School of Molecular and Life Science

Supplementing probiotics during early stages of mud crab (*Scylla paramamosain*) culture under various rearing systems

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DECLARATION

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:

Date: 07/12/2018

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PREAMBLE

All experiments were conducted in South Viet Nam under a facility of commercial mud crab hatchery.

The thesis is structured into two sections. The section I is based on the production of megalopa from zoea 1 stage, whereas section II is based on the production of crablets from megalopa stage. Section I comprises of 5 chapters, out of which chapters 3 to 5 are independent experiments based chapters aiming to supplement probiotics to the live food and finding the impact of most suitable existing rearing system. Section II comprises of three chapters, out of which two chapters 6 and 7 are experimental chapters focussing on investigating the most suitable locally available diet in most appropriate rearing system. The details of these two sections are provided in subsequent paragraphs.

Chapter 1 briefly highlights the status, gaps and bottlenecks of mud crab seed production research. This chapter also justifies and underlines the need to undertake the current research, and ends with the aim, objectives and significance of the study.

Chapter 2 provides an overview of existing literature on the general biology and status of mud crab hatchery techniques. This chapter also reviews literature on nutrition, prophylaxis and rearing systems on mud crab larviculture as well as describes the use of probiotics and biofloc in crustacean culture.

Chapter 3 describes the first experiment of the thesis that critically re-evaluates by conducting an experiment on the most frequently used five feeding regimes used in the existing literature. The research in this chapter compares five feeding regimes in order to select two best performing regimes. A version of this chapter has been published in “Modern Applied Science” (*Appendix 2*).

Chapter 4 researches on the interactions between the enrichment of live food with probiotics and extended inclusion of rotifers in the pre-selected diets from the chapter 3. The effects of enriching live food fed to crab larvae and extension of rotifer inclusion with *Artemia* until Z4 or Z5 are also researched in this chapter (Published in “American Journal of Applied Science”, *Appendix 2*).

Chapter 5 describes an experiment that compares the effects of four different rearing systems on the survival, growth performance, and quality of mud crab megalopa. As

biofloc water system recently has been one of the most argued systems in crustacean aquaculture, its role in a larval rearing system for mud crabs is used one to the test treatments in this chapter (Published in "Aquaculture International", *Appendix 2*).

Chapter 6 (will be submitted soon) and chapter 7 describe two independent experiments that investigate the effect of locally available diets and different rearing systems on the survival, growth performance, and duration of megalopa metamorphosis into crablets, respectively. The potential application of biofloc technology in the nursery phase is also used and researched in chapter 7.

Chapter 8 summarizes all findings and attempts to produce a coherent discussion on all-important outcomes of the research. The main conclusions from the present study and recommendations for future research are also included in this chapter.

ABSTRACT

Mud crabs of the genus *Scylla* are commercially important species in aquaculture owing to their high consumer demand and high prices in global market place. In Vietnam, *Scylla paramamosain* is the second most cultured species after shrimps in coastal areas. The demand for mud crab seed has recently increased as shrimp industry suffered from disease outbreaks. However, hatchery-raised mud crab seed fails to meet the high commercial demand for mud crabs. Sub-optimal feeding regimes, rearing conditions and disease are considered as crucial factors affecting the overall health and growth of mud crab larvae, leading to the low success rate in the production of crablets under hatchery conditions. Though the use of probiotics is practiced in shrimp industry, the published literature on their use in crab hatchery is limited.

A series of three experiments on zoeal stages of mud crab were conducted to investigate the effects of supplementing probiotics to several feeding regimes including a regime where rotifers were extended as a part of feeding regime under various rearing systems that were set up at a commercial crab hatchery in Viet Nam. Other two experiments were conducted on megalopa stage of mud crab using locally available diets under four rearing systems.

