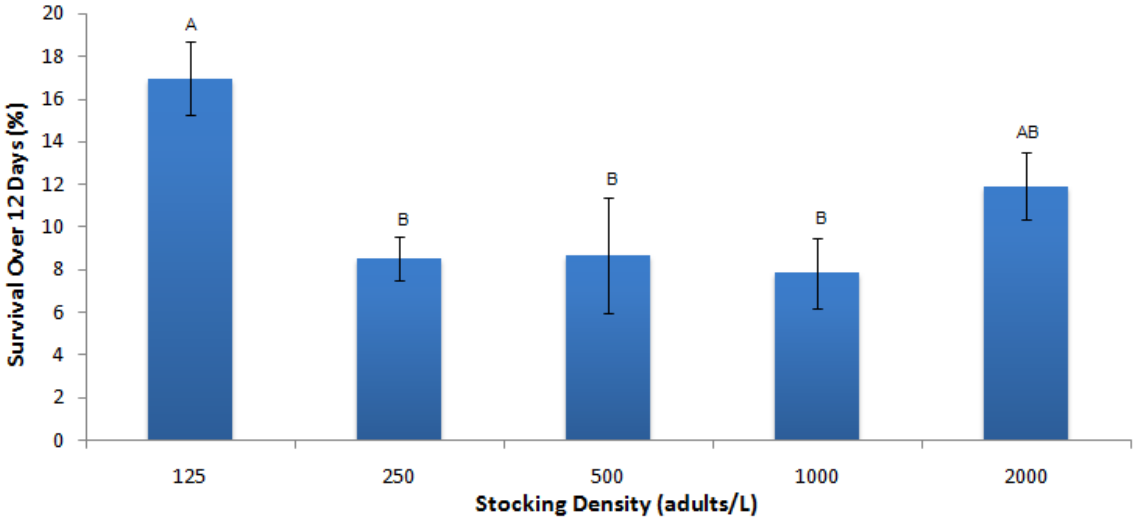


Figure 4.2: Percentage survival of *A. sinjiensis* adults over 12 days under different initial stocking densities. Data are presented as mean ± SE. Different letters on the tops of bars indicate significant differences ($p<0.05$).

More than 95% of the surviving *A. sinjiensis* on the final day of the 12 day experiment were females. Based on the number of surviving females within each replicate on the final day of the experiment, mean 24h egg production rates (#eggs/female/day) on the final day of the trial were calculated and presented, together with those of the first day (Fig. 4.3). Average daily egg production per female was neither statistically different among the tested densities for both the first and the last day of the experiment ($p>0.05$), nor was any significant changes in average egg

production found between the first and the final day of the experiment within each density treatment ($p<0.005$) (Fig. 4.3). This suggested that *A. sinjiensis* females reproductive capacity was unaffected by stocking density.

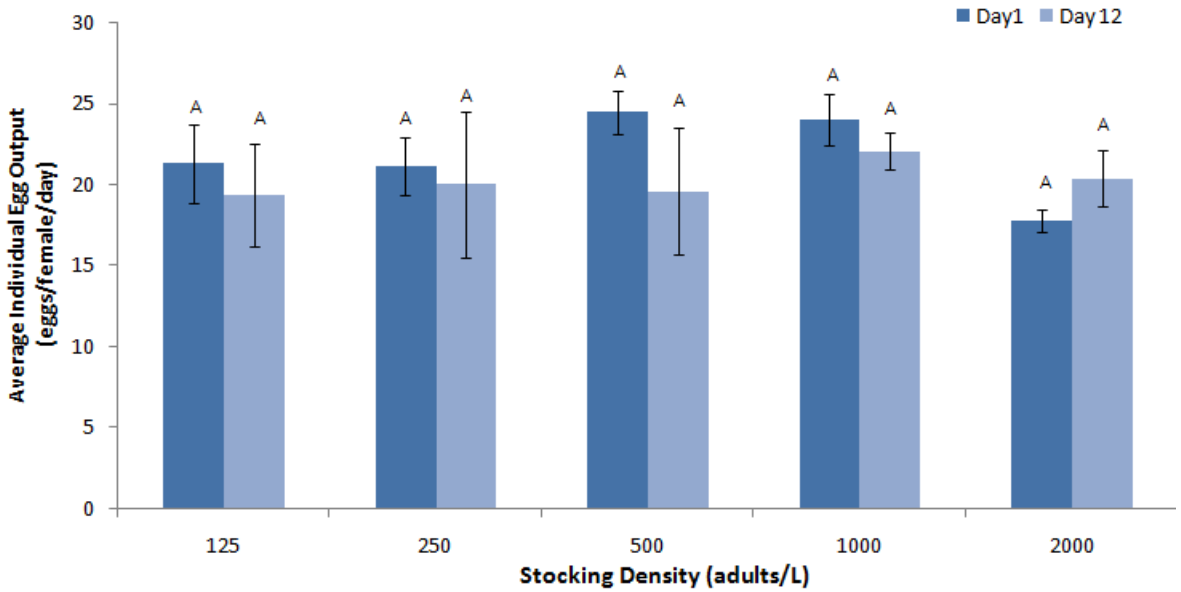


Figure 4.3: Under different initial stocking densities, average daily egg production per female *A. sinjiensis* on the first and the last day of a 12 day culture period. Data are presented as mean ± SE. Different letters on the tops of bars indicate significant differences ($p<0.05$).

4.3.2 Egg hatching success experiments

Hatching rates of *A. sinjiensis* eggs produced under different stocking densities were examined following 48 and 96 h incubation periods. Stocking density was found to significantly impact 48 h egg hatching success ($p<0.001$) (Fig. 4.4). The 48 h hatching rates increased steadily from 60.9 ± 2.3% for the highest density treatment to 87.2 ± 2.2% for the lowest stocking density treatment. Eggs produced at the highest stocking density (2000 adults/l) had a significantly lower hatching rate than all other treatments and egg hatching success of the second highest stocking density (1000 adults/l) was also significantly lower than that of the 125 and 250 adults/l treatments. However, no significant differences was found between 500 and 1000 adults/l treatments or among the 125, 250 and 500 adults/l treatments (Fig. 4.4).

In contrast to 48 h hatching data, no significant differences was detected among density treatments for 96 h egg hatching rates ($p>0.05$) although the lowest hatching was again recorded for the eggs produced under the highest density (2000 adults/l: 91.3 ± 3.4%) and the highest hatching rate found for the eggs produced under the lowest density (125 adults/l: 96.5 ± 0.8%) (Fig. 4.4).

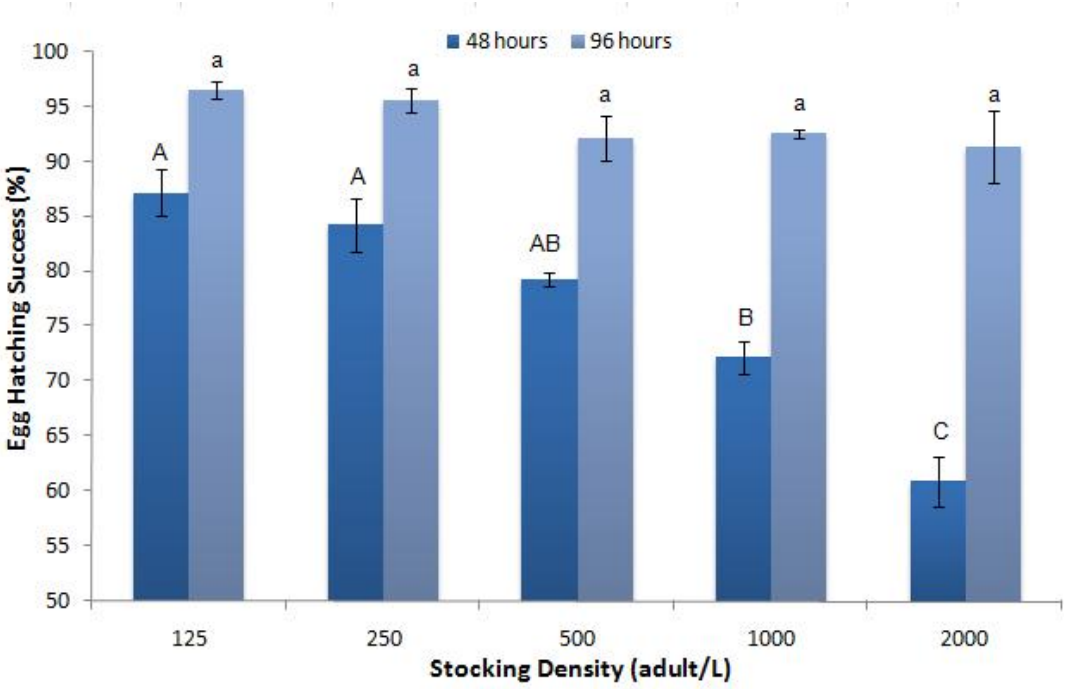


Figure 4.4: The 48 and 96 hours egg hatching success of eggs produced by *A. sinjiensis* females cultured at different stocking densities. Data are presented mean ± SE. Different letters on the tops of bars indicate significant difference ($p<0.05$).

4.3.3 Cannibalism experiment

Predation of *A. sinjiensis* adults and late copepodites (CV) on stage 1 and 2 nauplii (NI and NII) increased steadily with increasing stocking density ($p<0.05$) (Fig. 4.5). Statistical analysis showed that cannibalism rate on nauplii was significantly higher at the highest stocking density of 2000 ind./l when compared to that of the 125 and 250 ind./l treatments ($p<0.05$) while no significant difference was found among other treatments ($p>0.05$) (Fig. 4.5).

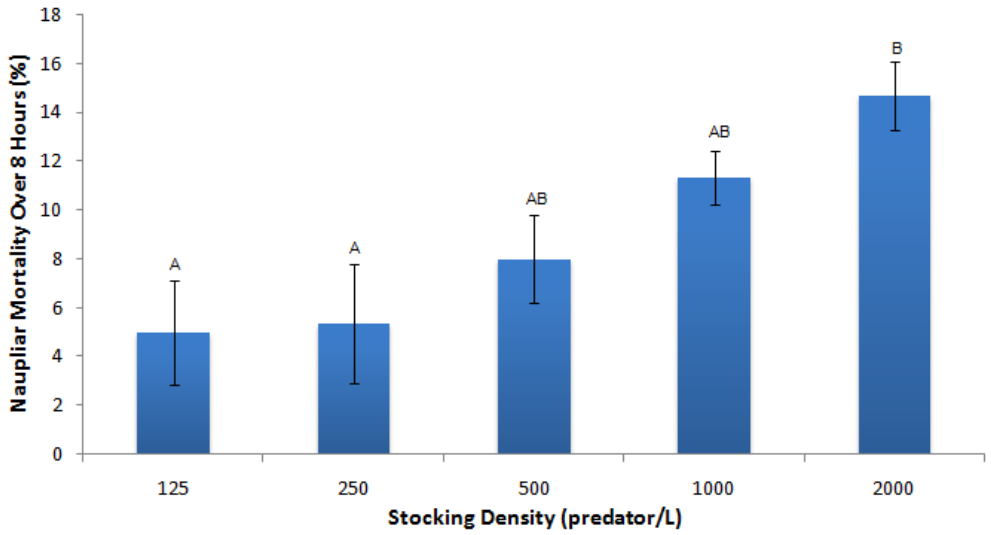


Figure 4.5: Naupliar mortality rates over 8 hour period at different *A. sinjiensis* stocking densities (n=5). Data are presented as mean ± SE. Different letters on the tops of bars indicate significant differences ($p<0.05$).

4.3.4 Sex ratio and population growth experiments

4.3.4.1 Sex ratio

No significant difference in sex ratio was found among all density treatments for the final populations of *A. sinjiensis* over the 12 day culture period ($p>0.05$, Fig. 4.6). For all stocking densities tested, a skewed sex ratio toward female was observed (Fig. 4.6).

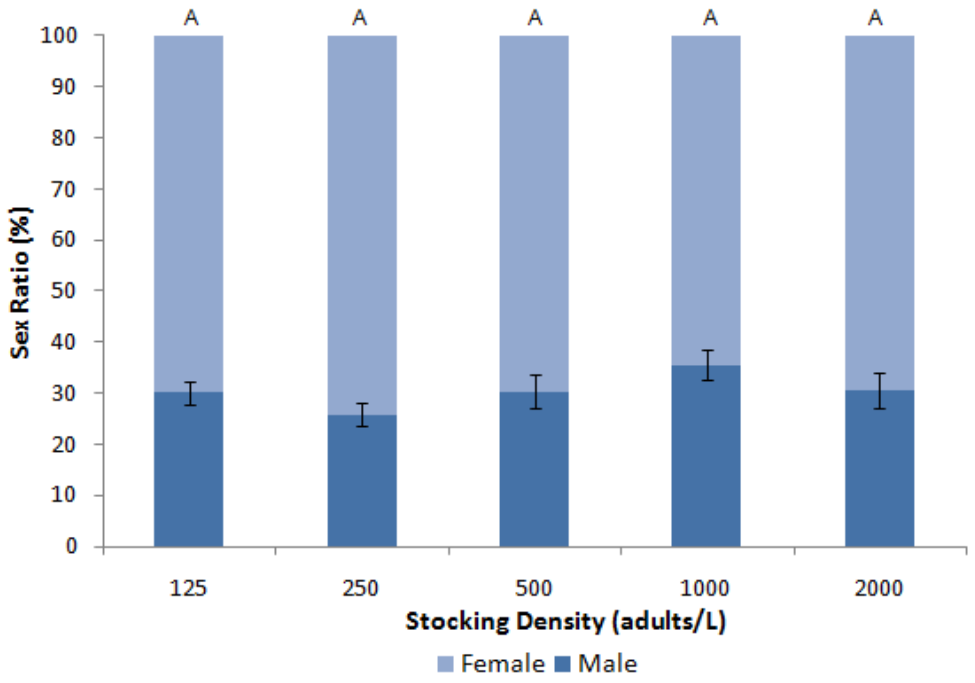


Figure 4.6: Sex ratio of *A. sinjiensis* at the end of a12 day culture period under different initial stocking densities (n=5). Data are presented as mean ± SE. No significant differences was detected among treatments ($p>0.05$).

4.3.4.2 Population growth

For all density regimes tested, adult populations had either increased from or sustained its initial level over the 12 day culture period, except for the 500 ind./l treatment in which a slight decrease (- 13%) in adult population was observed (Table 4.1).

When all post-egg-stages were considered, a substantial increase in population number was found across all density treatments with a clear trend of higher percentage increases at lower initial stocking densities. The percentage increases in population number at the two lowest stocking densities (470% and 408% for 125 and 250 adults/l respectively) were significantly higher than that of the highest stocking density at 2000 adults/l (171%) ($p<0.05$) (Table 4.1). However, if eggs were included in the final population counts, such a trend disappeared with more similar percentage increases, ranging from 1960% to 2584%, found among all treatments (Table 4.1). Overall, the population growth data suggested that *A. sinjiensis* is capable of sustaining a culture density as high as or higher than 2000 adults/l.

Table 4.1: Final population of *Acartia sinjiensis* cultured over a 12 days period at different initial stocking density.

Population Composition	Initial Stocking Density (adults/l)	Final Population Number mean ± S.E. (ind./l)	Percentage Increase Over 12 Days means ± S.E. (%)
Adult Only	2000	2472 ± 253	124 ± 13% ^{A}
	1000	1339 ± 371	134 ± 37% ^{B}
	500	436 ± 132	87 ± 26% ^{C}
	250	254 ± 58	102 ± 23% ^{C}
	125	137 ± 44	110 ± 39% ^{C}
All post-egg-stages (excluding eggs)	2000	3425 ± 333	171 ± 17% ^{a}
	1000	2439 ± 446	244 ± 45% ^{ab}
	500	1495 ± 151	299 ± 30% ^{bc}
	250	1020 ± 162	408 ± 65% ^{c}
	125	587 ± 150	470 ± 120% ^{c}
Population including all stages and eggs	2000	50540 ± 2562	2527 ± 128% ^{A}
	1000	19593 ± 1528	1960 ± 153% ^{A}
	500	10861 ± 1700	2172 ± 340% ^{A}
	250	6461 ± 523	2584 ± 209% ^{A}
	125	2915 ± 625	2332 ± 500% ^{A}

Based on final population data, population growth of *A. sinjiensis* over 12 day culture period was regressed against the initial stocking density (Fig. 4.7). Population growth was presented as the population of all stages including eggs, population of all post-egg-stages, and adult only (Fig. 4.7).

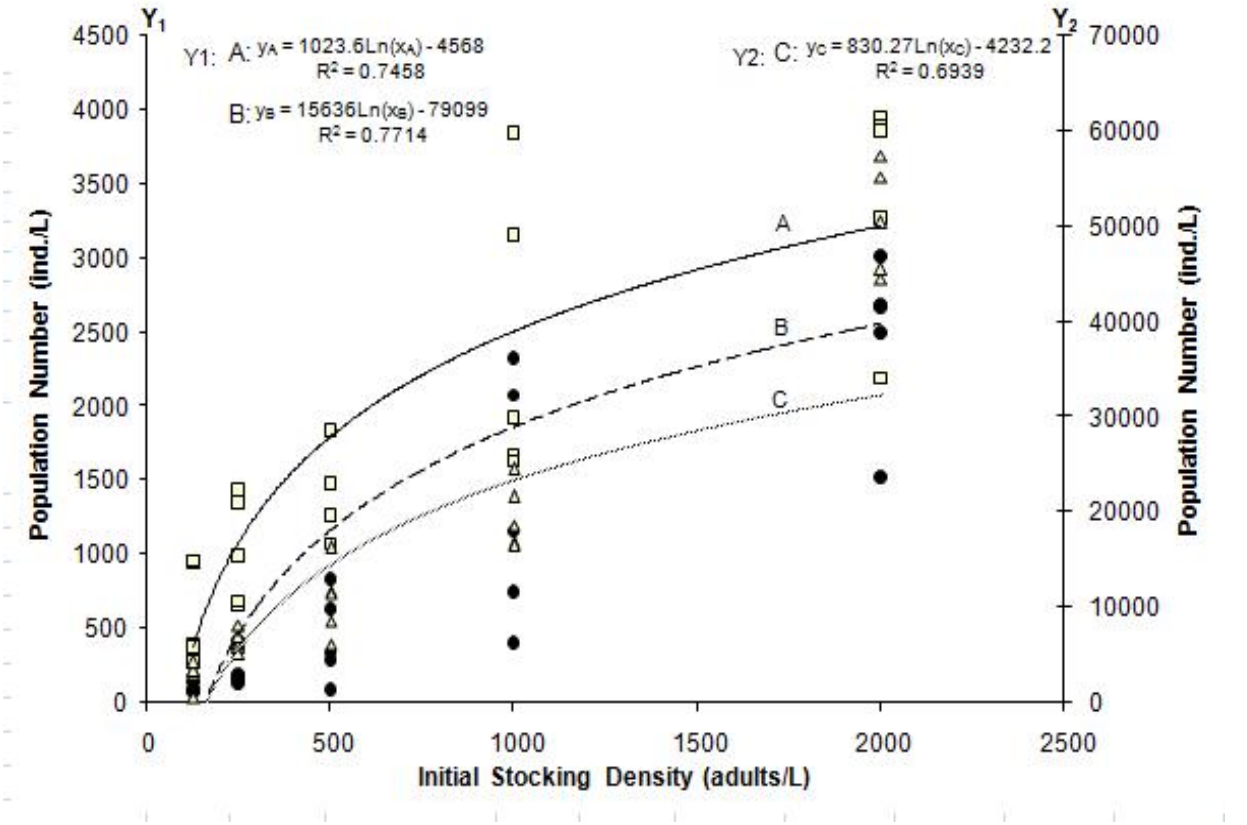


Figure 4.7: Population growth curves of *A. sinjiensis* over a 12 day period under different initial stocking densities. A - population of all post-egg-stages excluding eggs (●); B – population of adults only (□); and C - population including all stages and eggs (Δ). Due to substantial differences on total population numbers, A and B are shown on Y_1 axis while C is shown on Y_2 axis.

4.4 Discussion

The inability to culture copepods, particularly calanoid species, at a sustainable high density is a major bottleneck that hinders their utilization in aquaculture hatcheries, despite their perceived many advantages as larval prey. Yet, only a few studies so far have investigated the effects of stocking density on copepod productivity and aimed at their application in aquaculture (e.g. Calanoids: Medina and Barata, 2004; Jepsen et al., 2007; Peck and Holste, 2006). Other publications focused on the ecological implications of copepod density in their natural environments, which normally dealt with substantially lower densities (e.g.: Shahidul and Tanaka, 2007). Table 4.2 summarizes literature on copepod density studies that are pertinent to aquaculture.

Table 4.2: Summary of the past literature investigating the stocking density potential of various copepods species.

Species (order)	Stocking Density Tested	Culture Condition	Reference
Acartia plumosa (calanoid)	2000-5150 nauplii/l 1000 adults/l	Laboratory	Schipp et al., 1999
Acartia tsuensis (calanoid)	1136 nauplii/l 280 adults/l	Outdoor	Ohno & Okamura, 1988
Acartia tonsa (calanoid)	232 adults/l	Outdoor	Ogle, 1979
Acartia tonsa (calanoid)	50-400 adults/l	Laboratory	Peck & Holste, 2006
Acartia tonsa (calanoid)	50-100 adults/l	Laboratory	Støttrup et al., 1986
Acartia tonsa (calanoid)	2000 adults/l	Laboratory	Medina and Barata, 2004
Acartia tonsa (calanoid)	100-600 adults/l	Laboratory	Jepsen et al., 2007
Acartia sinjiensis (calanoid)	125-2000 adults/l	Laboratory	Present study
Gladioferens imparipes (calanoid)	520 nauplii/l	Laboratory	Payne & Rippingale, 2001
Bestiolina similis (paracalanid)	270-600 ind./l	Laboratory	VanderLugt & Lenz, 2008
Schizopear elatensis (harpacticoid) *Tisbe sp. (harpacticoid)*	37,000-100,000 ind./l	Laboratory	Kahan et al., 1982
Euterpina acutifons (harpacticoid)	20,000-50,000 adults/l	Laboratory	Kraul, 1989
Tisbe holothuriae (harpacticoid)	8,000 adults/l	Laboratory	Stottrup & Norsker, 1997
Tigriopus californicus (harpacticoid)	500-3000 nauplii/l	Laboratory	Voordouw et al., 2005
Tigriopus japonicus (harpacticoid)	10,000-22,000 adults/l	Semi-extensive outdoor tanks	Fukusho, 1980
Tisbe battagliai *Tisbe holothuriae* (harpacticoid)	10,000-20,000 ind./l	Laboratory	Sibly et al., 2000

High stocking density has been linked to reduction in female egg production for calanoid copepods (Zhang and Uhlig, 1993). For example, an inverse relationship between egg production and culture density has been reported for *A. tonsa* (Medina and Barata, 2004: 500 to 2000 ind./l; Peck and Holste, 2006: 50 to 400 ind./l). Similarly, VanderLugt and Lenz (2008) found that female fecundity was inversely related to stocking density for the paracalanoid *Bestiolina similis.* Such decreases in egg production at high stocking density have been attributed to increased competition for food and space, resulting in lower food intake and higher energetic costs associated with increased swimming activity (Sibly et al., 2000). However, in separated experiments, Voordouw et al. (2005) and Jepsen et al. (2007) found no evidence that stocking density significantly affected female egg production for *A. tonsa* (density range tested: 100 to 600 adults/l) and *Tigriopus californicus,* respectively. Similarly, our result showed that average egg production of *A. sinjiensis* females was not adversely affected by a stocking density as high as 2,000 adults/l. These contrasting results indicate that the effects of stocking density on female egg production are probably species-specific or strain-specific for copepods. Therefore the effects of stocking density on copepod egg production should probably be examined on a case by case basis by making sure that feeding rates are not a limiting factor among treatments. The fact that daily egg production of. *A sinjiensis* was not adversely affected by high stocking density suggests that *A. sinjiensis* is a very promising species for intensive culture for aquaculture hatcheries.