The first experiment was conducted to raise megalopa from Z1 by comparing five selected feeding regimes from the published literature. The results showed that prolonged inclusion of rotifers along with *Artemia* nauplii until the Z4 and Z5 stages, could improve the production of megalopa, which were used as two of the selected feeding regimes and subjected to a 4x2 experimental design where probiotic-enriched live food and extended inclusion of rotifers in the diet were used as two factors. The results of this experiment showed that the metamorphosis and survival of megalopa were improved when they were fed both probiotic-enriched rotifers and *Artemia*, however, there was no interactive effect between enriched live food and extended inclusion of rotifers in the diet.

In the third experiment, clear water (CW), green water (GW), recirculating water (RW) and biofloc water (BW) systems were compared and evaluated to select the best performing rearing system during the zoeal stages. The results showed that significantly increased megalopa survival and resistance to air exposure during the

simulated transport stress test was possible in the green water system. The biofloc water system performed similar to clear water system for rearing zoeal stages.

The fourth experiment investigated the use of locally available diets including live *Acetes* (LA), minced shrimp meat (MSM), locally formulated feed (LFF) and commercial feed (CF) while raising crablets from megalopa. The results demonstrated that LA, MSM, LFF and CF could be used in a similar manner to microbound diet (MBD) or *Artemia* during the megalopa stage, as reflected by the successful moults and high survival of crablets. However, LA was the most suitable diet for megalopa shown by the highest survival, synchronous moulting and short development time.

The final experiment was similar to the third experiment but was performed at the megalopa stage. The result showed that megalopa fed with local diets could be reared under recirculating or biofloc water systems without any adverse effects on the crablet survival. Conversely, a green water system resulted in low survival of crablets.

The study concludes that the mud crab seed production should be divided into two phases to improve crablet production. In the first phase, enriched rotifers mixed with enriched *Artemia* with probiotics should be fed to the late zoeal stage in a green water system. In the second phase, a clear water system can be used for raising crablets from megalopa. However, when seawater supply is limited biofloc water or recirculating water systems can be used to raise crablets from megalopa fed locally available diets.

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LIST OF ABBREVIATIONS

A	*Artemia*
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
BW	Biofloc water
BFT	Biofloc technology
C	Carbon
C1:	First crablet
CF	Commercial feed
CFU	Colony forming unit
CIPRS	Curtin International Postgraduate Research Scholarships
CP	Crude protein
CW	Clear water
DAH	Days after hatching
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EMS	Early mortality syndrome
EPA	Eicosapentaenoic acid
G	Gram
GW	Green water
H	Hour
HUFA	Highly unsaturated fatty acids
ITS-1	First internal transcribed spacer
L	Litre
LA	Live *Acetes*
LC_{50}	Lethal concentration 50
LFF	Locally formulated feed
M	Megalopa
Mm	Millimetre
MBD	Microbound diet
µm	Micrometre
mg	Milligram
MoET	Ministry of Education and Training
mL	Millilitre

MSM	Minced shrimp meat
N	Nitrogen
R	Rotifers
RW	Recirculating water
SE	Standard error
SPSS	Statistical package for social science
TAN	Total ammonia nitrogen
TSA	Tryptic soy agar
PL	Post-larvae
ppm	Parts per million
USA	United States of America
W	Wattage
WSSV	White spot syndrome virus
Z	Zoea

LIST OF COMMON AND SCIENTIFIC NAMES

Acetes shrimp, *Acetes* spp.

Atlantic cod, *Gadus morhua*

Blood cockle, *Anadara granosa*

Blue shrimp, *Penaeus stylirostris*

Brine shrimp, *Artemia franciscana*

Brown tiger shrimp, *Penaeus esculentus*

Common mud crab, *Panopeus herbstii*

Florida pompano, *Trachinotus carolinus*

Giant freshwater prawn, *Macrobrachium rosenbergii*

Gilthead seabream, *Sparus aurata*

Greasyback shrimp, *Metapenaeus ensis*

Indian white prawn, *Fenneropenaeus indicus*

Kuruma shrimp, *Marsupanaeus japonicas*

Mud crab, *Scylla paramamosain*

Mud crab, *Scylla serrata*

Mud crab, *Scylla olivacea*

Mud crab, *Sylla tranquebarica*

Mud shrimp, *Upogebia pusilla*

Mud worm, *Marphysa* spp.