Ohman and Hirche (2001) reported a non-linear, density dependent mortality for *Calanus Finmarchicus* in open-ocean, while on the other hand, Medina and Barata (2004) and Jepsen et al. (2007) found no clear links between mortality and stocking density for *A. tonsa* (500-2000 ind./l and 50-600 ind./l, respectively). With a wider range of density tested in the current experiment, survival over a 12 day period was significantly higher at the lowest density of 125 adults/l. However, no significant difference was found among higher stocking densities of 250, 500, 1000 and 2000 adults/l. It is not clear why the survival of the lowest and the highest densities tested was also not significantly different.

Stocking density significantly affected 48 h egg hatching success of *A. sinjiensis*. However, when eggs were allowed a longer incubation period of 96 h, hatching rates across all stocking densities were not significantly different. The hatching process in *A. sinjiensis* appears to be

delayed in eggs produced at higher stocking densities, since their hatchability over a longer incubation period was not affected. Such a delaying effect on hatching may have its evolutionary value in natural environment, as it may help newly hatched nauplii avoid temporal overcrowding condition (e.g. well known zooplankton 'patchy distribution' phenomenon (Omori and Hamner, 1982)) that could result in high competition for food and space. It also suggested that longer incubation period should probably be adopted for future *A. sinjiensis* hatching success experiments to account for such potential delaying effects.

Sex in calanoid copepods is probably determined genetically during meiosis through an X-Y chromosome mechanism (Gilbert and Williamson, 1983) and is also known to be influenced by temperature (Voordouw and Anholt, 2002) as well as food levels during larval development (Voordouw et al., 2005). *A. sinjiensis* adult sex ratio was not significantly affected by stocking density and was always skewed in favor of females. Similarly, Voordouw et al. (2005) found that stocking density had no significant impacts upon sex determination of *T. californicus*.

Productivity of *A. sinjiensis* in intensive culture is not only linked to daily egg production and hatching rate, but also to the subsequent recruitment success of nauplii. The genus *Acartia* is known to be cannibalistic (Gallucci and Ólafsson, 2007), which could in turn negatively impact culture productivity under controlled conditions. Despite this, no study has been conducted previously on the cannibalism behaviour of *A. sinjiensis*. Cannibalism rate is generally reported to increase with increased stocking density (Gallucci and Ólafsson, 2007). For example, Hada and Uye (1991) demonstrated that cannibalism rate increased asymptotically with naupliar and copepodite density (as prey) for *Sinocalanus tenellus* and Ohman and Hirche (2001) found that egg mortality of *C. finmarchicus* was related to the abundance of adults in the field. Results from current research confirmed such a trend for *A. sinjiensis,* with a significantly higher predation rates toward nauplii found at the densest predator treatment of 2000 ind./l when compared to that of the lowest density treatments of 125 and 250 ind./l. It is important to consider that egg cannibalism might have provided a slight bias in the data of this trial. However, the fact that no empty egg membrane were found throughout the trial is a strong indication that *A. sinjiensis* do not engage in egg cannibalism

To obtain a more complete picture on various interacting factors contributing to the final productivity of *A. sinjiensis* in intensive culture, population growth of *A. sinjiensis* over a 12 day

culture period with different initial stocking densities was assessed. The results showed that *A. sinjiensis* is capable of sustaining a high culture density of 2000 ind./l and likely higher. In fact, the *A. sinjiensis* strain used in present experiment had been isolated and cultured for more than 1.5 years in our laboratory. Based on samples counted from the stock cultures, peak densities as high as 5000-7000 ind./l for all post-egg-stages were sustained for many days. Few copepod species, particularly calanoids, have been tested so far to assess their potential for high density culture (O'Bryen and Lee, 2005). With the huge diversity of copepods species, it would not be surprising if more species that could tolerate crowding culture condition are discovered in future. For example, a naupliar density as high as 5150 ind./l for *Acartia plumosa* has been reported by Schipp et al. (1999). Clearly, more copepod species should be tested on this regard.

As a conclusion, it is worth noting that when considering percentage increase in population of all post-egg-stages of *A. sinjiensis* over 12 days, a trend of higher rates of increase at lower stocking densities was obvious. Such results probably could be attributed to higher cannibalism rate toward nauplii as well as a delayed hatching process under crowded culture condition. The highest adult survival rate found at the lowest density (125 adults/l) can also be considered as a likely contributor to the highest percentage increase registered under that density. Based on the present results, we recommend *A. sinjiensis* to be cultured at high stocking density to save space and labor. However, the optimal inoculation density to be adopted would obviously depending on the needs of a particular hatchery, taking into consideration various factors, such as quantity of available starter *A. sinjiensis* for inoculation and culture duration allowed prior to expected time of larval hatching. The population growth regression lines showed in Figure 4.7 provides a useful tool for such a decision making process.

CHAPTER 5

ADULT CANNIBALISM IN THE CALANOID COPEPOD *ACARTIA SINJIENSIS*: THE INFLUENCE OF PREY DEVELOPMENTAL STAGE, DENSITY, PREDATOR FEEDING CONDITION AND SEX

5.1 Introduction

Knowledge of copepod feeding habits is not only critical for understanding trophodynamics of marine ecosystems (Conley and Turner, 1985), but also to develop suitable dietary practices for their intensive cultivation as live feeds for marine hatcheries. Feeding studies conducted on calanoid copepods have shown that all common genera include omnivorous species (Daan et al., 1988) that ingest a variety of prey items, including phytoplankton (Basedow and Tande, 2006), tintinnid protozoans (Gifford and Dagg, 1988), planktonic nonloriate ciliates (Jonsson and Tiselius, 1990), bacteria (Kang and Poulet, 2000), rotifers (Williamson and Butler, 1986) and detritus particles (Stoecker and Egloff, 1987). Cannibalism, defined as intraspecific predation (Fox, 1975), was also observed in a wide variety of calanoids, including *Acartia* spp. (Conley and Turner, 1985; Daan et al., 1988; Lazzaretto and Salvato, 1992; Gallucci and Ólafsson, 2007), with highly variable rates reported between species (see, Bonnet et al., 2004).

Copepod nauplii are rich in nutrients such as saturated fatty acid and free amino acids, and are hence very attractive food items for adult copepods (Kang and Poulet, 2000). Although cannibalism can prevent the extinction of a copepod population during periods of severe food limitation in the wild (Daan et al., 1988), this behaviour is detrimental in artificial culture settings as it negatively affects recruitment and productivity (Støttrup et al., 1986; Ohno et al., 1990). It is therefore of primary interest to investigate the cannibalistic behaviour of all copepod species candidates for mass cultivation.

So far, most investigations of cannibalism in calanoid copepods were conducted in the field (Daan et al., 1988; Conley and Turner, 1985; Hada, 1991; Uye and Lang, 1998; Sell et al., 2001) or in laboratory conditions with prey densities representative of natural settings (Gifford and Dagg, 1988; Kang and Poulet, 2000; Basedow and Tande, 2006). To the author's knowledge, only one study has investigated the impact of cannibalism under culture condition representative of intensive culture conditions (i.e. 2000 prey^{-1} l^{-1} for *Acartia clausii*) (Landry, 1978).

Acartia spp. are considered the best copepod candidates for aquaculture live feeds as they are fully pelagic, of an appropriate size and relatively easy to grow (O'Bryen and Lee, 2005). *Acartia sinjiensis* is distributed in coastal waters and estuaries across the Indo-Pacific region, especially in the mangroves of Northeast of Australia (McKinnon and Klumpp, 1998) and is a suitable candidate for aquaculture hatcheries (Doi et al., 1997; McKinnon et al., 2003; Knuckey et al., 2005). Field studies have reported that *A. sinjiensis* was able to feed opportunistically, either as a carnivore or as a suspension feeder (McKinnon and Klumpp, 1998). The present study was thus designed to investigate if cannibalism was part of *A. sinjiensis* carnivorous tendencies, and the factors that might affect it. As *A. sinjiensis* possess a high potential as live prey for marine hatcheries, it is important to quantify its cannibalistic tendencies in order to properly manipulate the culture conditions in order to alleviate negative impacts of cannibalism in their culture.

Using feeding experiments, *A. sinjiensis* adult cannibalism toward nauplii was investigated to determine its impact under various culture settings. Understanding the factors influencing cannibalism allows for the manipulation of the relevant culture parameter in order to alleviate cannibalism and thus improves culture productivity overtime. Specifically, the following question were considered through four experiments: 1) Is adult cannibalism observed in adult male and adult female? 2) Is adult cannibalism restricted to some naupliar stages? 3) Is

cannibalism influenced by the presence of microalgae and/or by prey concentration and at what prey concentration did adult cannibalism rate saturate? 4) Is cannibalism influenced by predator starvation duration?

5.2. Materials and Methods

5.2.1 General procedure

All experiments were conducted at Marine MARFU (Aquaculture Research Facility Unit), James Cook University. The water temperature was maintained at 27±1°C and salinity at 33 ±1‰ in all trials. Although the optimal light regime for *Acartia sinjiensis* productivity was determined to be 18L: 6D (Chapter 3), *Acartia* spp. are known to increase feeding activity in darkness (Stearns, 1986) and so the photoperiod regime was decreased to 12L: 12D to encourage feeding.

Sexually mature adult *A. sinjiensis* (i.e. CVI stage) were used as predators in all experiments, and were obtained as follow: *A. sinjiensis* stock cultures were gently siphoned onto a 150 µm submerged mesh sieve before being re-suspended in 1 µm filtered seawater. Adults were isolated from other developmental stages and sorted by sex using a Leica stereoscopic microscope (model TN-PSE30)

Nauplius instars were used as prey for all trials and were obtained as follow: after stopping aeration in the stock cultures for 30 minutes, unhatched eggs were siphoned from the bottom of the cultures onto a 25 µm submerged mesh sieve. The mesh was then rinsed in fresh water for 5 minutes to kill all post-egg-stages, after which eggs were split among covered Petri-dishes containing 1 µm filtered seawater and fresh microalgal food. Following a 24 hour incubation period, early nauplii (NI to NIII) were selected using a stereoscopic microscope (Leica CME). When later nauplii were needed (NIV to NVI), the incubation period was increased to 48 to 72 hours.

Control containers holding nauplii without predators were setup in all experiments to check for naupliar survival and handling loss. Survival of *A. sinjiensis* naupliar stages is quite high (> 95%) when cultured using optimal water quality parameters and provided with an appropriate microalgae diet (cumulative survival from newly hatched first stage nauplii (NI) to naupliar stage

5 (NVI), the final naupliar stage is > 95%, (Chapter 4)). Nauplii disappearing from a replicate during the duration of a trial can safely be assumed to result from adult predation.

All trials were run for a 24 hours duration, after which the content from each replicate was fixed using 10% formalin. Adult cannibalism rate (# nauplii adult^{-1} day^{-1}) was calculated as the difference between the number of nauplii initially introduced and the number of nauplii recovered at the end of the experiment (Daan et al., 1988), using a Leica CME stereo microscope. Based on Daan et al. (1988), dead nauplii found with marks of damage were considered to be cannibalised.

Considering the development duration of *A. sinjiensis* at 27± 0.5ºC (McKinnon et al., 2003), all of the NI and NII nauplii initially introduced as prey will develop to NIII or further stages during a 24 hours period. As a result, NI and NII nauplii found at the end of the trial were not taken into account for statistical analysis as they must have been produced by the predator females during the trial and. The production of nauplii by the predator females was confirmed by the presence of empty egg membrane(s) in replicate containers (Kang and Poulet, 2000).

5.2.2 Experiment A: Effect of predator sex on cannibalism rate on nauplii

This trial was run to determine if adult sex affects cannibalistic behaviour in *A. sinjiensis*. Adult males or females, with intact swimming appendages, were individually introduced into a 60 ml cylindrical containers filled with 50 ml of filtered seawater. Following the introduction of the predator, thirty early nauplii (NI and NII) were subsequently introduced into each container as prey. Ten replicates were conducted for each treatment and no microalgal food was provided to the replicate containers in order to encourage cannibalism. Containers were then set up in a rotating plankton wheel (rotation rate=60 cycles/hour).

5.2.3 Experiment B: Effect of naupliar developmental stage on cannibalism rate

This experiment was run to determine if *A. sinjiensis* cannibalism was confined to the early (NI, NII and NIII), or late naupliar stages (NIV, NV and NVI). Only *A. sinjiensis* adult females with

intact swimming appendages were selected as predators for this experiment. Individual CVI females were introduced haphazardly inside 60 ml containers filled with 50 ml of fresh filtered seawater. *A. sinjiensis* nauplii were divided in groups of early (NI to NIII) or late nauplii (NIV to NVI) before haphazardly selected and introduced inside the experimental containers (30 prey per replicate). As for the previous experiment, ten replicates were conducted per treatment, and no microalgal food was provided to encourage cannibalistic behaviour. Containers were set up in a rotating plankton wheel (rotation rate=60 cycles/hour) for the duration of the experiment.

5.2.4 Experiment C: Interaction of phytoplankton concentration and prey density on cannibalism rate

5.3.3.1 Effect of prey concentration

Acartia sinjiensis adult females were exposed to different combinations of naupliar prey densities in the absence of microalgal food.

Eight levels of initial prey density (#nauplii l^{-1}) were tested: (1) 1; (2) 10; (3) 20; (4) 80; (5) 160; (6) 660; (7) 1320 and (8) 2000 nauplii l^{-1}, with 10 replicates conducted for each treatment. Controls consisting of prey without predators and predators without prey were run to check for prey survival as well as predator egg production, respectively.

Experimental containers (1l volume) were first filled with fresh filtered seawater at the various tested microalgae concentrations before introducing a single adult female (predator). Early nauplii (NI and NII) were then added to replicate containers at the various initial densities. Experimental containers were then placed on a rotating plankton wheel for the duration of the experiment.

5.3.3.2 Effect of phytoplankton food concentration

In this experiment, adult females were exposed to a fixed quantity of nauplius prey at different quantities of microalgae in order to investigate the influence of ambient microalgal food in *A. sinjiensis* cannibalism.

A microalgal diet of 134 µg AFDW ml^{-1} with a 40:60 ratio of T-iso:Tet achieved maximum productivity of *A. sinjiensis* (Knuckey et al., 2005). Accordingly, diets were calculated on the basis of cell counts of T-iso = 32,160 cells ml^{-1} and Tet = 3190 cells ml^{-1} to achieve this ration. Carbon quantity was converted to equivalent cells ml^{-1} for both algal species, based on average cell carbon content data in Knuckey et al. (2005). Average microalgae concentrations were determined using a FlowCAM™ . Microalgae cultures were subsequently diluted down to experimental concentrations using 1 µm filtered seawater. A mixed microalgal diet consisting of T-iso+Tet was offered to *A. sinjiensis* at the following ratios: (1) 0% (i.e. no algal food offered); (2) 50% of optimal microalgae concentration (3) 100% optimal microalgae concentration.

Experimental containers (1l volume) were first filled with fresh filtered seawater at the various tested microalgae concentrations before introducing a single predator (10 replicates/treatment). Early nauplii (NI and NII) were then added to all of the replicate containers at a concentration of 1320 prey l^{-1}. Experimental containers were then placed on a rotating plankton wheel for the duration of the experiment.

5.3.3.3 Interaction between prey density phytoplankton and food quantity

In this experiment, adult females were exposed to different combinations of naupliar prey densities as well as various microalgal concentrations in order to investigate the importance of these factors on *A. sinjiensis* cannibalism, as well as potential interactions between the two factors.

Following the methodology described in section 5.3.3.2, a mixed microalgal diet consisting of T-iso+Tet was offered in the following ratios: (1) 0% (i.e. no algal food offered); (2) 50% of optimal microalgae concentration (3) 100% optimal microalgae concentration. In addition to different rations of microalgae, eight levels of initial prey density (#nauplii l^{-1}) were also tested: (1) 1; (2) 10; (3) 20; (4) 80; (5) 160; (6) 660; (7) 1320 nauplii $l^{-1}$and (8) 2000 nauplii l^{-1}.

A total of 24 different treatments (8 initial prey density x 3 microalgae quantity) were hence prepared for this trial, with 10 replicates conducted for each treatment. As in the previous experiments, controls consisting of prey without predators and predators without prey were run to check for prey survival and predator egg production respectively.

Experimental containers (1l volume) were first filled with fresh filtered seawater at the various tested microalgae concentrations before introducing a single predator. Early nauplii (NI and NII) were then added to replicate containers at the various experimental densities. Experimental containers were then placed on a rotating plankton wheel for the duration of the experiment.

5.2.5 Experiment D: Effect of starvation status of predator on cannibalism rate

This trial was conducted to evaluate the effects of starvation status on adult female *A. sinjiensis* on adult cannibalism. Pilot studies conducted by the authors provided that *A. sinjiensis* females can survive for 72 hours without microalgal food. Females were thus starved for: (1) 0 hours; (2) 24 hours; (3) 48 hours and (4) 72 hours; before being offered naupliar prey. Starved adults (predators) were individually introduced into 60 ml containers filled with 50 ml of fresh, filtered salt water, after which 10 early nauplii (NI-NIII) (prey) were haphazardly selected and introduced to each replicate. A total of 34 replicates were run for every treatment and placed on a rotating plankton wheel for the duration of the experiment.

5.2.6 Statistical analysis

Cannibalism rate (nauplii adult^{-1} day^{-1}) was statistically analysed with several factors used as independent variables: predator sex (Experiment A); prey developmental stage (Experiment B); average initial microalgae concentration and average initial prey density (Experiment C) and predator starvation duration (Experiment D).

Data from both Experiments A and B did not meet parametric test assumption and were therefore analysed using the non parametric Wilcoxon rank-sum test. Data from experiment C and D were verified to meet parametric test assumptions. Data from experiment C was analysed using a 2-factorial ANOVA, the 2 factors being initial microalgae concentration (cells l^{-1}) and average nauplii density (nauplii l^{-1}). Data from Experiment D was analysed using a 1-way ANOVA. Upon detection of significant differences between treatments ($p<0.05$), Tukey's multiple comparison test was performed to determine specific differences among treatments. Predation rate data from experiment D were square root transformed prior to analyses to increase

homogeneity of variance. A 0.05 p-level of significance was used in all statistical analysis. All analyses were conducted using the Statistica software, version 7. Data are presented as mean ± standard error (SE).

The correlation between cannibalism rate and initial nauplii density in the absence of microalgae (0% microalgae ration) was assessed using nonlinear regression analysis in SigmaPlot™ (version 11). The relationship between adult cannibalism rate and initial naupliar concentration was described by the nonlinear Hill equation (3 parameters, (Holling, 1959)):

$$y = \frac{\mathrm{a} * \mathrm{x}^{\mathrm{b}}}{c^b + x^b}$$

Where x is the initial naupliar concentration (# nauplii l^{-1}), y is the adult cannibalism rate (nauplii $adult^{-1}$ day^{-1}), a is the maximum rate of cannibalism, b is the gradient of curve between 25% and 75% of maximum y value, c is the half saturation rate (initial naupliar concentration that produces 50% of the highest cannibalism value).

5.3. Results

5.3.1 Effect of predator sex

Acartia sinjiensis adult cannibalism on early nauplii (NI and NII) was significantly influenced by the sex of the predators (Fig. 5.1). Adult males exhibited only very little cannibalism with only 0.10± 0.10 nauplii $adult^{-1}$ day^{-1} in average. This was significantly different from the average cannibalism rate of adult females which was approximately 12 times higher at 1.20± 0.29 nauplii $adult^{-1}$ day^{-1} (Fig. 5.1).

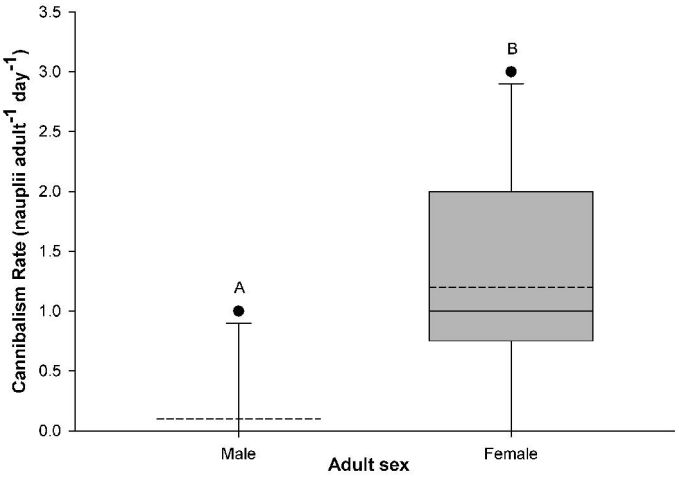


Figure 5.1: Effects of adult sex (n=8) on cannibalism rate by adult *Acartia sinjiensis* on early nauplii (NI, NII and NIII) over 24 hours. The boundary of the box closest to zero indicates the 25th percentile and the boundary farthest from zero indicates the 75th percentile. Vertical error bars above indicate the 90th and 10th percentiles. Average are indicated by the dashed line while median values are indicated with the full line. Black circles indicate outliers. Different letters on top of the box plots indicate significant differences ($p<0.05$) between the treatments.

5.3.2 Effect of naupliar developmental stage

Cannibalism rate in *A. sinjiensis* adult females was significantly affected by the developmental stage of the naupliar prey (Fig. 5.2). Average predation rate by females on early nauplii (NI, NII and NIII) was 2.2± 0.6 nauplii adult^{-1} day^{-1} and was 5.5 times higher than cannibalism rate on late stage nauplii (NIV, NV and NVI) (0.4± 0.2 nauplii adult^{-1} day^{-1}) (Fig. 5.2).

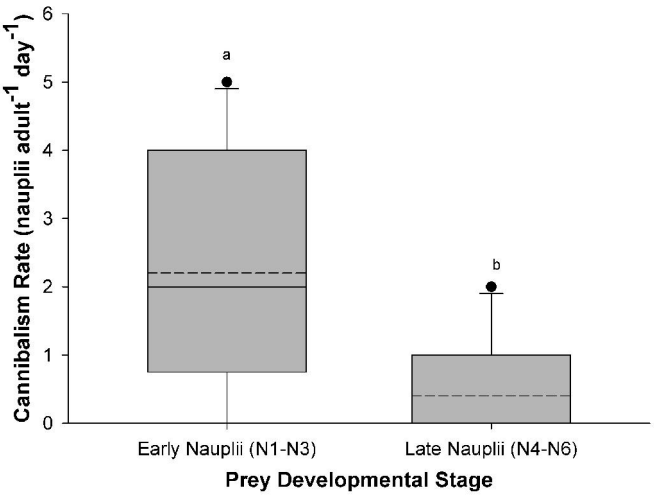


Figure 5.2: Average Predation rate of *A. sinjiensis* adult females on early (NI, NII and NIII) and late (NIV, NV and NVI) nauplius stages. The boundary of the box closest to zero indicates the 25th percentile and the boundary farthest from zero indicates the 75th percentile. Vertical error bars above indicate the 90th and 10th percentiles. Average are indicated by the dashed line while median values are indicated with the full line. Black circles indicate outliers. Different letters on top of the box plots indicate significant differences ($p<0.05$) between the treatments.

5.3.3 Influence of prey density and phytoplankton food concentration

5.3.3.1 Effect of prey concentration

A. sinjiensis cannibalism by adult females on early nauplii was significantly influenced ($p<0.01$) by the density of the naupliar prey (Fig. 5.3). Cannibalism rate increased with prey density: from 0 at the lowest prey concentration of 1 nauplii l^{-1} to 7.3± 0.6 nauplii $adult^{-1}$ day^{-1} for a prey concentration of 1320 nauplii l^{-1}. Average cannibalism rates observed at the 5 lower prey densities tested (1 to 80 nauplii l^{-1}) ranged from 0 to 1.1± 0.3 nauplii $adult^{-1}$ day^{-1} and were not significantly different from one another ($p>0.05$) (Fig. 5.3). Adult cannibalism rate increased to 3.5± 0.5 nauplii $adult^{-1}$ day^{-1} at a prey concentration of 660 nauplii l^{-1} and was significantly different from all other treatments except for 160 nauplii l^{-1} (2.6± 0.3 nauplii $adult^{-1}$ day^{-1}). The maximum cannibalism rate was observed at 1320 nauplii l^{-1} (7.3± 0.6 nauplii $adult^{-1}$ day^{-1}) and was not significantly different from the 2000 nauplii l^{-1} treatment (6.9± 0.7 nauplii $adult^{-1}$ day^{-1}) (Fig. 5.3).

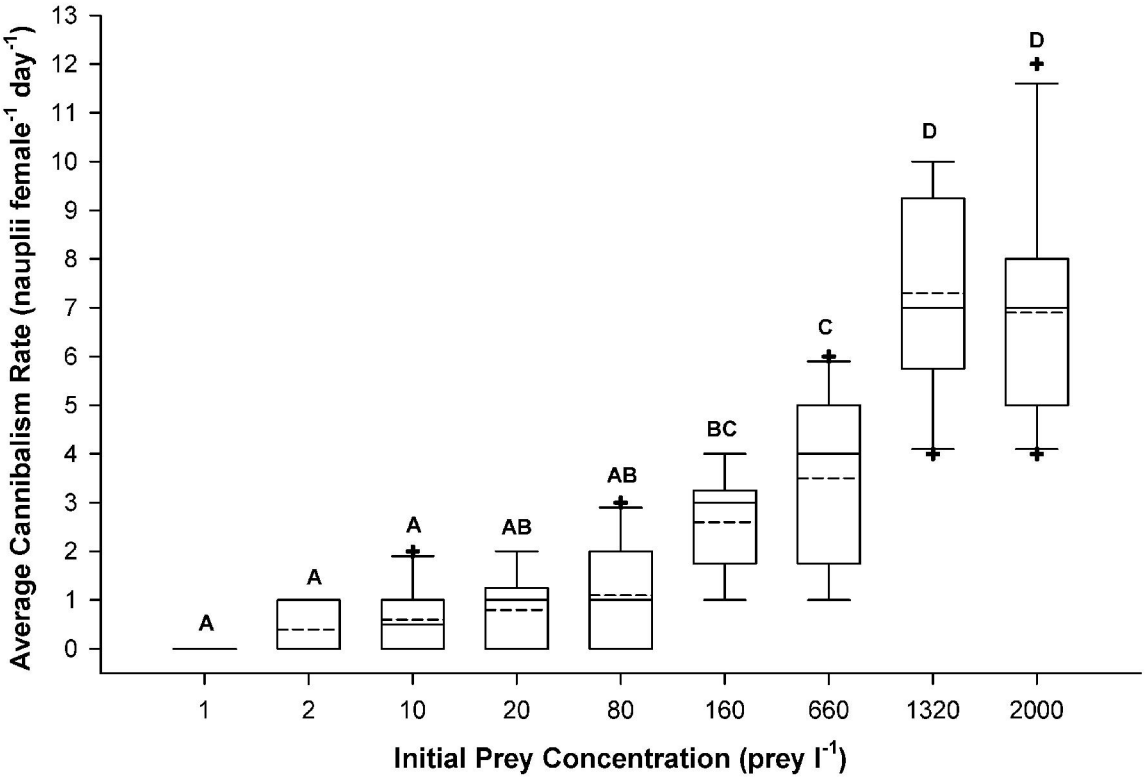


Figure 5.3: Average predation rate by *Acartia sinjiensis* CVI female under different initial prey densities and with no alternative microalgal food offered (0% microalgae). Trial was run for 24 hours. The boundary of the box closest to zero indicates the 25th percentile and the boundary farthest from zero indicates the 75th percentile. Vertical error bars above indicate the 90th and 10th percentiles. Average are indicated by the dashed line while median values are indicated with the full line. Different letters on top of the box plots indicate significant differences ($p<0.05$) between the treatments.

5.3.3.2 Effect of phytoplankton food concentration

With fixed prey concentrations, microalgal quantity was found to have a significant effect on cannibalism by adult females (Fig. 5.4). *A. sinjiensis* female cannibalism rate was 7.3± 2.0 nauplii adult^{-1} day^{-1} in the absence of microalgal food (0% optimal microalgal ration provided) but decreased significantly ($p<0.05$) to 4.1± 2.4 nauplii adult^{-1} day^{-1} when a 100% optimal concentration of phytoplankton was provided. When *A. sinjiensis* adult females were provided

with a 50% optimal microalgal ration cultured, cannibalism rate was 5.6± 2.2 nauplii adult^{-1} day^{-1} and was not significantly different from the 0% or the 100% treatments (Fig. 5.4).

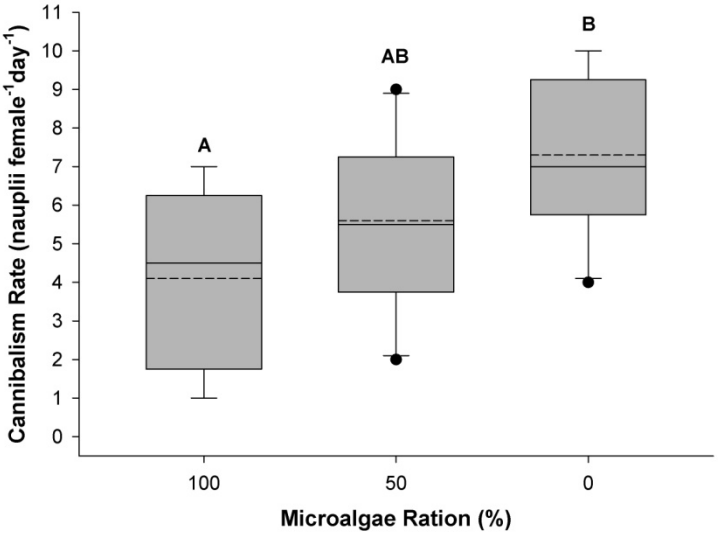


Figure 5.4: Average predation rate by *Acartia sinjiensis* female for 3 different microalgae quantity (0%, 50% and 100% optimal concentration). The boundary of the box closest to zero indicates the 25th percentile and the boundary farthest from zero indicates the 75th percentile. Vertical error bars above indicate the 90th and 10th percentiles. Average are indicated by the dashed line while median values are indicated with the full line. Black circles indicate outliers. Different letters on top of the box plots indicate significant differences ($p<0.05$) between the treatments.

5.3.3.3 Interaction between prey density phytoplankton and food quantity

A significant interaction ($p<0.01$) was found between initial prey density and phytoplankton concentration on *A. sinjiensis* adult cannibalism. The effect of different levels of microalgal food concentration is dependent on the level of initial prey density present, and as phytoplankton quantity decreased, female cannibalism rate tended to increase more substantially with increasing prey densities (Fig. 5.5).

There was no significant difference in cannibalism rate among the 3 feeding conditions tested when prey density ranging from 1 to 80 nauplii l^{-1}, but feeding condition had significant impact

on female cannibalism for initial prey density above 80 nauplii l^{-1} (Fig. 5.5). At prey densities of 160 and 660 nauplii l^{-1} female cannibalism rate was significantly higher in the absence of microalgal food (0% microalgal food treatment) when compared to female provided with a 100% optimal microalgal ration but was not significantly different from a 50% optimal food ration (Fig. 5.5). At the highest prey densities tested, 1320 and 2000 nauplii l^{-1}, female cannibalism rates were significantly different between the 3 feeding conditions tested, with significantly higher cannibalism rate found for the 0% optimal food ration treatment (Fig. 5.5).

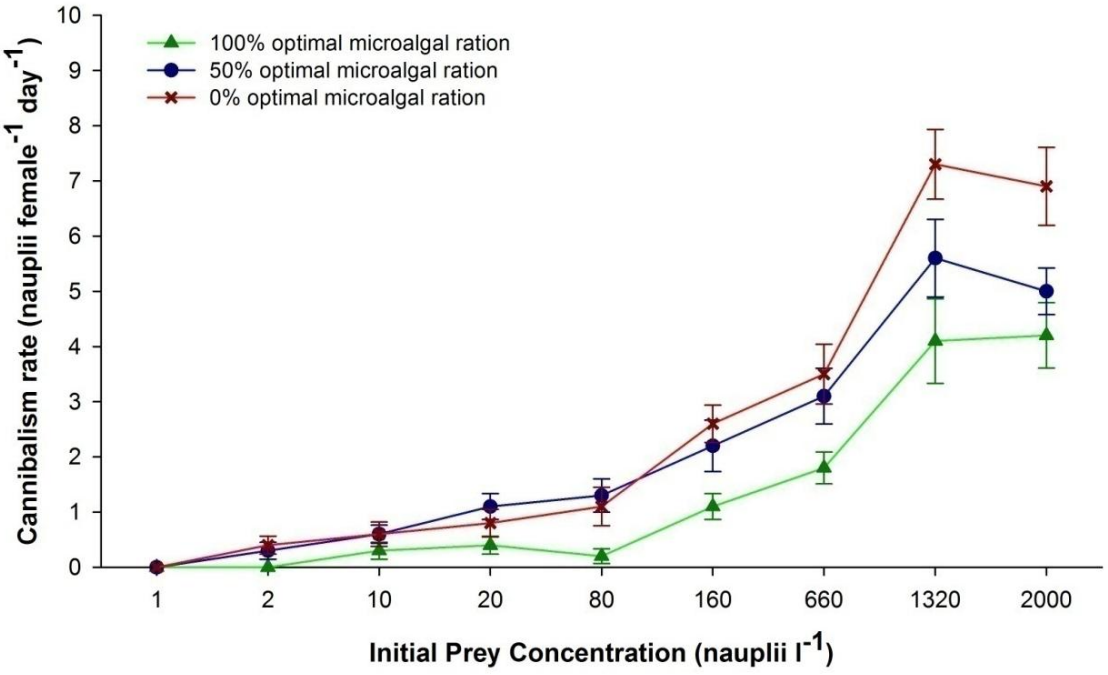


Figure 5.5: *A. sinjiensis* adult female cannibalism rate (# nauplii $adult^{-1}$ day^{-1}) as a function of (i) microalgae quantity and (ii) prey initial density. Data are presented as mean ± S.E. Letters to indicate significant differences between treatments were omitted for clarity.

5.3.3.4 Saturation of cannibalism as a function of prey density

The functional response of *A. sinjiensis* cannibalism rate as a function of initial naupliar density were determined at a 0% optimal microalgae concentration. Data was modelled using the non-linear Hill equation (Fig. 5.6).

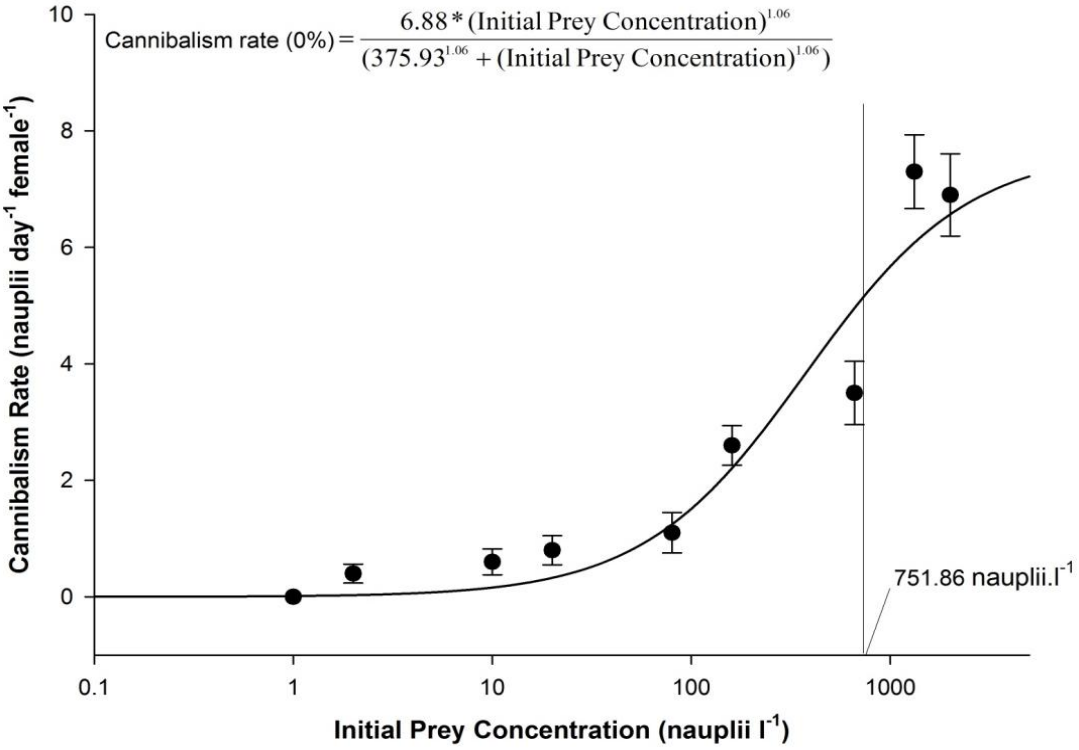


Figure 5.6: Saturation of cannibalism by *B. similis* adult females as a function of the initial prey concentration in the absence of microalgal food. Refer to Table 5.1 for equation details.

A Holling type III relationship (Holling, 1959) achieved a high goodness of fit (R^2= 0.93; $p<0.01$, Table 5.1). The saturation points in *A. sinjiensis* cannibalism, defined as the threshold above which adult cannibalism does not increase significantly with further increase in prey density, was determined to be at prey concentration of 752 nauplii l^{-1} when provided with a 0% optimal quantity of microalgal quantity (Fig. 5.6, Table 5.1).

Table 5.1: Summary of models parameters and results. Functional response parameters based on the Hill functions of adult female *A. sinjiensis*. Parameters of the nonlinear Hill equation were used to describe the relationship between initial nauplii density and cannibalism in the absence of microalgal food (0% optimal food ration). Also indicated are the coefficient of determination (R^2), the adjusted coefficient of determination (R^2 adj) and the significance level of the fit p.

Dependent variable	Function	Equation	a	b	c	Saturation Point (µg C l^{-1})	R^2	R^2 adj	p	Microalgal Condition
Initial nauplii density	Hill, 3 parameters	$y=(a*x^b)/(c^b+x^b)$	7.68	1.06	375.93	663.78	0.93	0.91	<0.01	no microalgal food (0%)

5.3.4 Effect of starvation status of predator on cannibalism rate

Cannibalism was observed on both starved and un-starved females (Fig. 5.7) and was significantly influenced by the starvation duration ($p<0.01$). Cannibalism in un-starved predators rate was significantly lower (1.4± 0.2 nauplii $adult^{-1}$ day^{-1}) when compared to predators starved for 24, 48 or 72 h (3.1± 0.2 nauplii $adult^{-1}$ day^{-1}, 3.5± 0.2 nauplii $adult^{-1}$ day^{-1} and 3.1± 0.3 nauplii $adult^{-1}$ day^{-1}, respectively). There was no significant difference in cannibalism rate between 24, 48 or 72 h starvation periods (Fig. 5.7).

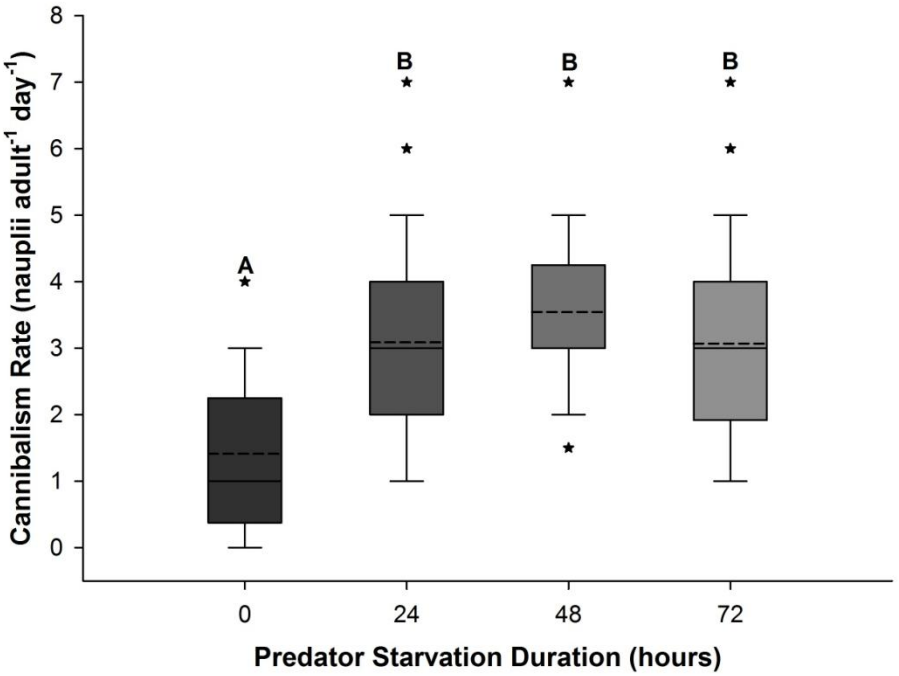


Figure 5.7: *A. sinjiensis* cannibalism as a function of predator starvation duration (0, 24, 48 or 72 hours). Average are indicated by the dashed line while median values are indicated with the full line. The boundary of the box closest to zero indicates the 25th percentile and the boundary farthest from zero indicates the 75th percentile. Vertical error bars above indicate the 90th and 10th percentiles. Black stars indicate outliers. Different letters on top of the box plots indicate significant differences ($p<0.05$) between the treatments.

5.4. Discussion

While calanoid copepods are known as excellent prey for the rearing of a wide variety of marine larvae, their utilization in commercial aquaculture remains somewhat limited due to lack of productivity under intensive culture condition (Støttrup, 2003), which could in turn be attributable to a lack of research. In the wild, cannibalism allows copepod populations to exploit food sources otherwise unavailable (van den Bosh and Santer, 1993). However, under artificial culture conditions, cannibalism is considered to be one of the various factors that could significantly reduce the productivity overtime. In the past, many species of calanoids were reported to be active cannibals and to ingest nauplii of their own and/or other species (Bonnet et

al., 2004; Gallucci and Ólafsson, 2007; Pierson et al., 2007). Copepods species with high cannibalism rates are clearly unsuitable candidates for the development of intensive culture protocols for commercial hatcheries. On the other hand, species with relatively mild cannibalistic behaviour need research to investigate how their cannibalistic behaviour might be alleviated under intensive culture condition.

The results from the present study showed that cannibalism did occur in the copepod *Acartia sinjiensis,* but at quite modest rates overall (i.e. > 1 nauplii adult^{-1} day^{-1} at prey density < 160 nauplii l^{-1}). *A. sinjiensis* cannibalism rates observed in this study were comparable to rates exhibited by the calanoid copepod *Acartia tonsa* at similar prey concentration (2.8 nauplii adult^{-1} day^{-1} at 278 nauplii l^{-1} (Lonsdale et al., 1979)). However, cannibalism rates reported for *Sinocalanus tenellus*, another calanoid species were considerably higher than the one reported for *A. sinjiensis* in this study: 99.2% naupliar mortality due to adult cannibalism at an adult density of 10 l^{-1} (Hada, 1991). This confirm the need for species-specific research when assessing cannibalism behaviour in calanoid copepods as significant variation exist between species.

Cannibalism by *A. sinjiensis* adult males on early stage nauplii (NI to NIII) was almost negligible and significantly different then cannibalism by adult females. This could be attributed to sex-specific difference in energy budget, or to dietary preferences, as females are likely to require higher energy reserves for egg production.

Early nauplii (NI to NIII) were significantly more susceptible to adult cannibalism when compared to later naupliar stages (NIV to NVI), with mortality of the later due to cannibalism almost negligible. Copepod nauplii are known to perform distinct escape responses upon detecting the hydrodynamic signal generated by an approaching predator (Titelman and Kiørboe, 2003b). When a nauplius is initiating an escape response, even a modest increase in swimming speed will reduce the risk of predation (Jonsson and Tiselius, 1990). Nauplii increases in both size and swimming speed with ascending developmental stages (Mauchline, 1998) and it has been suggested that more rapid swimming, rather than size, make late nauplii (NIV to NVI) less vulnerable to adult cannibalism (Daan et al., 1988; Sell et al., 2001; Bonnet et al. 2004). A similar trend of decreasing predation rate with increasing naupliar instars appeared to be common for calanoid copepods as it was reported for various species, including *Acartia tonsa*

(Lonsdale et al., 1979; Lemus, 2006), *Temora longicornis* (Daan et al., 1988), *Calanus finmarchicus* and *Pseudocalanus* spp. (Sell et al., 2001). It should also be noted that the lower cannibalism rate observed for the late naupliar prey when compared to the early naupliar prey might also be partly linked to higher individual nutritive contribution from these later developmental stages, due to their higher size and weight.

Cannibalism in copepod is often a reaction of adults to food scarcity and/or high population density (Gallucci and Ólafsson, 2007). In the present study, *A. sinjiensis* cannibalism increased significantly with increasing abundance of naupliar prey, and it occurred even when an optimal concentration of microalgal food was provided. This increase in cannibalism rate with increasing prey abundance is indicative of a opportunistic feeding strategy in *A. sinjiensis,* where cannibalism rate is determined by encounter rate (Hada and Uye, 1991; Gallucci and Ólafsson, 2007). Opportunistic cannibalism has similarly been reported in several calanoid species such as *Calanus finmarchicus* (Basedow and Tande, 2006), *Temora longicornis* (Daan et al., 1988) and *Acartia tonsa* (Lonsdale et al., 1979).

Although adult female cannibalism was shown to increase with nauplii density initially, it is expected that the predators will eventually become saturated at a specific level of prey abundance, above which no significant increase in cannibalism will be observed. In the absence of microalgal food, such a saturation threshold was determined to be 751.9 nauplii l^{-1} for *A. sinjiensis*, meaning that cannibalism rate in adult females was maximized at such a prey concentration and above. *A. sinjiensis* cannibalism of naupliar stages can hence be significantly decreased if the prey density in culture is prevented from reaching this critical threshold.

Algal food ration was also found to significantly affected cannibalism by adult females. When the optimal feeding ration was decreased from 100% to 50%, and further down to 0%, *A. sinjiensis* cannibalism rate augmented more rapidly with increasing prey density, reaching significantly different cannibalism rates at 1320 preys l^{-1}. Basedow and Tande (2006) proposed that calanoid copepods have the ability to switch their prey preference from microalgae to nauplii during episodes of high naupliar abundance and low algal concentration. It seems to be the case for *A. sinjiensis*, as microalgae feeding ration and prey density were found to significantly interact with one another. Several other studies on calanoid copepods have reported a similar reduction in cannibalism rate with the addition of algal food (Landry, 1981; Laabir et al., 1995;

Lemus, 2006; Gallucci and Ólafsson, 2007). Conversely, Lonsdale et al. (1979) reported no significant decrease in *A. tonsa* cannibalism rates in the presence of an alternative food source, suggesting possible species-specific difference in copepod cannibalism behaviour in relation to microalgal food conditions.