Oyster, *Crassostrea belcheri*

Pink shrimp, *Farfantepenaeus paulensis*

Pink shrimp, *Farfantepenaeus brasilliensis*

Pink shrimp, *Farfantepenaeus duorarum*

Portunid crab, *Thalamita crenata*

Rainbow trout, *Oncorhynchus mykiss*

Rock lobster, *Panulirus cygnus*

Rotifers, *Brachionus plicatilis*

Spider crabs, *Maja brachydactyla*

Swimmer crab, *Portunus pelagicus*

Swimming crab, *P. trituberculatus*

Tiger shrimp, *Penaeus monodon*

Tilapia, *Oreochromis niloticus*

White leg shrimp, *Litopenaeus vannamei*

Section I

Production of Megalopae

CHAPTER 1: Introduction

1.1 Background

Mud crabs of the genus *Scylla* are economically important in crustacean aquaculture (Waiho et al., 2018), helping to generate a source of income and fresh food for many coastal communities in the Asia-Pacific region (Petersen, Suc, Thanh, & Hien, 2011). The earliest research on mud crab larviculture was performed by Ong Kah Sin during the early 1960s (Ong, 1964). Subsequently, despite intensive research in Japan and South East Asia, mud crab seed production has remained a challenge and failed to meet the high commercial demand. The survival rate of newly hatched Z1 to megalopa is highly variable and ranges from zero to 70% (Baylon & Failaman, 1999; Dat, 1999; Heasman & Fielder, 1983; Jantrarotai, Temphakdee, & Pripanapong, 2004; Yi, Lee, & Lee, 2009). In a review of commercially important portunid crabs, the average survival rate from hatching to first crab stage was around 10% (Hamasaki, Obata, Dan, & Kitada, 2011). A more recent study showed that the survival of first crab stage has not yet improved, with a survival rate of 6.9% for crablets (Thirunavukkarasu, Nesakumari, & Shanmugam, 2014). Nutrition, disease and rearing conditions are crucial factors that affect the health and growth of larvae, and if not optimal, can result in mass mortality (Waiho et al., 2018).

An inappropriate feeding regime that results in poor nutritional status during the zoeal stages can affect the survival of megalopa (Zeng & Li, 1999). Feeding either rotifers alone (Baylon, 2009; Nghia, 2004) or exclusively *Artemia* (Ruscoe, Williams, & Shelley, 2004b; Zeng, Li, & Zeng, 2004) or starved *Artemia* (Dan, Oshiro, Ashidate, & Hamasaki, 2016b) resulted in low survival and an increase in larval development time. In contrast, a feeding regime combining rotifers and *Artemia* led to the higher overall zoeal survival in *Scylla serrata* (Baylon, Bachoco, Huyo, & Gallo, 1999), *S. paramamosain* (Zeng & Li, 1999) and *Thalamita crenata* (Godfred, Ravi, & Kannupandi, 1997). However, research findings on the actual timing of the withdrawal of rotifers from a mixed diet are inconsistent. Ruscoe et al. (2004b) and Nghia (2004) recommended that inclusion of dietary rotifers beyond the Z1 stage is not beneficial for the metamorphosis and survival rate of megalopa, whereas withdrawal of rotifers from a mixed diet at early larval stages may influence the nutritional links between consecutive larval stages that might result in mass

mortality (Zeng & Li, 1999). *Artemia* can starve in the larval culture tank due to their quick assimilation of yolk reserves; and the larvae fed such *Artemia* during the late zoeal stages might carry over adverse effects on survival and morphology to the megalopa stage and beyond (Dan et al., 2016b; Dan et al., 2016c). Therefore, the timing of the withdrawal of rotifers from the rearing system is crucial and needs to be investigated.