Copepod previous feeding history is known to influence their present feeding habits (Stoecker and Egloff, 1987). While starvation was not essential for initiating cannibalism in *A. sinjiensis*, females starved for 24, 48 or 72 hours did exhibit significantly higher cannibalism rates when compared to fed females. Although longer starvation periods were expected to produce higher cannibalism rates, no significant difference was observed among females starved for 24, 48 or 72 hours. A trade-off between energy and starvation might exist in *A. sinjiensis,* affecting their ability to track down and capture naupliar prey as a function of their recent feeding history. This would explain why cannibalism increase significantly between unstarved and starved females, and why no significant difference in cannibalism was found when females were starved for 24, 48 or 72 hours. Increase of cannibalistic tendencies in relation to deprivation of microalgal food was similarly reported in the calanoid copepod *Calanus helgolandicus* (Laabir et al., 1995).

In conclusion, cannibalism in *A. sinjiensis* was demonstrated to be relatively modest and to depend on multiple factors, including predator sex, developmental stage and density of naupliar prey, whether or not phytoplankton food is abundant and the degree of predator starvation. Maintaining microalgae feeding ration close to optimal quantities will significantly lessen the impacts of adult cannibalism on nauplii. The results of the present study showed that as long as quality and quantity of microalgal diets were well maintained, *A. sinjiensis* cannibalism in culture could be managed to become only a minor contributor to naupliar mortality. Meanwhile, as cannibalism was also showed to be closely related to the predator/prey encounter rate in culture, daily harvesting of nauplii could also reduce *A. sinjiensis* cannibalism, while preventing the prey density to reach the maximum saturation threshold.

CHAPTER 6

EGG PRODUCTION, EGG HATCHING SUCCESS AND POPULATION INCREASE OF THE CALANOID COPEPOD BESTIOLINA SIMILIS FED DIFFERENT MICROALGAL DIETS [3]

6.1 Introduction

Nowadays, most marine hatcheries rely on rotifers and *Artemia* as the main source of live feeds for fish and crustacean larvae (Shansudin et al., 1997; Marcus and Murray, 2001). Yet, for a variety of marine larvae, rotifers and *Artemia* nauplii are either not properly ingested or do not meet larval nutritional requirements (Schipp et al., 1999; O'Bryen and Lee, 2005; Chen et al., 2006). This represents a major challenge to the aquaculture industry (Chesney, 2005; O'Bryen and Lee, 2005) as this list of species include numerous high valued food fishes as well as many marine ornamental species (See Chapter 1, Table 1.2).

[3] Chapter 6 is adapted from two publications:
Camus T, Zeng C, McKinnon D (2009) Egg production, egg hatching success and population increase of the tropical paracalanid copepod *Bestiolina similis* (Calanoida: Paracalanidae) fed different microalgal diets. Aquaculture 297, 169-175.
Camus T, Zeng C (2010) Role of microalgae on total egg production over female lifespan and egg incubation time, naupliar and copepodite survival, sex ratio and female life expectancy of the copepod *Bestiolina similis*. Aquaculture Research 41, 1717-1726.

Copepods are probably the most numerous metazoan sub-class on the planet and usually dominate the mesozooplankton, constituting about 80% of its biomass (Verity and Smetacek, 1996). Marine copepods, particularly their nauplii, constitute an important source of natural food for marine fish larvae in the wild (Houde, 1973; McKinnon and Duggan, 2001; Payne and Rippingale, 2001; Sampey et al., 2007) typically make up more than 50% of their stomach contents (Støttrup, 2000). Copepods, especially calanoids, have been proven an ideal source of live feeds for many cultured marine larvae (Hernandez Molejon and Alvarez-Lajonchere, 2003) and are known to provide significant benefits to the cultured larvae when compared to rotifers and *Artemia* (Chen et al., 2006). Copepods vast species diversity, as well as their many larval stages provide a broad spectrum of sizes for marine larvae (O'Bryen & Lee, 2005). In addition, their biochemical composition is known to match requirements of most marine fish larvae (Hernandez Molejon and Alvarez-Lajonchere, 2003; van der Meeren et al., 2008). As a result of these advantages, calanoids have been proposed as valuable alternative to traditional hatchery live feeds (Lee et al., 2005; Buttino et al., 2009), providing opportunity to successfully culture larvae that cannot be reared using traditional live feeds (Marcus and Murray, 2001; O'Bryen and Lee, 2005). Furthermore, the inclusion of copepods as supplement to traditional live feeds diets could improve the survival, development and pigmentation of larvae of which the culture is already established commercially (Støttrup, 2000; Knuckey et al., 2005). Despite these promising features, copepods utilization in aquaculture remains sporadic (Marcus et al., 2004). This under-utilization is mainly attributed to their relative low productivity in intensive culture (O'Bryen and Lee, 2005) and the lack of standard production methods, which could in turn be partially attributed to the lack of research on this field.

Microalgal food quality is a key biotic factors affecting copepod culture productivity (Castro-Longoria, 2003). As such, the effects of different microalgal diets on egg production (Calbet and Alcaraz, 1996; Kleppel et al., 1998; Koski and Kuosa, 1999; Payne and Rippingale, 2000; Turner et al., 2001), egg hatching success (Shin et al., 2003; Milione and Zeng, 2007), mortality (Kang and Poulet, 2000; Lincoln et al., 2001) and development (Knuckey et al., 2005; Leandro et al., 2006) have been documented for several calanoid species (Payne and Rippingale, 2000; Lacoste et al., 2001; McKinnon et al., 2003; Shin et al., 2003; Knuckey et al., 2005; Holste and Peck, 2006; Milione and Zeng, 2007). In contrast, only few studies have investigated the response of

paracalanid copepods to different food sources (McKinnon et al., 2003; VanderLugt and Lenz, 2008) and none have considered the effects of mixed microalgal diets.

Copepods productivity in intensive culture is firstly linked to female egg production as copepods egg production rate is a direct indicator of population recruitment, as well as a measure of the net production rate of adult females (Shin et al., 2003). However, subsequent egg hatching rate, naupliar and copepodite survival as well as development rates all have an impact on the cultures productivity (Milione and Zeng, 2007). A comprehensive investigation, including assessment of the population increase after a period of culture, is therefore more likely to provide a more complete understanding of the effects of algal diets on copepod productivity.

Through a series of laboratory experiments, the present study examined the suitability of 10 microalgal diets (4 monoalgal diets and 6 mixed diets) for the intensive cultivation of *B. similis* in order to optimize its intensive culture protocol and improve its productivity overtime.

6.2 Materials and methods

6.2.1 General procedure

All experiments were conducted at MARFU, James Cook University, Queensland, Australia. The collection, separation and stock culture of *B. similis*, as well as the culture protocol for all microalgal species used for this experiment were described in the General Materials and Methods (Chapter 2).

A series of experiment were conducted to assess the influence of various microalgal diets and their combinations on major parameters related to *B. similis* culture productivity, i.e. (1) daily egg production rate, (2) total egg production over female lifespan, (3) egg incubation time, (4) egg hatching rate, (5) survival at the naupliar and copepodite stage, (6) population increase over a 12 day culture period, (7) adult sex ratio and adult female life expectancy. The same 10 microalgal diets (i.e. 4 mono-algal, 5 binary algal and 1 tri-algal diet) were used in all experiments. Details of the microalgal diet treatments were as follows (dimensions are expressed as equivalent spherical diameter (ESD)):

Diet 1: The Tahitian strain of *Isochrysis* sp. (T-Iso) (CS-177, 5±0.8 µm, carbon/cell≈ 17.8 pg/cell, Prymnesiophyceae)

Diet 2: *Tetraselmis chuii* (Tet) (CS-26, 13± 2 µm, carbon/cell≈ 89.9 pg/cell, Prasinophyceae)

Diet 3: *Pavlova salina* (Pav) (CS-49, 4.5± 0.7 µm, carbon/cell≈ 9.6 pg/cell, Prymnesiophyceae)

Diet 4: *Chaetoceros muelleri* (Chaet) (CS-176, 7± 1.3 µm, carbon/cell≈ 9.2 pg/cell, Bacillariophyceae)

Diet 5: *Isochrysis* sp. + *Tetraselmis chuii* (T-Iso+Tet) (1 :1)

Diet 6: *Tetraselmis chuii* + *Pavlova salina* (Tet+Pav) (1 :1)

Diet 7: *Isochrysis* sp. + *Pavlova salina* (T-Iso+Pav) (1:1)

Diet 8: *Chaetoceros muelleri* + *Tetraselmis chuii* (Chaet+Tet) (1:1)

Diet 9: *Chaetoceros muelleri* + *Pavlova salina* (Chaet+Pav) (1:1)

Diet 10: *Isochrysis* sp. + *Tetraselmis chuii* + *Pavlova salina* (T-Iso+Tet+Pav=1:1:1)

Microalgae were offered in fresh and live form to copepods and their concentrations were determined daily using a haemocytometer and a microscope (Leica CME, model TN-PSE30). *B. similis* were fed with the different microalgal diets on an equal biomass basis in all experiments, which was based on carbon concentrations calculated for each species according to Strathmann (1967). Algae were fed to copepod cultures at a level of about 1500 µgC l^{-1}, a carbon concentration known to satiate *A. tonsa,* a similar size planktonic copepod (Kiørboe et al., 1985). When *B. similis* were fed with a binary or a tri-algal diet, the carbon concentration was divided equally between the 2 or 3 algae offered based on their biomass. While pre-conditioning periods of 48 hours or less were commonly used in most dietary studies conducted on copepods (e.g. Kang and Poulet, 2000; McKinnon et al., 2003; Shin et al. 2003), a longer acclimatization period of 3 days was adopted in this study, based on Sedlack and Marcus (2005) to remove any residual effect from previous diets.

All experiments were carried with water temperature maintained at 26±1°C and a salinity of 30±1‰ while the photoperiod regime was 12L:12D. Observations and counting of eggs, nauplii,

copepodites and adults were made using a Sedgewick–Rafter counter and a Leica CME optical microscope (model TN-PSE30).

6.2.2 Egg production experiments

6.2.2.1 Egg production over 8 consecutive days

B. similis adults and late copepodites were obtained from four 20 l carboy stock cultures by draining culture water through a 150 μm sieve. Animals caught on the sieve were immediately resuspended in a Petri dish with a small amount of seawater. Groups of *B. similis* were then randomly captured using a broad-tipped pipette and distributed into thirty 1 l beakers, with groups of 3 beakers assigned to each diet treatment as replicates (approximately 1000 individual.L^{-1}). *B. similis* were then fed daily in excess for 3 days with the designated algal diets to acclimatize them to respective diets and to remove any potential residual effects of previous diets.

Following 3 days of acclimatization, individual *B. similis* females that were actively swimming with intact appendages were randomly selected from beakers of a given diet and carefully transferred to 30 ml Petri dishes in order to monitor their daily egg output. There were 6 replicates per tested diet and hence a total of 60 Petri dishes. After 24 h, *B. similis* females were removed from each Petri dish and eggs they produced were counted using a Sedgewick Rafter counting cell and a compound microscope (Leica CME).

Following the procedure described above, new females were randomly selected daily from the stock culture beakers and transferred into a new set of sixty 30 ml Petri dishes containing fresh filtered seawater and algal diets to obtain individual 24 h egg output for each of 4 consecutive days. Daily replacement of experimental females from the pre-conditioned mixed population ensured that experimental animals were fertilized and healthy prior to introduction to the Petri dishes for the egg production experiment. For each treatment, 24 hours female Egg Production Rate (EPR) was subsequently calculated by averaging data from all 6 females over 4 days.

6.2.2.2 Mean 24 h egg production

At least three conditioning culture beakers containing mixed *B. similis* population were similarly set up for each algal diet treatment for acclimatization. After 3 days of pre-conditioning, CV

copepodites were haphazardly selected from the conditioning beakers by gently draining parts of culture water through a 150 μm sieve which retained copepodites and adults. CV copepodites were then individually placed in 30 ml Petri dishes and fed with one of the 10 microalgae diets. Upon molting to adults, they were sexed and only females were kept to record daily and total egg production over their lifespan. This was done by that once a newly molted female was found, a mature male selected from a conditioning beaker of the same diet was added to the Petri dish to ensure proper fertilization. Pairs of adult *B. similis* were then transferred daily to a new Petri dish with fresh seawater and food and daily egg output of the female was recorded for every following day until the female eventually died. The total egg productions over female lifespan were then calculated for each diet treatment based on data obtained from 10 replicate females.

6.2.3 Egg hatching success experiment

As in the egg production experiment, groups of *B. similis* were randomly selected from the carboy cultures and transferred into 30 beakers of 1 l and fed with 10 different microalgal diets (3 replicate beakers per treatment, approximately 1000 individuals.L^{-1})). After 2 days, eggs produced over the period were discarded by thoroughly siphoning the bottom of each beaker with a siphon that had a 60 μm mesh attached to the end to prevent copepodites and adults being siphoned out. This procedure ensured that eggs presented in the beakers the next day were all produced within 24 h. The following day, freshly produced eggs in each beaker were carefully sieved out using a 25 μm mesh and counted before being randomly distributed into fifty 30 ml containers, sealed an labeled with a particular diet treatment. There were 5 replicates (containers) per diet treatment with each replicate containing 40 to 60 eggs.

Egg hatching success (%) was estimated for each microalgal diet by calculating the difference between the number of eggs introduced and the number of unhatched eggs after 48 and 96 h incubation period respectively (Chapter 3).

$$EHS\ (\%) = \frac{[(\text{No. of eggs introduced initially} - \text{No. of unhatched eggs}) * 100]}{\text{No. of eggs introduced initially}}$$

6.2.4 Egg incubation time, naupliar and copepodite survival and adult sex ratio experiment

Three pre-conditioning 1 l culture beakers were set-up for each microalgal treatment, each containing a mixed population of *B. similis* (i.e. containing all developmental stages) obtained from the stock cultures (stocking density approximately 1000 individual.L^{-1}). Copepods in the conditioning beakers were then fed daily in excess with the designated algal diet for 3 days. A pilot experiment conducted by the authors showed that > 95% of *B. similis* died after 3 days of starvation. A three days period of pre-conditioning was therefore considered to be sufficient to remove the potential residual effects of previous diets.

After 3 days of acclimatization, eggs released by *B. similis* females within past 24 h were collected (eggs produced earlier were removed by thoroughly siphoning the bottom of each beaker the day before) by gently siphoning the bottom of each pre-conditioning beaker using a 3 mm tube with a 100 µm mesh sieve attached to one end to prevent the removal of adults. Eggs produced from all replicate beakers of a same diet were carefully sieved out using a 25 µm mesh sieve and combined before being counted and haphazardly distributed into five 30 ml Petri dishes filled with 25 ml of fresh seawater and the designated diet. Each Petri dish served as a replicate (containing 80-120 eggs) and a total of 50 Petri dishes were used for the 10 diet treatments (i.e. five replicates per treatment). Petri dishes were covered at all time except during daily checking and transferring to prevent evaporation of the water and salinity increase during experiments.

All 50 replicate Petri dishes were checked daily for newly hatched nauplii and any nauplii found within a Petri dish was recorded before they had been gently transferred with a broad mouth pipette to a new Petri dish, containing the same diet and fresh seawater for further culture. After 96 h, average egg incubation time over 96 h was calculated based on hatching data obtained from five replicates of each diet treatment.

Newly hatched nauplii that had been transferred to separate 50 Petri dishes for culture were checked daily for mortality and molting to the first copepodite stage. Following daily checking, all surviving nauplii were carefully transferred to new Petri dishes containing fresh seawater and a designated diet. This ensured that water quality and food condition were maintained throughout the experiment. On each day, newly molted copepodites found in a replicate were transferred to a

new Petri dish, which allowed their development to be monitored precisely. Once all nauplii within a given replicate Petri dish had either molted to copepodites or died, overall survival at the naupliar stage was calculated for the replicate, which was then averaged among five replicates of each treatment.

A similar overall survival at the copepodite stage was obtained for each diet treatment, i.e. by culturing them until they either died or molted to adults, upon which they were sexed to obtain sex ratio data.

6.2.5 Female life expectancy experiment

A similar procedure as described in section 6.2.4 was followed initially, i.e. at least three conditioning beakers containing mixed populations of *B. similis* were set-up for each microalgal diet to acclimatize them to a designated diet for three days. After three days, stage five copepodites (CV) found inside the conditioning stocks were haphazardly selected and individually transferred to 30 ml Petri dishes filled with fresh seawater and the designated diet. These individually kept CV copepodites were monitored daily until they molted to adults (confirmed by the presence of an exuvia in the Petri dish) upon which they were sexed. The day CV molted to adults was considered to be day 0 of their adult phase. Adult females were then checked daily for mortality before being transferred to new Petri dishes with fresh designated food until mortality eventually occurred. There were 17 females (replicates) monitored for their adult lifespan for each diet treatment. Mean adult life expectancy of *B. similis* females was then calculated for each treatment by averaging the life expectancies of all replicates.

6.2.6 Population increase experiment

B. similis were acclimatized to each diet for 3 days in 1 l beakers before being used for the experiment. Upon completion of the acclimatization period, 10 mature *B. similis* adults (3 males and 7 females) were introduced into each 500 ml beaker, for the population increase experiment. Such a sex ratio was selected to ensure that all adult females could be fertilized by adult males. The low initial stocking density was chosen to remove all potential effect of stress attributable to crowding. A total of 50 replicate beakers were established with 5 replicates for each treatment. For the following 12 days, *B. similis* were fed daily with the designated diets and approximately 30% of the culture water was exchanged through gentle siphoning. The siphon had a 25 µm

mesh attached to the end to prevent the removal of any eggs or post-egg-stages of *B. similis* from the culture. After 12 days, content from each beaker was drained through a 25 µm sieve and all eggs, nauplii, copepodites and adults retained were fixed with 10% formalin and stored for later counting. *B. similis* eggs, nauplii, copepodites and adults were counted in each replicate and the final population estimated as the average of five replicates. Based on Fenchel (1974), the intrinsic rate of population increase r was then calculated for each treatment using the formulation:

$$r = \frac{\ln(\frac{N_0}{N_1})}{t}$$

Where N_0= population number at the beginning of the experiment, N_1 = population number at the end of the experiment while t (days) is the duration of the experiment.

6.2.7 Data collection and analysis

Data from egg incubation time, naupliar and copepodite survival, egg production and adult female life expectancy were tested and confirmed to meet the parametric test assumption (i.e. normally distributed, homogeneity of variance, independent and randomness of the data), and one-way ANOVA was used to look for any significant differences among treatments. When significant differences ($p<0.05$) were found, a Tukey's multiple comparisons test was used to determine specific differences among treatments. The sex ratio data was log transformed and pooled across all replicates before being analyzed for significant difference using the X^2 square test. All statistical analyses were conducted using Statistica, version 7. Data are presented as mean± standard error (SE).

6.3 Results

6.3.1 Egg production

6.3.1 Daily egg production rate

B. similis 24 h egg production rates (EPR; eggs female^{-1} day^{-1}) when fed 10 different microalgal diets during 4 consecutive days are presented in Table 6.1.

Table 6.1: Average Egg Production Rates (EPR; eggs female^{-1} day^{-1}) of *Bestiolina similis* females fed 10 different micro-algal diets over 4 consecutive days. Data are presented as mean ± standard errors.

Microalgal Diet	Average Daily Egg Production Rate			
	Day 1	Day 2	Day 3	Day 4
T-Iso+Tet+Pav	35.8± 1.9	37.4± 1.3	54.8± 2.1	48.4± 2.2
T-Iso+Pav	23.7± 3.8	28.5± 2.7	37.6± 3.5	31.2± 1.5
Tet+Pav	25.8± 1.5	32.3± 2.1	33.0±.0 1.4	30.2± 1.1
T-Iso+Tet	19.2± 1.3	27.5± 2.1	27.0± 1.2	20.5± 1.1
Chaet+Pav	22.2± 1.0	20.5± 1.5	17.8± 0.9	14.8± 1.3
Chaet+Tet	14.8± 1.0	14.8± 1.5	18.2± 0.9	18.4± 0.6
Pav	20.2± 0.9	19.3± 1.9	23.2± 1.5	21.3± 1.1
T-Iso	12.5± 1.1	19.8± 0.9	22.5± 1.2	19.8± 0.8
Tet	14.3± 1.4	12.7± 1.5	13.2± 0.3	11.8± 0.7
Chaet	9.6± 1.1	11.3± 0.6	10.6± 1.1	8.6± 0.6

As no significant differences in daily egg production was detected between days, for each diet treatment, data were pooled to calculate the overall mean 24 h EPR (Fig. 6.1). The results show that maternal food significantly impacted *B. similis* egg output ($p<0.001$): the highest EPR (44.1± 2.8 eggs female^{-1} day^{-1}) was produced by the tri-algal diet treatment (T-Iso+Tet+Pav), which was more than 4 times higher than that of the lowest EPR (10.0± 1.0 eggs female^{-1} day^{-1}) recorded for the diatom *Chaetoceros muelleri* (Chaet). EPR in the tri-algal T-Iso+Tet+Pav treatment was significantly higher than other diet treatments ($p<0.05$). Two binary algal treatments, T-Iso+Pav and Tet+Pav, were the next best diets, with EPR at 30.9± 3.1 and 30.4± 1.7 eggs female^{-1} day^{-1} respectively. In turn, these were both superior to the rest of the diet treatments ($p<0.05$), except that of T-Iso+Tet (23.2± 1.6 eggs female^{-1} day^{-1}) ($p>0.05$) (Fig. 6.1). The lowest EPR produced by the Chaet treatment was significantly lower than all other diet treatments, except that of Tet and Chaet+Tet treatments (12.9± 1.0 and 16.6± 1.1 eggs female^{-1} day^{-1} respectively) ($p>0.05$) (Fig. 6.1).

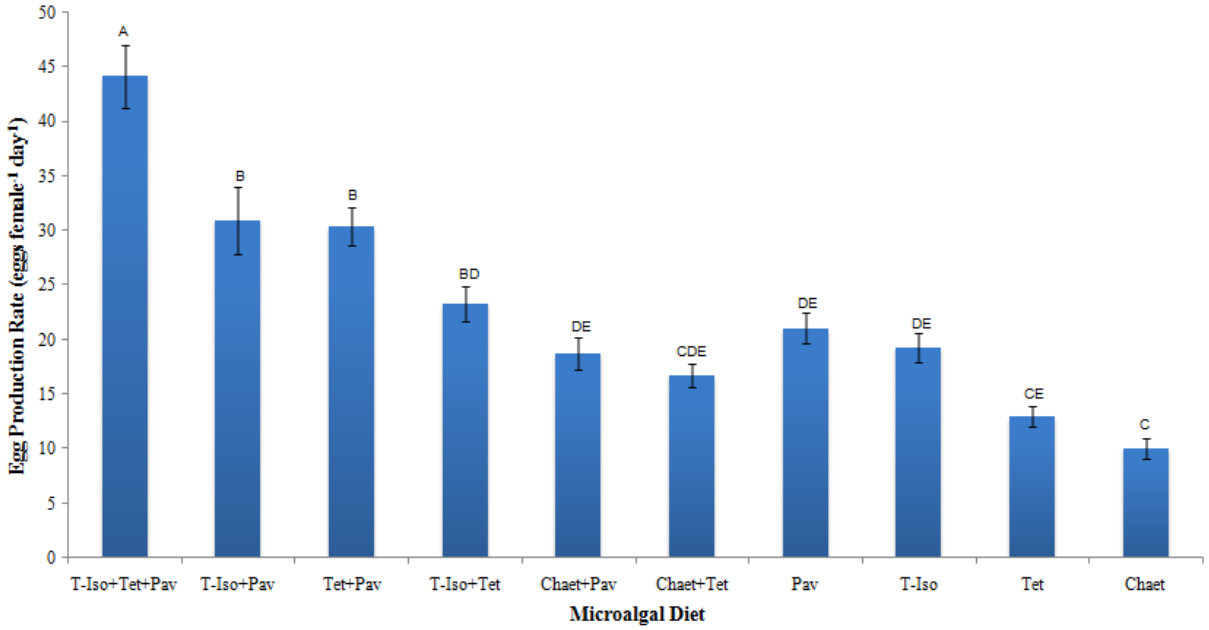


Figure 6.1: Effects of 10 microalgal diets on *Bestiolina similis* daily egg production rate. For each treatment, 24 hours egg production rates were averaged from 6 females over 4 days. Data are presented as mean ± S.E. Different letters on the tops of bars indicate significant differences ($p<0.05$).

6.3.1.2 Total egg production over female lifespan

Mean total egg output over female lifespan was highly dependent on microalgal diet ($p<0.01$) (Fig. 6.2). The highest total egg output over female lifespan were found in the tri-algal diet (156.4 ± 11.5 eggs female^{-1}), which was 7.5 times higher than the lowest output recorded for Tet (20.7 ± 2.8 eggs female^{-1}) and significantly higher than all diet treatments except that of the T-Iso+Pav (127.6 ± 11.1 eggs female^{-1}) ($p>0.05$) (Fig. 6.2). On the other end of the spectrum, the lowest total egg production produced by Tet (20.7 ± 2.8 eggs female^{-1}) was significantly lower than all other treatments ($p<0.05$) except that of Chaet (32.9 ± 4.4 eggs female^{-1}) and Chaet+Tet (46.3 ±4.9 eggs female^{-1}) treatments (Fig. 6.2). In between these, intermediate total egg outputs over female lifespan were recorded for Tet+Pav (118.0 ± 8.3 eggs female^{-1}), T-Iso+Tet (99.6 ± 5.8 eggs female^{-1}), T-Iso (86.1 ± 6.0 eggs female^{-1}) and Pav (84.2 ± 4.4 eggs female^{-1}) diets (Fig. 6.2).

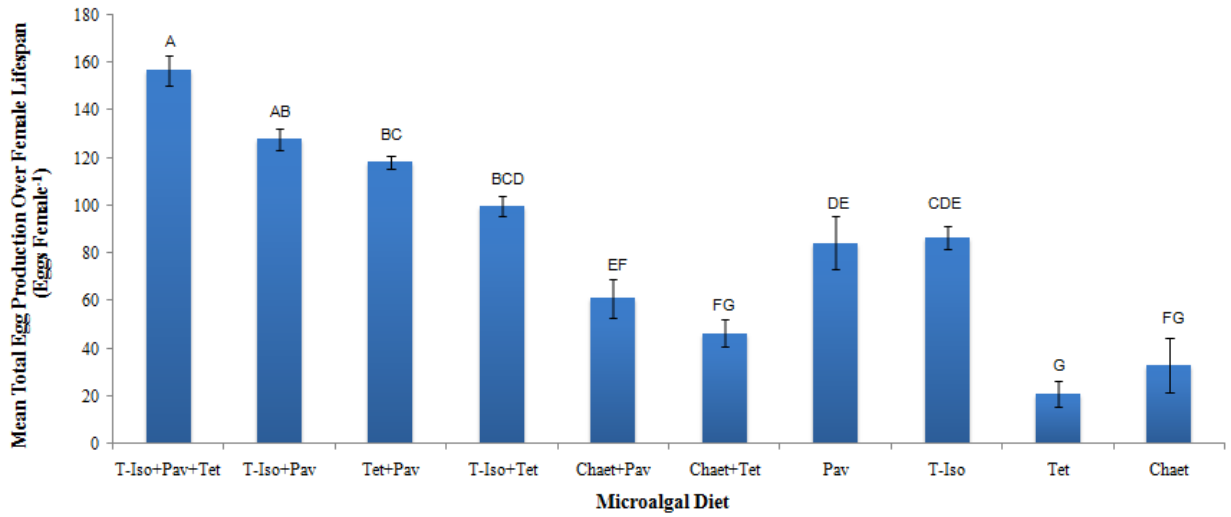


Figure 6.2: Effect of 10 microalgal diets on *Bestiolina similis* Total Egg Production over Female Lifespan. Data are presented as mean ± standard errors. Different letters on the tops of bars indicate significant differences.

6.3.2 Egg hatching rates

Microalgal diets significantly influenced both 48 and 96 h egg hatching rates (EHR) of *B. similis* ($p<0.001$) (Fig. 6.3). The 48 h EHR ranged from 65.4 ± 2.4% (Chaet) to 91.0 ± 2.0% (T-Iso+Tet+Pav) for the diets tested. The highest EHR recorded for the tri-algal T-Iso+Tet+Pav was significantly higher ($p<0.05$) than the rest of treatments except for the binary diets of T-Iso+Pav, Tet+Pav and T-Iso+Tet, as well as the monoalgal diet T-Iso ($p>0.05$). While the diatom Chaet produced the lowest 48 h EHR, it did not differ significantly from the Chaet+Tet and the Tet treatments (73.6 ± 3.2% and 70.1 ± 1.5% respectively) ($p>0.05$).

Compared to 48 h EHR, 96 h hatching rates improved across all diet treatments with EHR higher than 85% recorded for 8 of the 10 microalgal diet treatments (Fig. 6.3). The highest 96 h EHR was still the tri-algal diet T-Iso+Tet+Pav (96.3% ± 0.4), although now it was only significantly higher than the Tet and Chaet treatments ($p<0.05$). Indeed, no significant differences in 96 h EHR was found among 8 of the 10 diets tested, i.e. T-Iso+Tet+Pav, T-Iso+Pav, Tet+Pav, T-Iso+Tet, Chaet+Pav, Chaet+Tet, Pav and T-Iso ($p>0.05$). The monoalgal Chaet produced the lowest and significantly inferior 96 h EHR (73.4% ± 3.6), which was only not significantly lower than that of the Tet treatment (83.3% ± 2.7) ($p>0.05$) (Fig. 6.3).

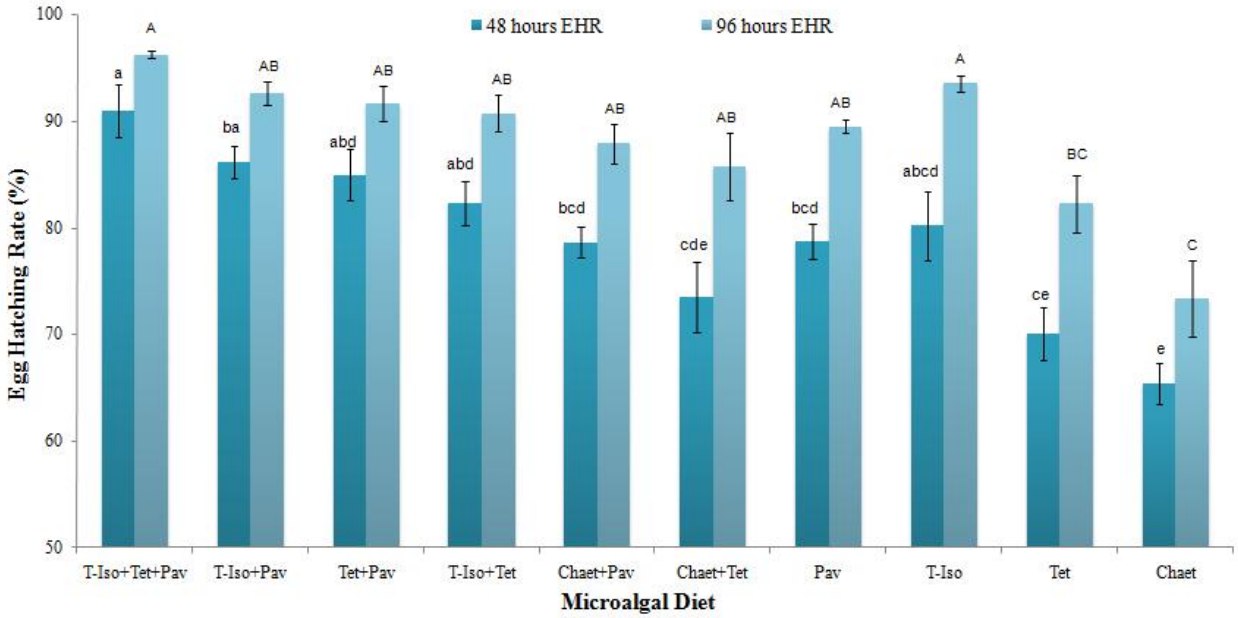


Figure 6.3: *Bestiolina similis* 48 h and 96 h Egg Hatching Rates (%) when fed with 10 different Microalgal Diets. Data are presented as mean ± SE. Different letters on the tops of bars indicate significant difference ($p<0.05$).

6.3.3 Egg incubation time, naupliar and copepodite survival and adult sex ratio

6.3.3.1 Mean egg incubation time

B. similis mean egg development time was significantly influenced by microalgal diets ($p<0.01$) (Table 6.2). The shortest average egg incubation time was recorded for the tri-algal diet T-Iso+Tet+Pav (1.91 ± 0.04 days), although it was not statistically different from the T-Iso+Tet (2.04 ± 0.04 days), Chaet (2.09± 0.05 days), Tet+Pav (2.10 ± 0.05 days) and T-Iso+Pav (2.12 ± 0.03 days) treatments ($p>0.05$) (Table 6.2). On the other hand, the monoalgal diet Tet produced the longest mean egg incubation time (2.41 ± 0.09 days), significantly longer than the tri-algal, T-Iso+Tet and Chaet treatments ($p<0.05$) but not significantly different from the rests of the diet treatments ($p>0.05$) (Table 6.2).

Table 6.2: Mean egg incubation time, average survival at naupliar and copepodite stage and sex ratio of the copepod *Bestiolina similis* fed 10 different microalgal diets. Data are presented as mean ± standard errors. Data in parentheses are only indicative and not subjected to statistical analysis as too few animals were found in these treatments. Different superscripts within a same column indicate significant difference ($p<0.05$).

Microalgal diet	Egg Incubation Time (days)	Naupliar Survival (%)	Copepodite Survival (%)	Percentage of Females (%)	Percentage of Males (%)
T-Iso+Pav+Tet	1.91 ± 0.04 ^{A}	86.3 ± 1.0 ^{b}	65.9 ± 2.9 ^{AB}	84.0 ± 2.7 ^{T}	16.0 ± 2.7 ^{t}
T-Iso+Pav	2.12 ± 0.03 ^{ABCD}	79.7 ± 0.7 ^{ab}	62.6 ± 3.3 ^{AB}	86.4 ± 1.9 ^{T}	13.6 ± 1.9 ^{t}
Tet+Pav	2.10 ± 0.05 ^{ABCD}	77.3 ± 3.5 ^{a}	53.7 ± 3.1 ^{AB}	76.6 ± 3.4 ^{T}	23.4 ± 3.4 ^{t}
T-Iso+Tet	2.04 ± 0.04 ^{AD}	85.7 ± 2.1 ^{ab}	52.5 ± 4.1 ^{B}	77.9 ± 2.5 ^{T}	22.1 ± 2.5 ^{t}
Chaet+Pav	2.27 ± 0.09 ^{BCD}	44.6 ± 1.8 ^{c}	19.2 ± 4.1 ^{C}	(79.8 ± 4.3)	(20.2 ± 4.3)
Chaet+Tet	2.33 ± 0.10 ^{BCD}	48.6 ± 2.3 ^{c}	8.8 ± 1.2 ^{C}	(82.7 ± 7.5)	(17.3 ± 7.5)
Pav	2.37 ± 0.06 ^{BC}	77.5 ± 1.0 ^{a}	60.3 ± 2.7 ^{AB}	73.9 ± 3.1 ^{T}	26.1 ± 3.1 ^{t}
T-Iso	2.21 ± 0.07 ^{BCD}	85.2 ± 1.3 ^{ab}	69.0 ± 4.3 ^{A}	79.4 ± 5.2 ^{T}	20.6 ± 5.2 ^{t}
Tet	2.41 ± 0.09 ^{B}	0.0 ± 0.0 ^{e}	N/A	N/A	N/A
Chaet	2.09 ± 0.05 ^{ACD}	13.5 ± 1.4 ^{d}	(0.0 ± 0.0)	N/A	N/A

6.3.3.2 Naupliar and copepodite survival

Microalgal diets drastically affected *B. similis* naupliar survival, which ranged from 0% (Tet) to 86.3% (trialgal) ($p<0.001$). Of the 10 diets tested, six produced relatively high naupliar survival (> 77%): the highest being the tri-algal T-Iso+Tet+Pav (86.3 ± 1.0%), followed by T-Iso+Tet (85.7 ± 2.1%), T-Iso (85.2 ± 1.3%), T-Iso+Pav (79.7 ± 0.7%), Pav (77.5 ± 1.0%) and Tet+Pav (77.3 ± 3.5%) (Table 6.2). Statistical analysis showed that the highest naupliar survival of tri-algal diet was significantly higher than all other treatments ($p<0.05$) except that of T-Iso+Tet, T-Iso and T-Iso+Pav treatments ($p>0.05$) (Table 6.2). On the other hand, the monoalgal diet Tet produced a total naupliar mortality while Chaet resulted in the second lowest survival (13.5 ± 1.4%), both of them were significantly lower than the rest of treatments ($p<0.05$).

Similarly, copepodite survival was significantly impacted by microalgal diets ($p<0.01$). The highest mean copepodite survival (69.0 ± 4.3%) was recorded for the T-Iso diet, but it was not statistically different from the tri-algal (65.9 ± 2.9%), T-Iso+Pav (62.6 ± 3.3%) and Pav (60.3 ± 2.7%) ($p>0.05$) (Table 6.2). The diatom Chaet and mixed diets containing Chaet appeared to

produce the worst copepodite survival, with total mortality recorded for Chaet and low survival obtained for Chaet+Tet (8.8 ± 1.2%) and Chaet+Pav (19.2 ± 4.1%), which were significantly lower than the rest of the diets ($p<0.01$).

Overall, *B. similis* appears to have higher mortality at the copepodite stage than at the naupliar stage, as the copepodite survival was consistently lower than that of the nauplii for all diets tested, (Table 6.2).

6.3.3.3 Sex ratio

Sex ratio of *B. similis* was strongly skewed toward females although it was not significantly affected by their diets ($p>0.05$) (Table 6.2). Proportions of *B. similis* females in the adult population were consistently ranging from 73.9± 3.1% (Pav) to 86.4± 1.9% (T-Iso+Pav) with an average female to male ratio of approximately 4:1 (Table 6.2).

6.3.4 Female life expectancy

In contrast, the average life expectancy of *B. similis* adult females was dramatically influenced by microalgal diets ($p<0.01$). The shortest adult female life expectancy was found in the Tet treatment (3.1 ± 0.3 days) and was only about half that of Pav (6.3 ± 0.6 days) or the tri-algal T-Iso+Tet+Pav treatment (5.9± 0.4 days) (Fig. 6.4). Statistical analysis showed that there was no significant differences among adult female life expectancy for the Pav, tri-algal, T-Iso+Tet (5.7± 0.4 days), T-Iso (5.7± 0.5 days) and T-Iso+Pav (5.3± 0.3 days) treatments ($p>0.05$) (Fig. 6.4). On the other hand, no significant difference was found among low life expectancy group of Tet (1.5± 0.3), Chaet+Tet (3.2± 0.3), Chaet+Pav (3.4± 0.3) and Chaet (3.6± 0.3) treatments ($p>0.05$) (Fig. 6.4).

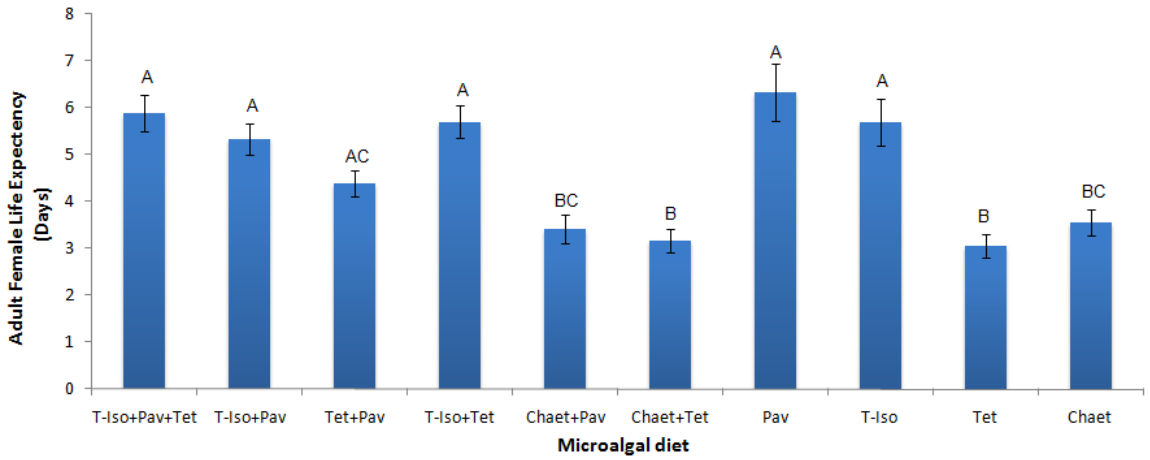


Figure 6.4: *B. similis* Female Life Expectancy as a function of 10 different Microalgae Diets. Data are presented as mean ± standard errors. Different letters on the tops of bars indicate significant differences.

6.3.5 Population increase

After 12 days of culture on different microalgal diets, the average final population numbers of *B. similis* are presented in three categories, i.e. 'All Stages Included' (i.e. including eggs); 'All Post-Egg-Stages' (i.e. excluding eggs); and 'Adult Only' (Table 6.3). When 'All Stages Included', the final number of *B. similis* population was highest for the T-Iso+Tet+Pav treatment (886.8 ± 139.4), significantly higher than all other diet treatments ($p<0.05$). The second most productive diet was T-Iso+Tet (541 ± 53.6) although it was not statistically different from T-Iso+Pav (479.0 ± 54.1), Chaet+Pav (285.4 ± 42.2) and the mono-algal diet T-Iso (334.4 ± 43.9) ($p>0.05$). The Tet and Chaet+Tet treatments produced the lowest population number at 26.0 ± 2.3 and 42.8± 8.4 respectively, significantly lower than all other treatments ($p<0.05$), except that of the Chaet, Pav, Chaet+Pav and Tet+Pav (Table 6.3). Excluding eggs from the final population counts resulted in substantially reduced final population number (i.e. All Post-Egg-Stages) for all treatments as eggs represented the highest counts among all life stages in all treatments. However, similar trend and statistics of dietary effects on the final population number remained (Table 6.3).

It is worth noting that while algal combinations generally provided higher population increase over 12 days, this was not always the case for some binary diet treatments. For example, the

mono-algal diet T-Iso was ranked 4th of all 10 diets tested, producing higher population increase than the binary algal diets Tet+Pav, Chaet+Tet and Chaet+Pav. Although the differences were not statistically significant for Tet+Pav and Chaet+Tet treatments ($p>0.05$), it was significantly higher than the Chaet+Pav treatment ($p<0.05$) (Table 6.3). Furthermore, no significant difference in population increase was found between the monoalgal diets Pav, Chaet and Tet when compared to the binary algal diets of Tet+Pav, Chaet+Tet and Chaet+Pav ($p>0.05$) (Table 6.3).

The intrinsic rates of population increase (r) of *B. similis* was calculated for all treatments (Table 2). When All-Stage-Included, it ranged from 0.08 ± 0.02 for Tet to 0.37 ± 0.03 for T-Iso+Tet+Pav. If eggs were excluded from final population counts, it ranged from 0.01 ± 0.02 (Tet) to 0.32 ± 0.02 (T-Iso+Tet+Pav) (Table 6.3).

Table 6.3: Final population and intrinsic rate (r) of population increase of B. similis cultured over a 12 days period fed on different microalgal diets. Data are presented as Mean ± S.E.; different superscript letters in a same column indicate significant differences ($p<0.05$).

Microalgal Diet	Final Population Number When All Stages Included	r (All Stage Included)	Final Population Number of All Post-Egg-Stages	r (All Post-Egg-Stages)	Final Number of Adults	r (Adults Only)
T-Iso+Tet+Pav	886.8 ± 139.4 ^{a}	0.37± 0.03 ^{a}	478.2 ± 63.4 ^{a}	0.32 ± 0.02 ^{a}	79.6 ± 16.2 ^{a}	0.17 ± 0.04 ^{a}
T-Iso+Pav	479.0 ± 54.1 ^{bc}	0.32 ± 0.02 ^{bc}	227.0 ± 41.7 ^{bc}	0.26 ± 0.03 ^{bc}	35.0 ± 5.2 ^{b}	0.10 ± 0.03 ^{b}
Tet+Pav	265.0 ± 34.9 ^{cde}	0.27 ± 0.03 ^{cde}	89.8 ± 9.2 ^{cde}	0.18 ± 0.02 ^{cde}	18.0 ± 1.2 ^{bc}	0.05 ± 0.02 ^{bc}
T-Iso+Tet	541.0 ± 53.6 ^{b}	0.33 ± 0.02 ^{b}	260.2 ± 36.1 ^{b}	0.27 ± 0.03 ^{b}	33.6 ± 5.5 ^{bc}	0.10 ± 0.05 ^{bc}
Chaet+Pav	285.4 ± 42.3 ^{bcde}	0.28 ± 0.03 ^{bcde}	125.4 ± 22.4 ^{bcde}	0.21 ± 0.03 ^{bcde}	18.2 ± 4.8 ^{bc}	0.04 ± 0.05 ^{bc}
Chaet+Tet	42.8 ± 8.4 ^{e}	0.11 ± 0.04 ^{e}	26.6 ± 5.6 ^{e}	0.07 ± 0.04 ^{e}	2.6 ± 0.5 ^{bc}	0.12 ± 0.04 ^{bc}
Pav	213.4 ± 37.5 ^{cde}	0.25 ± 0.03 ^{cde}	132.8 ± 29.7 ^{bcde}	0.21 ± 0.03 ^{bcde}	16.6 ± 4.5 ^{bc}	0.03 ± 0.04 ^{bc}
T-Iso	334.4 ± 43.9 ^{bcd}	0.29 ± 0.03 ^{bcd}	175.4 ± 26.4 ^{bcd}	0.23 ± 0.03 ^{bcd}	32.6 ± 5.6 ^{bc}	0.10 ± 0.04 ^{bc}
Tet	26.0 ± 2.3 ^{e}	0.08 ± 0.02 ^{e}	10.8 ± 1.0 ^{e}	0.01 ± 0.02 ^{e}	1.2 ± 0.3 ^{c}	0.13 ± 0.08 ^{c}
Chaet	110.2 ± 25.1 ^{de}	0.19 ± 0.04 ^{de}	55.6 ± 16.0 ^{de}	0.13 ± 0.05 ^{de}	6.8 ± 1.8 ^{bc}	0.05 ± 0.06 ^{bc}

A breakdown of *B. similis* final population composition based on 4 groups of life stages (i.e. eggs, nauplii, copepodites and adults) is shown in Figure 6.5. The results show that the T-

Iso+Tet+Pav treatment produced the highest numbers across all life stages and the differences among treatments were often statistically significant ($p<0.05$) (Fig. 6.5).