The replacement of antibiotics and chemicals with probiotics is considered as a promising approach in addressing the problems on malnutrition and diseases in mud crab larval rearing (Cholik, 1999; Lavilla-Pitogo & De la Peña, 2004). The addition of probiotics suppressed the growth of necrosis bacteria (Dan & Hamasaki, 2015) and pathogenic green colonies of *Vibrios* (Talib, Onn, Chowdury, Din, & Yahya, 2017) in culture water, which resulted in improved survival of larvae. Similarly, the probiotics effectively improved the survival, enzyme activities, and water quality of swimming crab, *Portunus pelagicus,* larvae (Talpur, Ikhwanuddin, Abdullah, & Ambok Bolong, 2013). However, information on the effects of using probiotic-enriched live food on crab larvae is lacking and the beneficial effects of probiotics are not apparent in commercial-scale seed production (Waiho et al., 2018).

The use of a closed rearing system with limited water exchange is important for minimizing the threat of pathogens and disease outbreaks in hatcheries. Careful management of incoming water, and limitation of water discharge from closed rearing system can reduce, or even eliminate, the transfer of pathogens from the environment into the hatcheries or vice versa (Samocha et al., 2007). Reducing the amount of water required for the hatchery phase also guarantees a year-round supply of seed (Mallasen & Valenti, 1998), allowing the establishment of hatcheries in areas far from the coast or away from unsuitable water conditions. The use of closed rearing systems such as recirculating water (Mallasen & Valenti, 1998), green water (Izquierdo et al., 2006; Tendencia, Bosma, Verdegem, & Verreth, 2015) and biofloc water systems (De Lorenzo et al., 2016a; Khanjani, Sajjadi, Alizadeh, & Sourinejad, 2017) is well documented for shrimp hatcheries and nurseries. However, there is no information on the use of the biofloc water system and very limited information on the use of recirculating and green water systems for mud crab seed production.

Live food accounts for more than 50% of the variable costs of production (Quinitio, Parado-Estepa, Millamena, Rodriguez, & Borlongan, 2001), and thus many studies have investigated the replacement of *Artemia* with fresh food (Jantrarotai et al., 2004; Marichamy, 1996; Quinitio et al., 2001; Quinitio, Parado-Estepa, & Rodriguez, 2002; Rodriguez, Quinitio, Parado-Estepa, & Millamena, 2001; Shelley & Lovatelli, 2011; Williams, Wood, Dalliston, Shelley, & Kuo, 1999) and microbound diets (MBD) (Genodepa, Zeng, & Southgate, 2004; Holme, Zeng, & Southgate, 2006b). Fresh food and MBD have the potential to replace 50–100% of *Artemia* while rearing megalopae; however, the findings regarding fresh food remain generalized, and ingredients to formulate MBD such as squid meal and dried rotifer meal are expensive and locally unavailable. Hence, more reliable and locally available diets should be derived and/or formulated with either globally acceptable or locally available ingredients (Kovalenko, D'Abramo, Ohs, & Buddington, 2002; Nguyen, Chim, Lemaire, & Wantiez, 2014) for the successful operation of mud crab aquaculture.

Based on the above background, this thesis investigates the live food based feeding regimes and locally available diets to culture megalopae and crablets respectively under various rearing systems by addressing the effects of probiotics supplied in enriched live food on the nursery production of mud crab megalopae and crablets.

1.2 Aim

The major aim of this study was to improve the production of the megalopae and crablets of *Scylla paramamosain* through the supplementation of probiotics and utilization of available feed under various rearing systems.

1.3 Objectives

To achieve this aim, the following specific objectives were formulated:

1. To re-evaluate the performance of selected published feeding regimes during larval stages of the mud crab and select a minimum of two best performing feeding regimes for subsequent experiments.