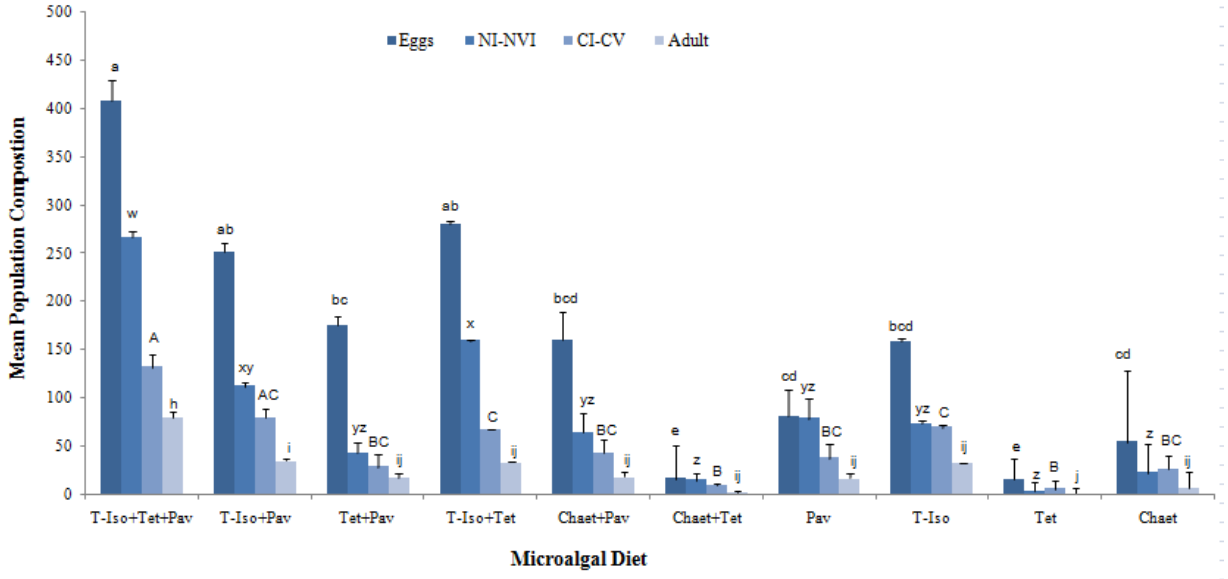


Figure 6.5: Influence of microalgal diets on average population composition of *Bestiolina similis* over a 12 day culture period. The experiment started with 10 adult (7 females and 3 males) of *B. similis*. Data are presented as mean ± standard errors. Different letters on the tops of bars indicate significant difference ($p<0.05$). NI-NVI: naupliar stage 1 to 6; CI-CV: copepodite stage 1 to 5.

6.4 Discussion

Previous research have demonstrated a clear link between food quality and the productivity of calanoid copepods culture (Kleppel et al., 1998; Morehead et al., 2005; Milione and Zeng, 2007). The selection of an appropriate microalgal diet is hence a crucial step in the improvement of *Bestiolina similis* intensive culture protocol.

In this study, a four day average of 44.1 eggs female $^{-1}$ day $^{-1}$ and a maximum of 54.8 eggs female $^{-1}$ day^{-1} on a single day was found for *B. similis* when fed with the diet T-Iso+Tet+Pav. These rates are comparable to the 48 eggs female^{-1} day $^{-1}$ reported by McKinnon et al (2003) when culturing *B. similis* under laboratory conditions with *Heterocapsa niei* (Dinophyceae),

although this algal species is known to be difficult and unreliable to culture. However, *B. similis* egg production rates (EPR) found in this study were substantially higher than those reported in the wild: 8.5 to 17.3 eggs $^{-1}$ female $^{-1}$ day $^{-1}$ for in waters adjacent to Australia's North-West Cape (McKinnon and Duggan, 2001) and a mean EPR of 25 eggs female^{-1} day $^{-1}$ was reported from the waters of the Great Barrier Reef (McKinnon et al, 2003).

Today, while the importance of microalgal food to copepod productivity in culture is well established, our knowledge of copepods specific nutritional requirements is still very limited (Lee et al., 2005). Copepod productivity has been linked to maternal nutrition (Castro-Longoria, 2003), especially to n-3 polyunsaturated fatty acids (PUFAs), such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) (Kleppel et al., 2005). PUFAs are known to affect hatching success and embryonic development as well as to play important roles in the cell membrane functions of crustaceans and mollusks (Lacoste et al., 2001; Anderson and De Silva, 2003). However, it is important to note that PUFAs dietary functions were largely established for decapod crustaceans and subsequently only assumed to be applicable to copepods (Lacoste et al., 2001). Hence, the definite roles of PUFAs in copepods nutrition remain unclear, with several species reported to have the ability to synthesize DHA and EPA *de novo* (Lacoste et al., 2001) while other studies reported no clear correlations between fertility and copepod dietary EPA and DHA concentrations (Koski et al., 1998; Lee et al., 1999). In this sudy, although *Pavlova* 50 (Pav) possessed a relatively balanced EPA to DHA profile (Table 6.4), it did not necessarily lead to significantly higher EPR or improved hatching rate for *B. similis,* when compared to T-Iso. This result supports the hypothesis that there are no clear correlation between copepod fertility and their dietary EPA and DHA concentrations.

While progressing through their 12 developmental stages (6 naupliar, 5 copepodites and the adult stages), copepods undergo substantial morphological and physiological changes which are likely to lead to variations in their nutritional requirements (Knuckey et al., 2005). There were indications that it was the case for *B. similis* in this study as, for instance, the binary diet T-Iso+Tet supported a high naupliar survival as well as a female life expectancy but produced modest total egg output over female lifespan and low copepodite survival. Similarly, the monoalgal diet Pav produced the longest female life expectancy but only about half of the cumulative egg production observed with the tri-algal diet T-Iso+Pav+Tet.

Among the 10 microalgal diet tested, the tri-algal diet T-Iso+Tet+Pav was determined to be the most adapted microalgal diet for the intensive cultivation of *B. similis* and provided the best egg production rate, total egg output over female lifespan , egg hatching rates, population increase, the shortest egg incubation time as well as the best naupliar survival and the highest copepodite survival. This is not unexpected, as calanoids are capable of efficiently utilize broad and diverse diets (Mauchline, 1998), and so a combination of 3 microalgae is likely to provide a more comprehensive and better balanced diet likely to improve the nutritional reserves and condition of the females and in turn to increase culture productivity overtime. In addition, the trialgal diet also produced superior 48h EHS which indicates the possibility of enhanced nutrition provided by the tri-algal diet, which could in turn lead to faster embryonic development when compared to the rest of the diets. Among the binary diets, T-Iso+Tet and T-Iso+Pav performed relatively well when compared to the others binary diets.

As for the mono-algal diets, T-Iso stood out, while Pav also achieve reasonably good results. McKinnon et al (2003) reported that a mono-algal diet of *Pavlova salina* led to low EPR for *B. similis.* In the present study, a different strain of *Pavlova, Pavlova* 50, was found to support reasonably good EPR and EHS as a monoalgal diet. However, dead *B. similis* were sometimes found next to their molt during culture, indicating that Pav might be nutritionally deficient in promoting successful molting, especially for the molt from NVI to the first copepodite stage. This was not observed when *B. similis* were fed the monoalgal T-Iso, and may be explained by the lower DHA to EPA ratio of Pav (Table 6.4), which could impede molting when offered as the sole food source. As copepod reproduction is highly nutritionally and energetically demanding, a monoalgal diet can potentially deficient of certain nutrients, therefore become a limiting factor if copepods are only offered a single microalgae species (Buttino et al., 2009). Limitation of monoalgal diets may be further reflected in the quality of eggs produced, as eggs produced with both T-Iso and Pav required significantly longer incubation times than the ones produced with the tri-algal diet T-Iso+Pav+Tet.

Table 6.4: Major features and fatty acid composition of the four microalgae used as diets for Bestiolina similis in the present study. Content in DHA, EPA and Linolenic acid are specified as % of Total fatty acids. Microalgae dimensions are expressed as equivalent spherical diameter (ESD).

Species Name (class)	Acronym	Strain Code	Cell Size (µm)	Total fatty Acids (pg/cell)	DHA 22:6n-3	EPA 20:5n-3	Linolenic Acid 18:3n-3	Reference
Chaetoceros muelleri (Bacillariophyceae)	Chaet	CS-176	7	2.23	0.8	12.8	0.3	Zhukova and Aizdaiche, 1995 Pernet et al., 2003
Tetraselmis chuii (Prasinophyceae)	Tet	CS-26	13 ± 2	2.7–4.5	Trace	4.3-10.8	11.1–21.7	Mueller-Fuega et al., 2003
Tahitian strain of *Isochrysis* sp. (Prymnesiophyceae)	T-Iso	CS-177	5 ± 0.8	1.2-1.46	6.4–25.9	0.2-0.5	3.6–7	Renaud et al. 1999 Fernandez-Reiriz et al., 1983 Pernet et al., 2003
Pavlova 50 (Prymnesiophyceae)	Pav	CS-50	4.5 ± 1	1.0-2.7	8.4-9.2	23.5-25	1.4-2	Volkman et al., 1991

The other two monoalgal diets, Chaet and Tet, were clearly inferior diets for *B. similis* and produced very low naupliar and copepodite survival as well as low female egg production rates. The diatom *Chaetoceros muelleri* provided significantly lower EPR and EHR when compared to T-Iso and Pav. Interestingly, McKinnon et al. (2003) found that *C. muelleri* produced the best EPR when compared to *Pavlova, Isochrysis* and *Tetraselmis*. This could be explained by different feeding ration or experimental protocols. Diatoms have recently been revealed to be potentially toxic to copepods (Miralto et al., 1999), and are now associated with negative effects on reproduction for several species, although it is often difficult to differentiate diatom toxicity from their low nutrition quality (Irigoien et al., 2005; Jones and Flynn, 2005). Whether the inferior performance of the diatom Chaet is related to their toxicity or low nutritional quality warrants further research. It appears that the diatom Chaet may have affected egg formation of *B. similis*, as improperly formed eggs, often in batches of 3 instead of the usual 4, with smaller diameter or more transparent appearance were regularly observed during the egg production experiment, which may have contributed to the lowest hatching rate observed for the diet among

all others. The poor performance of Tet observed here confirms the finding by McKinnon et al. (2003) that it is a poor diet for paracalanid copepods. Tet provided the second lowest EPR and EHR, as well as the poorest population increase, with on average less than 2 adults and fewer than 20 post-egg-stages remaining after 12 days of culture. This suggests that a total crash might happen if Tet is offered as a sole food source for *B. similis*. Such a poor result may be attributed to the inability for *B. similis* first feeding nauplii to properly ingest relatively large cell sizes of Tet might explain the total naupliar mortality observed in *B. similis*. Milione and Zeng (2007) reported that Tet as a mono-algal diet did not lead to mass naupliar mortality when fed to *Acartia sinjiensis* and could produce reasonably good population increase over a 8 day culture period. Such a clear difference confirms that the suitability of micro-algal diets for various copepods is species-specific, and that feeding experiments are required for each copepod species candidate for mass cultivation. It should be noted that the experiments presented in this chapter were conducted using Petri-dishes as replicate containers, with potential settlement of the food particles.

Sex ratio was the only parameter investigated in the present study that was not significantly affected by microalgal diets. Past studies on another tropical copepod *Acartia sinjiensis* have also shown that neither photoperiod (chapter 3) nor stocking density (chapter 4) significantly affected the sex ratio of calanoid copepods. Thus, sex determination in these copepod species appears less likely to be influenced by environmental factors. Skewed sex ratios, similar to those observed in current paper, have been reported in past literature for a variety of copepod species (Voordouw and Anholt, 2002). The cause for these biased sex ratios remains largely unclear although some studies have highlighted the influence of environmental conditions, such as stocking density, temperature or food availability, on copepod sex ratio (Voordouw et al., 2005).

Regular culture dilution was suggested as a way to produce high number of *B. similis* nauplii (VanderLugt and Lenz., 2008). These authors suggested that high adult density impaired the production of nauplii and led to subsequent population crash within a few days. However, *Isochrysis* was the only alga offered to *B. similis* during their study and hence, nutritional deficiencies might also have contributed to crashes of populations at high culture densities. A combined algal diet may help improve the situation.

In summary, the best suited diet for calanoid copepods may vary at different life stages or for different biological functions. On this basis, a monoalgal diet may become nutritionally limiting whereas appropriate combinations of microalgae are likely to offer a better balance of required nutrients, but this does not necessarily mean that a mixed diet is always more adapted than a monoalgal diet. As illustrated in the present study, some mono-algal diets were actually equally or even more productive than some of binary algal diets used for *B. similis* culture; combination of Chaet with other microalgae consistently performed inferiorly to the monoalgal Pav or T-Iso. Hence, comprehensive research is clearly needed to improve our understanding of the best feeding practice for any candidate copepod species that is considered having potential in aquaculture hatcheries. For example, biochemical analyses of copepods different developmental stages may help to provide a more complete picture on how microalgal diets affect their development and survival. This study clearly served the purpose of identifying the optimal microalgal diets for culturing *B. similis*. Based on its findings, *B. similis* should be cultured using the tri-algal diet T-Iso+Pav+Tet in order to achieve maximum productivity in culture for aquaculture purposes.

CHAPTER 7

EFFECTS OF MICROALGAE CONCENTRATION ON THE SURVIVAL, DEVELOPMENT AND PRODUCTIVITY OF THE CALANOID COPEPOD *BESTIOLINA SIMILIS*

7.1 Introduction

Marine copepods are the most abundant metazoans throughout the world's ocean (Boxshall and Halsey, 2004) and constitute the majority of plankton biomass in the epipelagic zone (Bunker and Hirst, 2004). In the wild, copepods mediate energy flow between primary producers and secondary consumers (Frost, 1972; Xu and Wang, 2001) and their naupliar stages often make up fifty percent or more of the stomach contents of early fish larvae (Sampey et al., 2007). Several commercially important zooplanktivorous fish also rely on adult copepods for their nutrition, commonly making up as much as 39% of their stomach content (e.g. *Pampus argenteus*, Dadzie et al., 2000). The ubiquitous occurrence of copepods in the marine environments and their importance as natural prey items for fish larvae has prompted an increasing interest in culturing them as a source of live feeds for marine hatcheries (Shansudin et al., 1997; O'Bryen and Lee, 2005).

Calanoid copepods are known to provide a range of crucial benefits to a variety of commercial and ornamental fish species when compared to traditional live feeds such as *Artemia* and rotifers (Payne and Rippingale, 2001; Hernández Molejón and Alvarez-Lajonchère, 2003; Drillet et al., 2006). Because of their excellent track record in significantly improving the health and fitness of cultured species, calanoids are considered as the solution for larvae that cannot be reared on traditional live feeds (Marcus and Murray, 2001; O'Bryen and Lee, 2005). Yet, despite their obvious advantages as larval live prey over traditional live feeds, copepod utilization in commercial hatcheries remains sporadic at present. This under-utilization is mainly attributed to their relatively low productivity in intensive culture (Støttrup, 2000), which in turn could be partially attributed to a lack of research in the field. For instance, most of the research efforts are focused on a handful of primarily coastal calanoid species and even after several decades of study, it was estimated that fewer than 4% of marine planktonic calanoid species have had their fecundity measured (Bunker and Hirst, 2004; Marcus et al., 2004; Hopcroft et al., 2005).

Although salinity and temperature are important culture parameters, copepod productivity is mainly dependent upon food quality and quantity, within the metabolic constraints set by temperature (Uye, 1981; Kleppel, 1992; Dam et al., 1994; Escribano et al., 1997; Dutz, 1998). The small size, natural abundance and the fact that it is a favourite prey for a broad assemblage of marine larvae makes *B. similis* an excellent candidate as live prey for fish larvae reared in aquaculture (McKinnon et al., 2003). While an optimal microalgal diet consisting of the tri-algal diet T-Iso+Tet+Pav was determined to be the optimal diet for *B. similis* (chapter 6), it is also of paramount importance to evaluate the influence of microalgal quantity on its culture productivity. Hence, this study was set out to investigate the effects of various concentration of this optimal microalgal diet on major productivity-related parameters of *B. similis* with the objective to provide guidelines for optimal feeding ration for *B. similis* under intensive culture conditions.

7.2 Materials and methods

7.2.1 General procedure

All experiments were conducted at MARFU, James Cook University, Queensland, Australia. The collection, separation and stock culture of *B. similis*, as well as the culture protocol for the three algal species used for this experiment were described in the General Materials and Methods (Chapter 2).

The experimental microalgae concentrations were as follow: 1800, 1500, 1200, 900, 600, 300,150 and 0 µg C l^{-1} .They were chosen to reflect a wide variety of food conditions, ranging from limiting to saturating as concentrations of 300 µg C l^{-1} and lower are generally characterized as limiting for calanoid copepods (Koski and Klein Breteler, 2003) while 1500 µg C l^{-1} and above are known to satiate *A. tonsa,* a similar sized planktonic copepod (Kiørboe et al., 1985). To achieve experimental microalgal concentrations, cell concentrations (cells/ml) of each microalgal species were first determined using a FlowCAM™ particle analyser, before being converted to absolute carbon concentration (µg C/l^{-1}) based on McKinnon et al. (2003). Microalgal cultures were subsequently combined in a 1:1:1 carbon ratio and diluted using filtered seawater to make for each of the experimental food concentrations.

A pre-conditioning period of at least one generation was ensured for each experiment. Copepods were cultured in 1L beaker with a stocking density of approximately 1000 individuals.L-1 and fed daily. Pelagic copepods are known to acclimate to their food condition on a time scale of hours to days (Mayzaud et al., 1998), and a pre-conditioning period of at least one generation was hence more than sufficient to eliminate any potential residual effect from previous feeding history. Depending on water quality condition, between 10 to 30% of culture water was exchanged every 2 days using a siphon with a 25 µm mesh attached to the end to prevent the removal of any *B. similis*.

Three experiments were conducted to test for the influences of microalgae concentration on major parameters related to *B. similis* productivity in culture, i.e. (1) daily egg production rate (EPR; egg $female^{-1}$ day^{-1}), egg diameter (µm), egg hatching rate (%), and faecal pellet production rate (FPPR; faecal pellet.$female^{-1}$.day^{-1}); (2) naupliar and copepodite survival (%), median development time from eggs to nauplii, copepodites and adult females (days), adult female life expectancy (days) and cumulative egg production over total female lifespan (eggs $female^{-1}$); (3) population growth and sex ratio.

Throughout all experiments, water temperature was maintained at 27±1°C and salinity at 30±1‰ while the light regime was set at 12L:12D. Observations and counting of eggs, nauplii, copepodites and adults were made using a Sedgewick–Rafter counter and a Leica CME optical microscope (model TN-PSE30).

7.2.2 Daily egg and faecal pellet production experiment

Following a pre-conditioning period, adult females with ripe ovaries were randomly selected and individually incubated in 60 ml containers filled with 50 ml of fresh seawater and microalgal food added at the designated concentrations except for the controls, in which females were starved for 48 h and incubated in filtered seawater without microalgal food (i.e. food ration= 0 µg C l^{-1}). Each containers was labelled according to its experimental food concentrations, sealed and mounted on a plankton wheel (rotation rate = 60 cycles/hour). Six replicates were set up daily for each treatment. Female egg production rate (EPR) and faecal pellet production rate (FPPR) were assessed after 24 h. In the cases of finding a dead female after 24 h, the replicate was discarded and its egg and faecal pellet production were not taken into account. New replicates containers were set up daily, with fresh seawater and a new female pre-conditioned to the particular experimental food concentration. The experiment was run for 9 consecutive days. Due to variation in female survival under different food concentrations, an uneven number of replicates was obtained daily for egg production and faecal pellet production data for each treatment. However, with the replacement of invalid replicates, data from a total of 33 valid replicates (n=33) were eventually achieved for each treatment.

In addition to determine daily egg and faecal pellet production, the diameters of all eggs produced on the final day of the experiment (6 replicates/treatment) were measured with a Leica CME optical microscope (model TN-PSE30). After measurement, the eggs were then incubated for hatching rates calculations. Egg hatching rate (%) was estimated by calculating the difference between the initial number of eggs and the number of unhatched eggs observed after 48 and 96 h of incubation.

$$EHS\ (\%) = \frac{[(\text{No. of eggs introduced initially} - \text{No. of unhatched eggs}) * 100]}{\text{No. of eggs introduced initially}}$$

7.2.3 Nauplii and copepodites survival and development, adult female life expectancy and cumulative lifespan egg production experiment

Groups of 15 sexually mature females were randomly selected and incubated inside containers filled with 500 ml of fresh seawater and fed at each of the 8 designated microalgal concentration. Two males were also added to each container to insure that female fecundity would not be affected by the absence of male. All containers were labelled with diet concentration treatment before being sealed and mounted on a rotating plankton wheel. After 24 h, all adult copepods were discarded so that only eggs and newly hatched nauplii (<24 h) remained in each of the containers. The number of newly hatched nauplii appearing on the first day was then recorded before gently transferring them to a separate 500 ml container filled with fresh seawater and with the same experimental food concentration. Containers containing nauplii were labelled to allow identification of the microalgae concentration treatment and the date of the nauplii hatching. Nauplii were then cultured in these containers with food concentration checked daily and adjusted accordingly for each treatment. Following the same procedure, separate containers were set up daily for nauplii hatching in the subsequent days from egg produced by the females. Daily, newly hatched nauplii were set up in separated containers until all eggs had hatched and no more nauplii were found. In this way, the exact hatching date of each nauplius was known, allowing precise estimation of the duration of development. A total of 4 replicates, each containing at least 20 nauplii were conducted for each treatments

Nauplii development was closely monitored and as they started to develop into copepodites, new containers were similarly set up daily for the newly appearing copepodites. As a result, every copepodites appearing on a similar day were cultured in the same container, to allow precise recording of their median development duration. The same procedure was applied for the newly appeared adult females in order to allow for the estimation of median development duration from egg to adult.

Nauplii survival was calculated by dividing the total number of nauplii that molted successfully into copepodites by the initial number of nauplii for each replicates and averaged for each algal concentration treatment. Average copepodite survival was similarly calculated by dividing the total number of copepodites that molted successfully to become adults by the initial number of copepodites for each treatment.

B. similis median development time from eggs to nauplii/copepodites/adult females is defined as the time when 50% of the eggs had hatched as nauplii or when 50% of population had molted to become copepodites or adult females. Median development duration was calculated using the following formula, (Peterson and Painting, 1990):

$$MDT(nauplii / copepodites / adultfemales) = \frac{\sum_{n}^{n+1} N(\text{development stage})_n * n}{\sum N(\text{development stage})}$$

Where N is the number of nauplii, copepodites or adult females found on a given day n.

Adult female life expectancy and their cumulative egg production over total lifespan were determined as follow: Upon noticing the appearance of a mature females (CVI) in a treatment, they were individually transferred to a 500 ml containers with the same experimental algal concentration. One adult male (CVI) preconditioned to the food concentration was also added to ensure that female fecundity was not limited by fertilization. The containers were then labelled, sealed and placed on the rotating plankton wheel. Every 24 h, each pair of male and female *B. similis* was gently transferred to a new container filled with fresh seawater and the same experimental algal concentration. The seawater in the original container was drained onto a mesh to enable the counting of eggs produced over the past 24 h. Egg output was determined daily for each female in all treatments until the death of the female, at which point lifespan and cumulative egg production over the female lifespan was calculated. During the experiment, any dead males found were replaced by a new male preconditioned to the same experimental food concentration. A total of 32 replicates were conducted for each treatment to determine cumulative egg production and average female lifespan.

7.2.4 Population growth and sex ratio experiment

For the population growth experiment, following the preconditioning period, 7 sexually mature females and 2 males were introduced to a 500 ml container and cultured under one experimental microalgae concentration (4 replicates/treatment). All containers were labelled, sealed and mounted on a rotating plankton wheel for a duration of 14 days, which should allow a second generation to be produced (Chapter 6). The microalgal concentration in each container was adjusted daily by adding appropriate quantity of the trialgal diet, while the build-up of detritus

was gently removed using a siphon with a 25 µm mesh sieve attached to its end to prevent the removal of any of any *B. similis*. After 14 days, all replicates were drained through a 25 µm sieve and all retained eggs, nauplii, copepodites and adults were fixed using a 10% buffered formalin fixative for later counting and sexing of all adults. The intrinsic rate of population increase r was then calculated for each treatment using the formulation:

$$r = \frac{\ln(\frac{N_0}{N_1})}{t}$$

Where N_0= population number at the beginning of the experiment, N_1 = population number at the end of the experiment while t (days) is the duration of the experiment (Fenchel, 1974).

7.2.5 Data collection and analysis

Data are presented as mean ± standard error (SE). Egg production rate, faecal pellet production rate, egg hatching rate, female proportion, female live expectancy, female total egg production, population growth and median development data were confirmed to meet the parametric test assumptions (i.e. balanced study design, normally distributed, homogeneity of variance) and were analysed using one-way ANOVA. When a significant difference ($p<0.05$) was detected, the Tukey's multiple comparisons test was used to determine specific differences among treatments ($p<0.05$). Egg diameter size data did not meet the parametric test assumptions and a Kruskal-Wallis test was used for statistical analysis. If a significant difference ($p<0.05$) was detected, a multiple comparison of mean ranks was used to determine specific differences among treatments ($p<0.05$). Data on egg hatching rate data were log transformed and pooled across all replicates for each treatment before being analysed for significant difference between treatments, using the Chi square test. All statistical analyses were conducted using Statistica™ version 8.

The correlation between faecal pellet production rates and microalgal diet concentrations, and the correlation between cumulative egg production rate and microalgal diet concentration were both assessed using nonlinear regression analysis in SigmaPlot™ (version 11).

Firstly, the non-linear Michael-Menten equation (2 parameters; Holling, 1959) was used to describe the relationship between female faecal pellet production rate (faecal pellets female^{-1} day^{-1}) and microalgae concentration (µg C l^{-1}):

$$y = \frac{a * x}{c + x}$$

where y is the faecal pellet production rate and x is the microalgae concentration, a is the maximum rate of faecal pellet production and c is the half saturation rate (microalgae concentration that produce 50% of the highest y value).

Secondly, the nonlinear Hill equation (3 parameters, Holling, 1959) was used to describe relationship between cumulative egg production rate over female lifespan (eggs female^{-1}) and microalgae concentration (µg C l^{-1}):

$$y = \frac{(a * x^b)}{(c^b + x^b)}$$

where y is the rate of egg production/cumulative egg production and x is the microalgae concentration, a is the maximum rate of egg production, b is the gradient of curve between 25% and 75% of maximum y value and c is the half saturation rate (microalgae concentration that produces 50% of the highest y value).

7.3 Results

7.3.1 Daily egg and faecal pellet production, egg size and hatching success

Microalgal diet concentration had a significant effect ($p<0.05$) on *B. similis* egg production rate (EPR) and faecal pellet production rate (FPPR), as well as 48 and 96 h egg hatching rate (EHR) but no significant effect ($p>0.05$) was detected for egg diameters (Table 7.1). Females fed with a 1500 µg C l^{-1} food concentration exhibited the highest EPR (22.6 ± 1.4 eggs female^{-1} day^{-1}), which was significantly higher than all other treatments except for the 1800 and 1200 µg C l^{-1} treatments (18.6 ± 1.4 and 20.1 ± 1.2 eggs female^{-1} day^{-1} respectively). The result also showed that *B. similis* fecundity was significantly limited when daily food ration was < 1200 µg C l^{-1} (Table 7.1). When result from the unfed control is excluded, the lowest EPR was found at the lowest food concentration treatment of 150 µg C l^{-1} (2.2 ± 0.2 eggs female^{-1} day^{-1}), significantly lower than all other treatments except for the 300 µg C l^{-1} treatment (4.4 ± 0.5 eggs female^{-1} day^{-1}).

Table 7.1: Female *Bestiolina similis* average daily egg and faecal pellets production rate when fed a trialgal diet (T-Iso+Tet+Pav) at different concentrations. Values in blanket were not included for statistical analysis due to few replicates retrieved. n/a indicates that not enough data were obtained to calculate reliable average. Different superscript letters indicate significant difference within a column ($p<0.05$). Data are presented as mean ± standard errors.

Food concentration (µg C l^{-1})	Daily Egg Production Rate (eggs $female^{-1}$ day^{-1})	Daily Faecal Pellets Production Rate (faecal pellets $female^{-1}$ day^{-1})	Egg Diameter (µm)	48 h Egg Hatching Rate (%)	96 h Egg Hatching Rate (%)
1800	18.6 ± 1.4 ^{a}	182.8 ± 10.2 ^{A}	81.64 ± 0.93	83.0 ± 2.0 ^{fg}	90.7 ± 1.7 ^{F}
1500	22.6 ± 1.4 ^{a}	206.4 ± 11.5 ^{A}	83.48 ± 1.05	85.2 ± 2.1 ^{f}	93.7 ± 1.0 ^{F}
1200	20.1 ± 1.2 ^{a}	197.9 ± 10.1 ^{A}	83.89 ± 0.85	85.5 ± 2.4 ^{fg}	89.3 ± 1.2 ^{F}
900	12.7 ± 0.8 ^{b}	140.0± 6.7 ^{B}	84.16 ± 0.74	86.8 ± 1.9 ^{f}	92.8 ± 1.0 ^{F}
600	6.9 ± 0.6 ^{c}	125.5 ± 6.0 ^{B}	87.04 ± 1.23	84.3 ± 1.7 ^{fg}	90.7 ± 1.2 ^{F}
300	4.4 ± 0.5 ^{cd}	106.1 ± 5.9 ^{BC}	85.94 ± 1.35	83.3 ± 2.1 ^{fg}	90.7 ± 0.7 ^{F}
150	2.2 ± 0.2 ^{d}	80.3 ± 5.9 ^{C}	84.08 ± 1.08	76.0 ± 1.8 ^{g}	83.3 ± 1.0 ^{G}
0	(0.3 ± 0.2)	(13.8 ± 1.3)	n/a	n/a	n/a

B. similis faecal pellet production rate was significantly influenced by microalgae concentration (Table 7.1). The highest FPPR was found at 1500 µg C l^{-1} (206.36 ± 11.50 faecal pellets $female^{-1}$ day^{-1}) but not significantly different from FPPR of the 1800 and 1200 µg C l^{-1} treatments (182.76 ± 10.14 and 197.88 ± 10.14 faecal pellets $female^{-1}$ day^{-1}) (Table 7.1). On the other hand, relatively low FPPR were found when *B. similis* was reared using a 150 µg C l^{-1} food ration (80 ± 6 faecal pellets $female^{-1}$ day^{-1}), significantly different from all other treatments except the 300 µg C l^{-1} treatment (106 ± 6 faecal pellets $female^{-1}$ day^{-1}), indicating that the ingestion of *B. similis* females was limited when reared using these low food concentrations. FPPR of *B. similis* female as a function of microalgae concentration was plotted to determine the microalgae threshold at which FPPR started to saturate (Figure 7.1; see Table 7.2 for details). *B. similis* FPPR was found to saturate at a food concentration of 783.40 C l^{-1} (Figure 7.1).

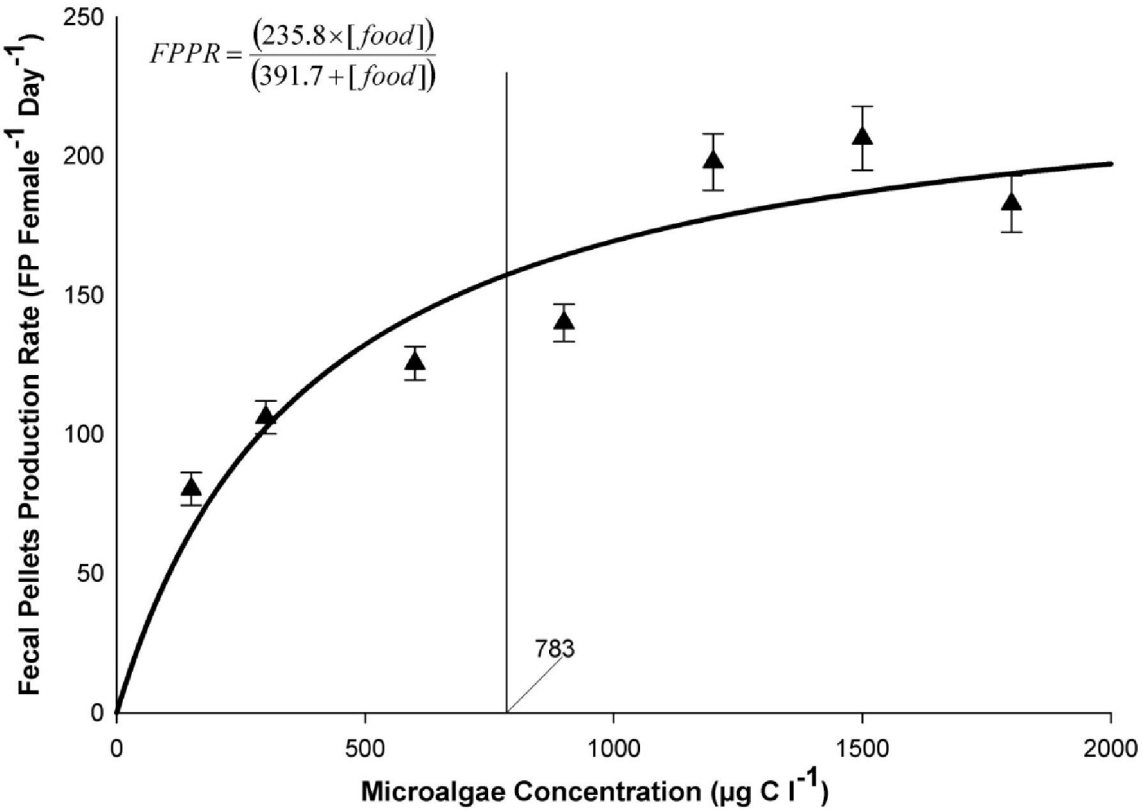


Figure 7.1: Relationship between faecal pellet production rates and concentration of a mixed microalgal diet T-Iso+Tet+Pav in the calanoid copepod *B. similis*. Each data point represents average ± standard error. The vertical line is the carbon concentration to which faecal pellet production saturates. Curves were fitted using SigmaPlot™, version 11.0 using an iterative process.

Table 7.2: Summary of models parameters and results for *B. similis* female. The nonlinear Hill equation were used to describe the relationship between microalgae concentration (µg C l^{-1}) and female cumulative egg production (eggs $female^{-1}$). The nonlinear Michaelis-Menten equation was used to describe the relationship between microalgae concentration (µg C l^{-1}) and faecal pellet production rate (faecal pellets $female^{-1}$ day^{-1}). 'R^2' is the coefficient of determination, 'R^2 adj' is the adjusted coefficient of determination and 'p' is the significance level of the fit.

Dependent variable	Function	Equation	a	b	c	Saturation point (µg C l^{-1})	R^2	R^2 adj	p
Female FPPR	Michaelis-Menton, 2 parameters	$y=(a*x)/(b+x)$	235.82	391.70	n/a	783.40	0.86	0.83	<0.03
Female Cumulative EP	Hill, 3 parameters	$y=(a*x^b)/(c^b+x^b)$	137.84	1.46	331.89	663.78	0.89	0.84	<0.02

Finally, egg hatching rate (EHR) was also significantly affected by microalgal concentration. Food ration of 300 µg C l^{-1} and higher had an higher EHR overall, with 48 h EHR>80% and 96 h EHR>89%. Egg produced at the 150µg C l^{-1} treatment had the lowest 48 hours and 96 hours egg hatching rates although they were only significantly different from the rest of the treatments for 96 hours (83 ± 1%) (Table 7.1).

7.3.2 Median development time from eggs to nauplii, copepodite and adult females and survival of nauplii and copepodites

The median development time (MDT) from egg to nauplius, copepodite and adult female were all significantly influenced ($p<0.05$) by microalgal concentration (Table 7.3).

Eggs produced at the two lowest microalgae concentrations tested (150 and 300 µg C l^{-1}) had the longest egg incubations times (2.94 ± 0.02 and 2.35 days, respectively) significantly longer ($p<0.05$) than the rest of the treatments, while the 150 µg C l^{-1} treatment was significant longer than the 300 µg C l^{-1} treatment (Table 7.3). There was no significant different difference in egg incubation time among the other treatments (Table 7.3)

Table 7.3: Average survival of nauplii and copepodites and median development time from egg of the naupliar, copepodite and adult female stage of the paracalanoid copepod *Bestiolina similis* when fed a trialgal diet (T-Iso+Tet+Pav) at different concentrations. Data are presented as average +/- standard errors. Different superscript letters indicate significant differences within the column. No enough data were retrieved from the controls as development was not observed beyond the second nauplius stage (NII) due to starvation.

Microalgal concentration (µg C l^{-1})	Median development time from eggs to nauplii (days)	Naupliar survival (%)	Median development time from egg to copepodite stage (days)	Copepodite survival (%)	Median development time from egg to adult female (days)
1800	2.14 ± 0.04 ^{a}	95 ± 2 ^{d}	3.80 ± 0.08 ^{f}	92 ± 2 ^{h}	5.93 ± 0.07 ^{k}
1500	2.16 ± 0.01 ^{a}	94 ± 2 ^{d}	3.78 ± 0.08 ^{f}	90 ± 2 ^{h}	5.94 ± 0.08 ^{k}
1200	2.16 ± 0.04 ^{a}	94 ± 3 ^{d}	3.80 ± 0.13 ^{f}	91 ± 3 ^{h}	5.96 ± 0.10 ^{k}
900	2.17 ± 0.03 ^{a}	92 ± 5 ^{d}	3.74 ± 0.04 ^{f}	81 ± 2 ^{h}	5.91 ± 0.05 ^{k}
600	2.14 ± 0.01 ^{a}	96 ± 3 ^{d}	3.86 ± 0.06 ^{f}	80 ± 2 ^{h}	6.00 ± 0.05 ^{k}
300	2.35 ± 0.01 ^{b}	85 ± 1 ^{d}	3.83 ± 0.08 ^{f}	52 ± 5 ^{i}	6.18 ± 0.08 ^{k}
150	2.94 ± 0.02 ^{c}	65 ± 3 ^{e}	4.52 ± 0.12 ^{g}	39 ± 3 ^{j}	7.46 ± 0.10 ^{l}

Naupliar survival was high (≥ 85%) for microalgae concentrations higher than 300 µg C l^{-1} and no significantly difference was detected among those treatments ($p>0.05$). However, a significantly lower survival of only 65% was found when a microalgal concentration as low as 150 µg C l^{-1} was used to fed *B. similis* (Table 7.3).

B. similis copepodites started to appear on day 3 and the average MDT from eggs to copepodites was shorter than 3.86 days when food rations was ≥ 300 µg C l^{-1} and no significant difference was detected among these treatments. However, when microalgae concentration were as low as 150 µg C l^{-1}, the development time was found to increase significantly to 4.52 ± 0.12 day (Table 7.3).

As for nauplii, copepodites survival was also reasonably high (≥ 80%) when reared at concentrations ranging from 600 to 1800 µg C l^{-1}, with no significant difference detected among these treatments. However, a significant decrease to 52 ± 5%. in copepodite survival was observed when food ration was decreased to 300, µg C l^{-1}. Reduction of microalgae

concentration to 150 µg C l^{-1} further significantly decreased copepodite survival to 39 ± 3%. In comparison to nauplii, copepodite survival was consistently lower under a similar microalgal ration (Table 7.3).

B. similis adult females started to appear on day 5 of the trial. MDT from eggs to sexually mature females was also significantly influenced by microalgal concentration. The longest MDT from eggs to females was produced at the lowest food ration of 150 µg C l^{-1} (7.46 ± 0.10 days), which was significantly longer than the rest of the treatments (Table 7.3).

7.3.3 Female lifespan and total EP over female lifespan

Food quantity had a significant influence ($p<0.05$) on average adult lifespan of *B. similis* females (Fig. 7.2). The longest average female lifespan for *B. similis* was found with food rations ranging from 300 to 900 µg C l^{-1} (6.93 ± 0.57 to 7.19 ± 0.39 days) although they were not significantly different ($p>0.05$) from those of the 1200, 1500 or 1800 µg C l^{-1} treatments (Fig. 7.2). The shortest average female lifespan (4.75 ± 0.80 days) was recorded at the lowest food ration treatment of 150 µg C l^{-1}, which was significantly shorter than the 300, 600 and 900 µg C l^{-1} treatments but not significantly different ($p<0.05$) from those found at microalgae concentrations ranging from 1200 to 1800 µg C l^{-1} (Fig. 7.2).

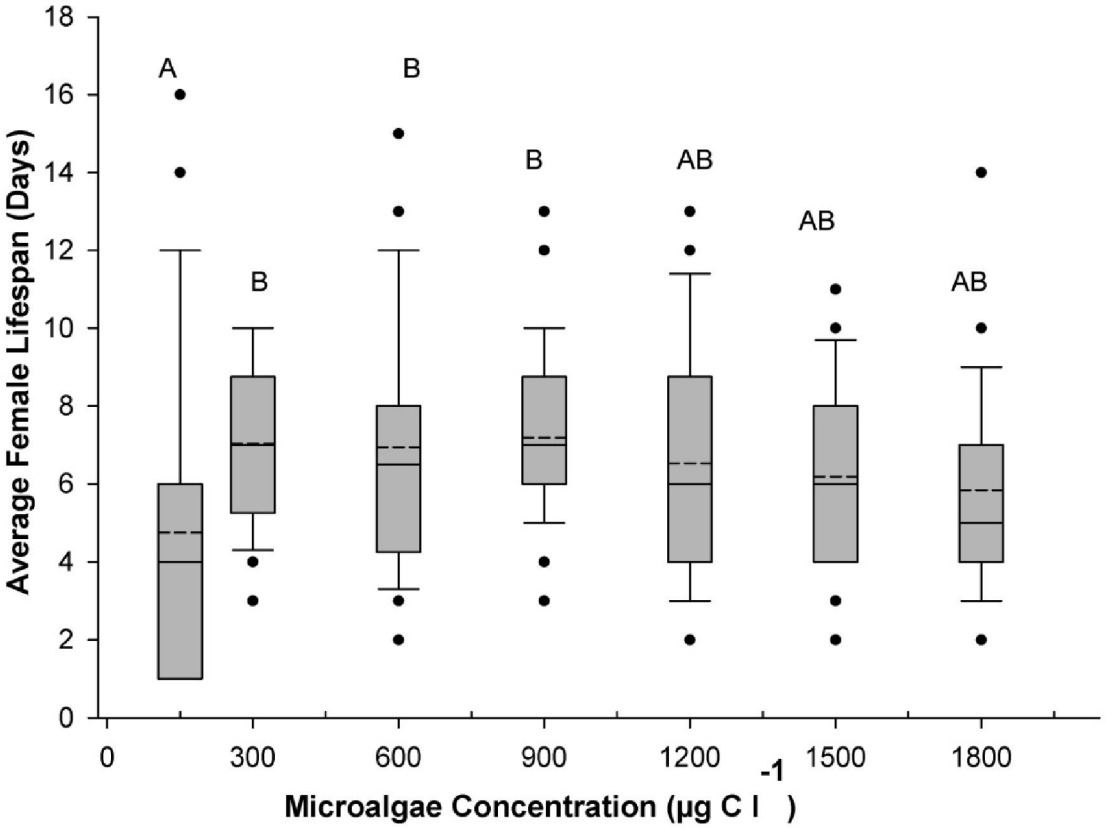


Figure 7.2: Average *B. similis* female lifespan as a function of concentration of a trialgal diet T-Iso+Tet+Pav. The boundary of the box closest to zero indicates the 25th percentile and the boundary farthest from zero indicates the 75th percentile. Vertical error bars indicate the 90th and 10th percentiles. Average are indicated by the dashed line while median values are indicated with the full line. Black circles indicate outliers. Different letters on top of the box plots indicate significant differences ($p<0.05$) between the treatments.

B. similis cumulative egg output over female lifespan was also significantly ($p<0.05$) influenced by food ration (Fig 7.3) with the higher total egg productions found at algal concentrations of 1200, 1500 and 1800 µg C l^{-1} (140.6 ± 10.4; 130.6 ± 10.0 and 132.3 ± 8.6 eggs $female^{-1}$, respectively), significantly higher than the 600, 300 and 150 µg C l^{-1} treatments ($p>0.05$). Females reared under 900 and 600 µg C l^{-1} food rations produced intermediate total egg output (100.8 ± 7.3 and 79.6614 ± 6.4 eggs $female^{-1}$ respectively) while the lowest average cumulative egg output was found with females reared on the lowest 150 µg C l^{-1} food ration (38.3 ± 3.7 eggs $female^{-1}$), which was significantly different ($p<0.05$) from all other treatments tested except for 300 µg C l^{-1} treatment, which was the second lowest ($p>0.05$) (Fig 7.3).

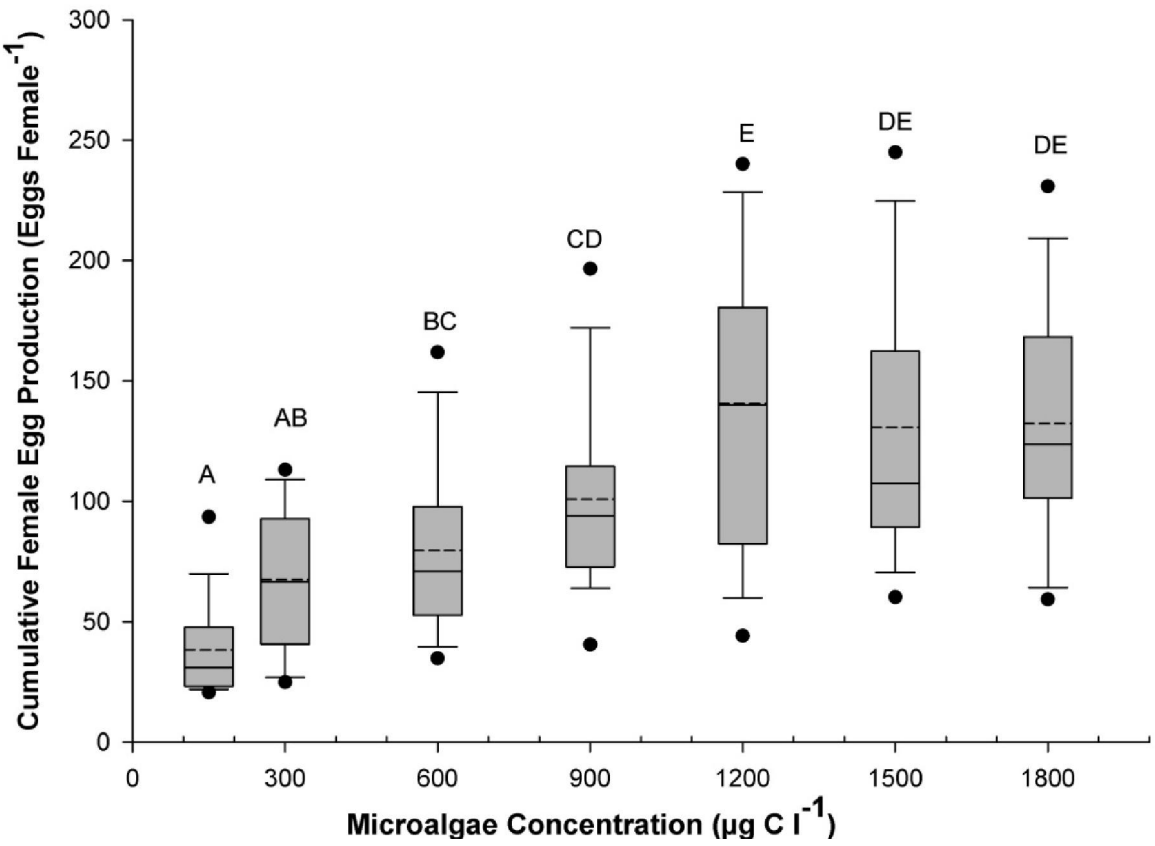


Figure 7.3: Average total egg production over *B. similis* female lifespan as a function of concentration of a trialgal diet T-Iso+Tet+Pav. The boundary of the box closest to zero indicates the 25th percentile and the boundary farthest from zero indicates the 75th percentile. Vertical error bars indicate the 90th and 10th percentiles. Average are indicated by the dashed line while median values are indicated with the full line. Dark circles indicate outliers. Different letters on top of the box plots indicate significant differences ($p<0.05$) between the treatments.