2. To evaluate the effect of selected probiotics on the survival, growth performance, metamorphosis and development time from zoea to megalopa stage when fed probiotic-enriched live food.

3. To examine any interactions between the enrichment of live food and extended inclusion of rotifers during rearing of zoeal to megalopa stage.

4. To investigate the effect of locally available diets in the nursery phase.

5. To compare the effects of four different rearing systems including clear water, green water, recirculating water and biofloc water systems on the survival, growth performance and quality of mud crab megalopae and crablets.

1.4 Significance

The output of this study will contribute in the improvement of mud crab seed production. The specific significance of the current research is as follows:

1. The research will provide new information about the benefit of extended rotifer inclusion with *Artemia* in the feeding regime for mud crab larviculture.

2. The research will provide novel knowledge on the application of probiotics *via* live food enrichment in mud crab larvae.

3. The research will contribute to understanding the interactive effects between enriched/un-enriched live food with probiotics and extension of rotifer inclusion in mud crab larviculture.

4. The research will provide an insight into the current practices of using locally available and commercial feed during mud crab hatchery production.

5. The research will provide new information about using a biofloc water system in a mud crab larval nursery.

CHAPTER 2: Literature Review

2.1 Mud crab species

Mud crabs, belonging to the genus *Scylla* and commonly known as mangrove crabs, are generally associated with mud flats of supralittoral, intertidal and subtidal zones in and in close proximity to mangrove forest habitats throughout the Indo-Pacific (Keenan, Davie, & Mann, 1998). Species identification of the mud crab has been quite confusing for a long period. Estampador (1949) correctly identified three species and new subspecies in the genus *Scylla* for the first time based on colour patterns, relative size, cheliped spination, chromosome 'form' and the process of gamete development. However, this nomenclature was not well accepted due to the lack of reference of material (Keenan et al., 1998). The confusion in the taxonomy of the genus *Scylla* was not resolved until 1998, when Keenan et al. (1998) recognised four distinct *Scylla* species, *viz. Scylla serrata* (Forskal, 1775), *S. olivacea* (Herbst, 1796), *S. tranquebarica* (Fabricius, 1798) and *S. paramamosain* (Estampador, 1949), by using two independent genetic methods, allozyme electrophoresis and mitochondrial DNA sequencing of two mitochondrial DNA genes. Most recently, identification of four mud crab species (genus *Scylla*) has been confirmed using the first internal transcribed spacer (ITS-1) and 16S rDNA markers (Imai, Cheng, Hamasaki, & Numachi, 2004).

2.2 General biology

2.2.1 Taxonomy and distribution of *Scylla paramamosain*

The taxonomy of *S. paramamosain* is as follow:

Phylum: Arthropoda
Class: Crustacea
Subclass: Malacostraca
Order: Decapoda
Suborder: Reptantia
Family: Portunidae
Genus: *Scylla*
Species: *S. paramamosain* (Estampador, 1949)

Among four mud crab species, *S. paramamosain* is widely distributed along the continental coast of South East Asia from the South China Sea to south Java Sea (Keenan et al., 1998). *S. paramamosain* is an abundant species in northern Japan (Obata, Imai, Kitakado, Hamasaki, & Kitada, 2006; Walton, Le Vay, Lebata, Binas, & Primavera, 2006), China (Ma, Zhang, Ma, & Qiao, 2006) and the Mekong Delta of Vietnam (Lindner, 2005; Petersen et al., 2013).

2.2.2 Life cycle and habitat

The life cycles of all four species of the genus *Scylla* are similar (Moser, Macintosh, Laoprasert, & Tongdee, 2005). Gravid females briefly migrate to the sea and spawn only in the place where salinity is high and stable (Hyland, Hill, & Lee, 1984; Kulasekarapandian & Panigrahi, 2005). Newly hatched larvae metamorphose and pass through five pelagic zoeal stages (Le, 2010). The fifth zoeal stage develops into the megalopa stage which appears and settles in shallow waters where macro algae, sea grasses and mangrove roots are in abundance (Davis, 2003). Megalopa metamorphose into crablets which migrate from intertidal to subtidal areas (Le Vay, Ut, & Jones, 2001). The crablets undergo 15–17 metamorphoses to attain full maturity (Davis, 2003; Robertson, 1996).