B. similis cumulative egg production as a function of algal concentration was estimated by fitting the data with a Type III Holling functional response (Hill equation, $R^2=0.89$, $p<0.02$) (Fig. 7.4, Table 7.2). *B. similis* cumulative egg production was found to saturate at a food concentration of 663.78 µg C l^{-1} (Fig. 7.4).

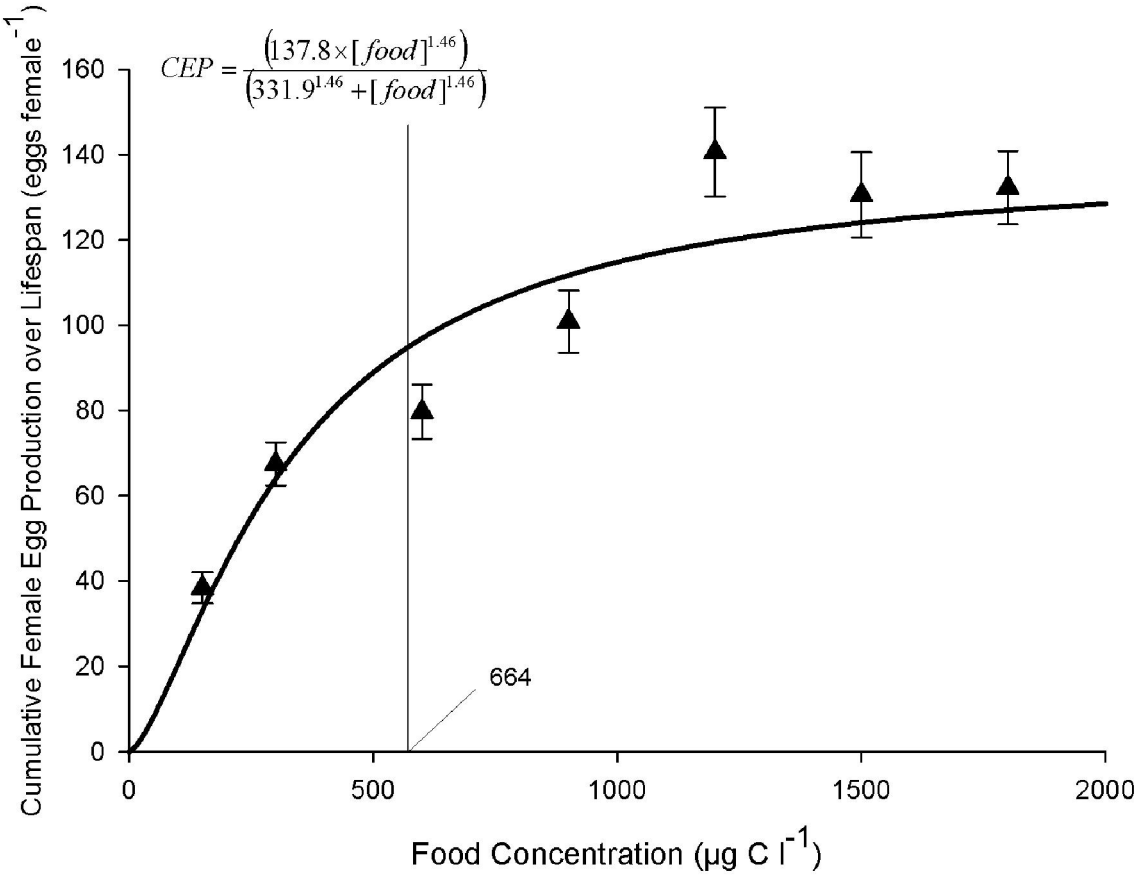


Figure 7.4: Relationship between female total egg production and concentration of a mixed microalgal diet T-Iso+Tet+Pav in the calanoid copepod *B. similis*. The data points represent average while vertical error bars represent standard errors (n=32). The vertical line is the carbon concentration at which total egg production saturates. Curves were fitted using SigmaPlot™, version 11.0 using an iterative process.

7.3.4 Population growth and composition

After 14 days of culture, population increase of *B. similis* was significantly ($p<0.05$) affected by microalgae concentration, with or without taking into account of unhatched eggs in the final count (Table 7.4). Copepods reared at the 1200 µg C l^{-1} food ration had the highest intrinsic rate of population increase (0.27 ± 0.01) although it was not significantly different from the other treatments except for the 150 µg C l^{-1} treatment, which was the only treatment to produce a negative intrinsic rate of population increase (-0.03 ± 0.03) (Table 7.4).

Among *B. similis* adults of the final population, sex ratios were consistently heavily skewed toward females with no significant effect of food ration on sex ratio detected ($p>0.05$) (Table 7.4).

Table 7.4: Final population and intrinsic rate (r) of population increase of *B. similis* cultured over a 14 days period fed on different concentration of a trialgal diet T-Iso+Tet+Pav. Values in blanket were not included for statistical analysis due to few replicates retrieved. No enough data were retrieved from the controls as development was not observed beyond the second nauplius stage (NII) due to starvation. Different superscripts indicate significant difference between microalgae concentrations.

Microalgal Concentration (µg C l-1)	Intrinsic Rate of Population Increase	Final Population Number When All Stages are Included	Final Population Number of All Post-Egg-Stages	Final Female Proportion (%)
1800	0.25 ± 0.01 a	323.5 ± 45.88	243.5 ± 35.32	0.91 ± 0.03
1500	0.25 ± 0.01 a	297 ± 26.89	254.75 ± 23.47	0.92 ± 0.01
1200	0.27 ± 0.01 a	433.25 ± 76.88	345 ± 71.51	0.94 ± 0.04
900	0.25 ± 0.01 a	327.25 ± 40.31	241.5 ± 38.21	0.87 ± 0.03
600	0.23 ± 0.01 a	237.5 ± 27.02	179.5 ± 28.00	0.83 ± 0.01
300	0.21 ± 0.02 a	182.5 ± 49.11	162 ± 49.48	0.80 ± 0.02
150	-0.03 ± 0.03 b	7.75 ± 3.77	7.25 ± 3.28	(0.95 ± 0.04)

7.4 Discussion

Microalgal concentration is known to be a key factor in determining copepod productivity (Klein Breteler and Gonzalez, 1982; Tirelli and Mayzaud, 2005). In an effort to improve *Bestiolina. similis* intensive culture protocol, the present study examined the influences of a wide range of microalgae concentrations of a trialgal diet (T-iso+Tet+Pav at a 1:1:1 carbon ratio) on major indices relating to *B. similis* productivity in culture.

A consumer functional response (FR) is the relationship between its consumption rate and the abundance of its food (Jeschke et al., 2004). Several studies have demonstrated that planktonic copepods ingestion rates were seldom saturated in the wild, even during events of phytoplankton blooms (Mayzaud & Poulet, 1978; Ayukai, 1987; Liang et al., 1994; Hirst and Lampitt, 1998), which makes it quite difficult to accurately characterize their FR *in situ*, especially at high food concentrations. Contrary to *in situ* experiments, food conditions can be precisely controlled during laboratory studies, and saturation of ingestion was commonly observed in laboratory-cultured copepods (Corner et al*.,* 1972; Frost, 1972; Uye, 1981; Tirelli and Mayzaud, 2005; Gusmão and McKinnon, 2009a).

Functional responses referred to as 'dome-shaped' responses take into account a decrease in consumption rate at very high food abundance (Jeschke et al., 2004). Dome-shaped functional responses result from consumer confusion, clogging of consumer filter and/or accumulation of toxic substances produced by high concentrations of prey items (Jeschke et al., 2004). These mechanisms can diminish ingestion rate during episodes of increased food abundance and are not always taken into account when modeling copepod functional response. Indications of decreased egg production rates, limited faecal pellet production rates and lower cumulative egg production were provided at the highest food concentrations tested, although never significant. Having conducted more analyses at very high food concentration (>1800 µg C l^{-1}) could have provided a more precise representation of a dome-shaped response for *B. similis* at high food rations. Laboratory studies are hence a relevant alternative to gain an understanding of the full spectrum of copepod FR (Støttrup and Jensen 1990), which could in turn provide useful information about their performances in the wild.

B. similis total egg production over female lifespan was determined to saturate at a food quantity of 664 µg C l^{-1}, meaning that any increase in microalgal concentration above this threshold will not provide further significant increase in total egg production. Cumulative egg production arguably offers a more complete picture of females productivity when compared to the daily egg production rate because it accounts for the total fecundity of *B. similis* females over lifespan and not just a daily average. To put into operation such a minimal optimal microalgal concentration as part of a culture protocol for *B. similis* is highly desirable for commercial hatcheries and would provide several significant advantages overtime. Firstly, it would insure that *B. similis*

productivity in culture is not limited by microalgae concentration. Secondly, such a minimal optimal food concentration would limit the accumulation of excess uneaten microalgae that can potentially negatively impact water quality parameters overtime. Finally, feeding such a food concentration to cultures of *B. similis* will save time and effort culturing unneeded additional algal biomass, ultimately cutting down operational costs overtime.

Copepod faecal pellet production rates is a good proxy of their ingestion rate (Ayukai, 1987) and food concentration is known to affected the physical characteristics of the faecal pellets (Besiktepe and Dam, 2002). Ingestion rate is not simply related to food quantity but rather to the combined interactions of food quality and quantity with ingestion, gut transit time and assimilation efficiency (Mitra and Flynn, 2007). High food concentration episodes tend to increased gut residence time and to produce large and densely packed faecal pellets (Dagg and Walser, 1986). However, other studies have reported a decrease in gut transit time associated with increasing concentration of certain food types such as diatoms (Tirelli and Mayzaud, 2005). Decrease in gut residence time during episodes of low food concentrations is believe to save the energy cost of ingestion, as copepods are unable to extract much from the ingested materials, and to result in the production of smaller, less dense and more fragile faecal pellets (Paffenhofer and Van Sant, 1985; Mitra and Flynn, 2007). This was confirmed in the present study as visual inspection of the faecal pellets revealed that smaller, less dense pellets of inconsistent shape were produced at food rations of 150 and 300 µg C l^{-1}, whereas comparatively larger and denser faecal pellets of consistent shape were found at food rations of 900 µg C l^{-1} and higher. Evidences based on faecal pellet production rate and their physical characteristics indicate a limiting food ration for *B. similis* at the 150 and 300 µg C l^{-1} treatments. Finally, the variable production of faecal pellets observed in the controls (no microalgal food offer) might be the result of opportunistic coprophagy.

Acartia spp. are known to contain mostly lipids such as triacylglycerol that only reflect recent nutritional conditions (Koski and Kuosa, 1999). The negligible amount of eggs, as well as very low faecal pellets production observed for *B. similis* in the unfed control confirmed that its fecundity is largely dependent on recently ingested food rather than on energy reserve from previous feeding history.

Experimental results demonstrated that a microalgal concentration of 150 µg C l^{-1} produced significantly shorter median development times from egg to nauplius, copepodite and adult female. This is consistent with reports of *B. similis* generation time increasing with decreasing food quantity, as reported in previously studies (Arnott et al., 1986; Ban, 1994).

Although no significant different in sex ratios were detected among the microalgae concentration tested, *B. similis* sex ratio was consistently strongly biased in favour of females. While calanoid copepods sex-determination mechanisms remain largely unknown, the highly skewed sex ratios observed in all treatments in the present study could be explained by the intersexuality mechanism postulated by Gusmão and McKinnon (2009b) in which under certain environmental conditions, a sex change occurs during the late copepodite development.

A population growth experiment conducted over 14 days provided positive intrinsic rates of population increase for all microalgae concentrations tested, with the exception for the 150 µg C l^{-1} treatment. Such a low food concentration should hence be avoided in *B. similis* culture as it was too low to support any growth in population over a 14 days period. Interestingly, food concentration ranging from 300 to 1800 µg C l^{-1} did not produced significant different in final intrinsic rates of population increase.

In light of the results presented in the present study, it is suggested that marine hatcheries should pay closer attention to optimizing food condition for calanoid intensive cultivation. The quality and quantity of a microalgal diet could both significant impact on the culture productivity of calanoid copepods. Identifying a minimal microalgal ration that would ensure maximum productivity would provide numerous advantages, including better water quality parameters maintained overtime, improved control over outbreak of contaminants such as protozoa or decomposers, and finally save time and effort in culturing microalgae. Based on the present study, for the intensive culture of *B. similis* using the trialgal diet of T-Iso+Tet+Pav, at a concentration between 664 µg C l^{-1} should insure that its rate of ingestion as well as total egg production over female lifespan, are maximized.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Calanoid copepods, especially their nauplii, are an important part of diet for a variety of marine fish larvae in the wild (Chen et al., 2006; Chesney, 2005), typically making up fifty percent or more of their stomach contents (Støttrup, 2000). In aquaculture settings, calanoids have also been proved to substantially improve larval fitness and survival in a wide range of species (see Chapter 1). Yet, calanoids are not used routinely in commercial hatcheries, and only a few species have so far been investigated for potential use in aquaculture. As a matter of fact, Bunker and Hirst (2004) reported that out of more than 2300 known calanoid species, only about 70 had their fecundity measured under laboratory conditions.

Acartia sinjiensis and *Bestiolina similis* are two tropical calanoid species that are commonly found in coastal waters of northern Queensland, Australia, and they possess a great potential as hatchery live feeds (McKinnon et al., 2003). Obviously, as a single copepod species is unlikely to be suited for the rearing of all fish species (Payne and Rippingale.,2001), it is beneficial to screen as many potential calanoid species as possible and assess their potential as live feeds, so that nauplii of different sizes and different biochemical contents can be provided as a live prey for the cultivation of various marine larvae.

The development of efficient, reliable and cost-effective intensive culture methods for calanoid copepods is critical for the advancement of their utilization in commercial hatcheries. Research presented in this thesis provides rigorous scientific evidence to drive the improvement of calanoid copepod intensive cultivation as well as recommending strategies to improve their

culture management. This thesis is hence an important contribution, not only to the optimization of intensive culture for these two calanoid copepod species in particular, but also for calanoid copepods culture in general.

8.1 Major findings from this thesis

The first three data chapters of this thesis (Chapter 3, 4 and 5) investigated the effects of photoperiod regime, stocking density and cannibalism on a wide range of parameters linking to culture productivity of *A. sinjiensis* and provide key suggestions to improve and maximize its productivity in intensive culture. These three parameters only received limited attention in the literature for copepods and had never been investigated for *A. sinjiensis*.

Photoperiod regime was shown to significantly impact on A. sinjiensis productivity and a clear trend of increasing egg production with longer illumination period was demonstrated. Based on the results, a 18L : 6D photoperiod was recommended to maximize culture productivity of A. sinjiensis **(Chapter 3).**

The influence of photoperiod on calanoids has received only very limited attention in the past (Peck and Holste, 2006) with a majority of past publications investigating its role in inducing diapause egg production (Chinnery and Williams, 2003). The present study showed that the 18L:6D and the 24L:0D photoperiods produced the best daily egg production rate, the highest 48 h egg hatching rate and accelerated the development of *A. sinjiensis*. Nauplii development duration was found inversely related to illumination duration, which allow for manipulation of the photoperiod regime in order to slow down their development and keep nauplii available for fish larvae for longer periods. This study was the first to investigate *A. sinjiensis* adult lifespan. A 12L:12D photoperiod regime produced the longest female lifespan (10.9 ± 0.1 days) which was significantly shorter than that of a 24L:0D photoperiod. A 18:6 photoperiod regime was hence recommended as the best photoperiod regime *A. sinjiensis* intensive culture methods. This study was also the first to report on *A. sinjiensis* sex ratio, which was strongly skewed in favor of females but not significantly affected by photoperiod regimes.

A relatively high 2000 adults l^{1} stocking density did not significantly limit A. sinjiensis daily egg production rates, 96 hours egg hatching rates, nor adult survival. A. sinjiensis possess a good tolerance for high stocking densities when compared to other calanoid species and is suitable to be intensively cultured at a stocking density of 2000 adults l^{1}, and possibly higher. **(Chapter 4).**

The difficulty in achieving high culture density of copepods greatly limits their utilization in commercial hatcheries. Only few studies have investigated the effects of stocking density on calanoids productivity from an aquaculture perspective (Peck and Holste, 2006; Jepsen et al., 2007), and no studies have so far evaluated its influence on *A. sinjiensis.* Based on results from this study, it is recommended to culture *A. sinjiensis* at high stocking density to save space and labor. However, the optimal inoculation density to be adopted would obviously depending on the needs of a particular hatchery, taking into consideration various factors, such as quantity of available starter *A. sinjiensis* for inoculation and culture duration allowed prior to expected time of larval hatching. The population growth regression lines (Fig. 4.7) provides a useful tool for such a decision making process.

Adult cannibalism in A. sinjiensis was revealed to be relatively modest and can be significantly decreased by preventing the accumulation of early naupliar stages within the culture and ensuring that optimal microalgae feeding ration are maintained **(Chapter 5).**

Though this study was not the first one to describe cannibalism in the genus *Acartia* (see Lonsdale et al., 1979), it was the first to focus on cannibalism from the perspective of maintenance of intensive copepod cultures for aquaculture hatchery purposes. Cannibalism in *A. sinjiensis* did not occur in adult males and was largely confined to early naupliar stages in terms of prey. It was also shown that cannibalism was more pronounced when predators were under conditions of food limitation. Starved females had significantly higher cannibalism rates than fed ones. Cannibalism rate also increased with increasing prey concentration, underlying *A. sinjiensis* opportunistic feeding strategy. It is further revealed that significant interactions exist between microalgae ration and prey density on cannibalism rate. The low microalgae rations resulted in significant increased differences in cannibalism rate when the prey density was 160 nauplii l^{-1} and above. Based on the results, it is important to maintain optimal microalgae ration

to alleviate cannibalistic tendency of adult females. Furthermore, daily removal of eggs and nauplii should also help to limit cannibalism.

The last two data chapters of this thesis (Chapter 6 and 7) focused on a second calanoid copepod with very good potential as aquaculture live feed: *Bestiolina similis*. These two chapters investigated the effects of microalgal diets and of microalgae concentration on productivity-related parameters of this copepod. Throughout these two chapters, key suggestions are made to significantly improve *B. similis* productivity in intensive culture conditions. These studies were the first to investigate the influence of microalgae quality and quantity on *B. similis* culture.

Among ten microalgal diets tested, the trialgal diet T-iso+Tet+Pav (1:1:1) was found to maximize egg production rate, cumulative egg output over female lifespan, egg incubation time and hatching success and population growth of B. similis and was therefore recommended as the best diet for B. similis intensive cultivation **(Chapter 6).**

Calanoid copepods undergo profound changes during ontogenetic development from eggs to adults and as a result, the best suited diets are likely to vary as they develop through different life stages. On this basis, a single species of microalgae can become nutritionally limiting whereas appropriate combination of algae are likely to offer a better balance of nutrients. However, it does not mean that any combination of microalgae will lead to higher culture productivity. Mixed diets including the diatom Chaet consistently performed inferiorly, even worse than the monoalgal diets of Pav and T-Iso illustrated the important study such as of chapter 6..With the identification of optimal microalgal diet conducted in this chapter, the need to find out suitable feeding ration is highlighted, which is dealt with in Chapter 7.

It was shown that the feeding ration lower than 600 µg C l^{-1} of the trialgal diet T-iso+Tet+Pas significantly limited B. similis productivity, while a low concentration of 150 µg C l^{-1} produced negative population growth and significantly delayed its development. B. similis total egg production over female lifespan was found to saturate at a concentration of 664µgC l^{-1} and such a ration is recommended as the minimal microalgae concentration for B. similis maximum productivity in culture **(Chapter 7).**

It is crucial to known the minimal microalgae feeding ration that would not affect the culture productivity for intensive culture of copepods and incorporated it in culture protocols as

it saves time and effort in culturing microalgae, as well as allows improved water quality parameters overtime. A trialgal diet T-i*so+Tet+Pav* concentration of 664 µg C l^{-1} was found to saturate the total egg production over female lifespan of *B. similis* and was hence recommended for optimal culture.

Through chapters 6 and 7, a clear picture of *B. similis* optimal feeding regime was revealed, both in term of microalgal quality and quantity. Based on results from both chapters, a mixed microalgal diet consisting of T-iso+Tet+Pav (1:1:1) at a concentration of 664 µg Cl^{-1} is recommended to maximize the productivity of *B. similis* under intensive culture methods. In the absence of evidence to the contrary, these results could be used as a baseline to determine the microalgal diet of other paracalanid species.

8.2 Future directions

Research presented in this thesis confirms the importance of conducting small-scale laboratory experiments to evaluate and identify the optimal culture parameters for the culture of calanoid copepods. Small scale experiments allow for more replicates to be conducted and for more precise data to be collected, down to individual level, providing a more complete understand the underling mechanisms investigated.

Clearly, more research is urgently needed to assess the culture potential of more candidate calanoid copepods as prey for culture of early fish larvae. Given the huge species diversity of calanoid copepods, it is highly possible more species will be identified as suitable for intensive cultivation in the near future. To identify more of such copepod species will mean that potentially more marine larvae could be cultured commercially, which would greatly benefit the aquaculture industry.

Additional knowledge on calanoids biochemical composition and its influence on their values as larval prey is also needed. Vitamins, protein and amino acid are known to play a significant role in the optimal performance and survival of copepods but have received very limited attention in the past literature, with most of the research focused on lipids and fatty acids composition (Ajiboye et al., 2011).

Research efforts should also be directed toward upscaling copepod cultivation to commercial levels in order to ensure a reliable and continuous production, especially for calanoid and paracalanid species with high potential as hatchery live feeds. Many families of copepods from these genera remain un-studied or under-studied. Design and engineering aspects of cost-effective mass cultivation systems for calanoid copepods are largely lacking, and a sustained research effort is needed to promote development of efficient and reliable culture systems for calanoids in aquaculture settings. Some recent publication have investigated the multi-generations cultivation of calanoid copepods through the use of re-circulating systems (Carotenuto et al., 2012) which have the benefits of limiting cannibalism and facilitating eggs and nauplii collection through automatic harvesting. Such research is valuable as it provides culture systems that maximize productivity while reducing labour costs.

Experiments conducted in this thesis provide numerous new findings applicable to improve culture productivity of *A. sinjiensis* and *B. similis*, two calanoid copepods with excellent potential as live feeds for aquaculture hatcheries that play important roles in mesoplanktonic ecosystems. Practical recommendations were made in each chapters with respect to different culture factors, including photoperiod, stocking density, cannibalism, food quality and quantity. Results provided in this chapter contribute to substantially improved our knowledge of intensive culture methods for these two important copepod species and provided valuable information about their general biology and ecology.

While made specifically for *A. sinjiensis* or *B. similis* these recommendations might be transferable to other calanoid species and could provide useful clues and general guideline information for the culture of other calanoid species with similar ecological requirements (Payne and Rippingale, 2001). Thus, the significance of the results presented in this thesis go far beyond the improvement of culture techniques for the two species.

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APPENDIX 1

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