2.2.3 Reproduction

Mud crab, *S. paramamosain* can reach maturity within 5.5 months (Le, 2010). After successful mating, the mature female stores spermatophores in their spermathecae (Robertson & Kruger, 1994) in order to fertilise their eggs for up to 6 months (spawning two or three times) without mating again (Nghia, Wille, & Sorgeloos, 2001). Spawning takes place after full ovarian maturation. In captive conditions, gravid females spawn within 8–95 days depending on the food (Millamena & Bangcaya, 2001), temperature and salinity (Dat, 1999). All extruded eggs are attached to the setae of pleopods of the female abdomen. The colour of the egg mass changes from yellow to orange then to brown and black prior to hatching. The eggs hatch into zoea (Z) after 9–13 days post spawning at a mean temperature of 28 ± 2 °C (Dat, 1999; Nguyen, 2014). The number of newly hatched Z1 from a single spawner is reported to be 3.92 ± 1.00 million per batch (Nguyen, 2014).

2.2.4 Larval development

Mud crab larvae pass through five distinct developmental stages of zoea (Z1 to Z5) and one megalopa stage before moulting into crablet. Detailed descriptions of each larval stage have been reported for closely related species of *S. olivacea* (Jantrarotai, Sirisintruwanich, Pripanapong, & Chayarat, 2006). The description of each larval stage was based on compound eyes, carapace, antennule, antenna, mandible, maxillule, maxilla, maxilliped and abdomen. The duration of moulting in each zoeal stage depends on species, spanning between 2 and 6 days (Table 2.1). Similarly, moulting duration at the megalopa stage lasts six to nine days.

Table 2.1: The duration of moulting (days) in each larval stage of the genus *Scylla*

Larval developmental stages	Duration of moulting (days)			
	S. paramamosain	*S. serrata*	*S. olivacea*	*S. tranquebarica*
Z1 to Z2	5–6	4 (3–5)	6	4
Z2 to Z3	4	2 (3–4)	4	3
Z3 to Z4	3–4	3 (3–4)	3	3
Z4 to Z5	3	3 (3–4)	4	3
Z5 to M	5	3 (4–5)	5	3
M to C1	-	7 (7–9)	7	6
Total	-	22 (22–31)	29	22
References	Thach and Thai (2004)	Yi et al. (2009) (Heasman & Fielder, 1983)	Gunarto, Parenrengi, and Septiningsih (2016)	Thirunavukkarasu et al. (2014)

Key: Z1: zoeal stage 1; Z2: zoeal stage 2; Z3: zoeal stage 3; Z4: zoeal stage 4; Z5: zoeal stage 5; M: megalopa; C1: first crablet; "-": no data. Column 3 has two reference, number in () is referred by from the second reference.

2.3 Status of mud crab hatchery technology

Research on the breeding of mud crabs has been widely undertaken in Hawaii (Brick, 1974), Africa (Davis, 2003; Hill, 1974), Australia (Heasman & Fielder, 1983), Japan (Hamasaki, Suprayudi, & Takeuchi, 2002a, 2002b), India (Thirunavukkarasu et al., 2014), Malaysia (Fazhan, Waiho, Wan Norfaizza, Megat, & Ikhwanuddin, 2017; Ong, 1964), Philippines (Baylon & Failaman, 1999; Quinitio, Parado-Estepa, & Alava, 1999; Quinitio et al., 2001), Indonesia (Cholik, 1999), Thailand (Jantrarotai et al., 2004), and Vietnam (Nghia et al., 2007a; Nguyen, 2014). However, seed supply

(crablets) for mass scale mud crab culture is currently an unmet demand (Petersen et al., 2013). Most crab farmers rely on wild collection of seeds which have declined due to over-exploitation, diminishing mangrove habitats (Le Vay et al., 2001) and pollution (Ma et al., 2014). Meanwhile, hatchery-raised seed is limited due to low success rates in hatchery production of first stage crablets (around 10%) (Hamasaki et al., 2011). The inconsistent survival rate of larvae and unsatisfactory production of crablets may be affected by sub-optimal conditions of any one of the following interrelated factors: nutrition, disease and rearing conditions (Waiho et al., 2018).

2.3.1 Nutrition

2.3.1.1 Live food

a) Rotifers and Artemia

Most hatcheries currently rely heavily on rotifers and *Artemia* as live food for the larvae due to the ease of mass culture, convenience and their suitable biochemical composition (Kostopoulou, Vasilakis, & Divanach, 2012; Sorgeloos, Coutteau, Dhert, Merchie, & Lavens, 1998). As a result of their small size (125–300 μm) (Daintith, 1996), low swimming movement, rapid reproduction and content of desired nutrients (Dhert, 1996), rotifers are used as the ideal first food for larval stages of mud crab, *Scylla* spp. (Davis, Wille, Hecht, & Sorgeloos, 2005a; Ruscoe et al., 2004b; Zeng & Li, 1999) and swimming crab, *Portunus pelagicus* (Ikhwanuddin, Azra, Redzuari, Aizam, & Abol-Munafi, 2012). In mud crab hatcheries, rotifers alone can meet the nutritional requirements for larval development until Z2 (Redzuari et al., 2012; Zeng & Li, 1999). However, if rotifers alone are used as food for Z3 onwards, heavy mortality can occur due to failure to develop to megalopa stage (Baylon, 2009; Zeng & Li, 1999).

Like rotifers, *Artemia* spp. have also been adopted as a standard food in the commercial larviculture of crustaceans (Sorgeloos et al., 1998). In mud crab culture, *Artemia* nauplii are considered as a nutritious type of food (Brick, 1974) that contribute to build-up of tissue in larvae enabling them to develop successfully to the next stage and leading to increased larval survival (Baylon, Bravo, & Maningo, 2004; Harvey & Epifanio, 1997). In contrast, a previous study suggested that *Artemia* nauplii are not a suitable food for early crab larvae (Zeng & Li, 1999), probably due to their large size (428–517 μm) (Daintith, 1996) and fast movement (Baylon, 2009;

Nghia, 2004; Zeng & Li, 1999). Thus, poor larval survival could be associated with a prey capture problem rather than a nutritional issue (Ruscoe et al., 2004b). In addition, Baylon (2009) reported that *Artemia* alone as food could not provide enough nutrients required for post-moulting, resulting in high mortality during metamorphosis of Z5 to megalopae.

Previous studies have reported that unfavourable feeding conditions, such as feeding rotifers alone or feeding exclusively *Artemia* in later larval stages, could lead to the appearance of an additional larval stage Z6 that resulted in low survival (Zeng et al., 2004). In morphological observation, Z6 are similar to Z5 except for numerical variation in spines and setae on the maxillula, maxilla and maxilliped (Zeng et al., 2004). A similar study (Dan, Kaneko, Takeshima, Ashidate, & Hamasaki, 2013) reported that poor nutrient accumulation during zoeal stages of *P. trituberculatus* led to great variation in megalopa morphology and eventually resulted in high mortality. For example, feeding newly hatched *Artemia* and starved *Artemia*, which contain low eicosapentaenoic acid (EPA) levels, produced abnormal megalopae and first crablets with close-set eyes (Dan et al., 2013; Dan et al., 2016b).

A combination diet of rotifers and *Artemia* is an ideal feeding regime that may satisfy the requirement of the optimal nutrient content and suitable size of live food for each larval stage. To date, all research results indicate that a combination of live food results in increased survival and improved development time of mud crabs, swimming crabs and other species of crab larvae. However, there have been some contradictory suggestions regarding the time of withdrawal of rotifers from the larval feeding regimes (Table 2.2). Ruscoe et al. (2004b) and Nghia (2004) found that extension of rotifer inclusion with *Artemia* until the last larval stages did not benefit the larvae. In contrast, Baylon et al. (2004) reported that inclusion of rotifers until last zoeal stages was considered to be the main food source that provided the nutrition necessary for larval survival after moulting. Furthermore, Dan et al. (2016b) found that presence of rotifers and their excrement could overcome the starvation of *Artemia,* leading to cancel the negative effects of *Artemia* starvation. Thus, the important role and the time of withdrawal of rotifers inclusion with *Artemia* in mud crab larval feeding regimes is described in details in Chapter 4 and Chapter 5 of this research.

Table 2.2: Summary of reports on the combination of rotifers and *Artemia* in the feeding regimes of brachyuran larvae

Species	Withdrawal of rotifers in feed at larval stage	Why withdrawal of rotifers in feeding regime?	Why extending inclusion of rotifers in feeding regime?	Reference
S. serrata	Z5	-	Nutritional content of rotifers and *Artemia* complement each other	Baylon and Failaman (1999)
S. serrata	Z1	No additional financial benefits	-	Ruscoe et al. (2004b)
S. serrata	Z5**	-	Best overall zoeal survival	Zeng and Li (1999)
S. paramamosain	Z1*	Not really necessary	-	Nghia (2004)
S. tranquebarica	Z3	Adversely effected metamorphosis into megalopa	-	Baylon (2009)
S. olivacea	Z5	-	High survival rate of Z5	Jantrarotai et al. (2004)
P. pelagicus	megalopa	-	Increased survival and growth rate, shortened development time.	Redzuari et al. (2012)
Thalamita crenata	Z5	Not necessary.	High survival rate of Z5	Godfred et al. (1997)
Eriocheir sinensis	Z3 or Z5**	-	-	Sui, Wille, Wu, Cheng, and Sorgeloos (2008)

* *Artemia was introduced in feeding regime at Z2 onwards*
** *Artemia was introduced in feeding regime at Z3 onwards*

Key: Z: zoea; number after the letter represents the larval stage.

b) Copepods

Apart from rotifers and *Artemia*, copepods are excellent live food for first feeding of larvae (Waiho et al., 2018) as they have a higher protein and lipid content compared to *Artemia* (Hamre et al., 2013) and higher taurine, astaxanthin and zinc content compared to rotifers (Karlsen et al., 2015). Previous studies have reported that marine copepods are potential live food that increase the growth and survival of tiger shrimp (*Penaeus monodon*) larvae (Santhanam et al., 2011) and marine fish, such as Florida pompano (*Trachinotus carolinus*) (Cassiano, Ohs, Weirich, Breen, & Rhyne,

2011) and Atlantic cod (*Gadus morhua*) (Karlsen et al., 2015). Jantrarotai et al. (2004) reported that survival and development time of *S. olivacae* larvae from Z1 to Z5 fed a combination of *Artemia* and frozen copepods were similar to those fed a combination of rotifers and *Artemia*. However, research on the use of copepods as live food in mud crab hatcheries is very limited due to specify technical difficulties and low productivity in mass production compared to rotifers and *Artemia* (Conceição, Yúfera, Makridis, Morais, & Dinis, 2010; van der Meeren, Karlsen, Liebig, & Mangor-Jensen, 2014).

c) Prey density

Prey density during rearing of the zoeal stages of the genus *Scylla* has a direct influence on survival and development time. A low density of rotifers and *Artemia* resulted in increased cannibalism and prolonged development time by the additional zoeal stage (Z6), resulting in low survival of crab larvae (Suprayudi et al., 2002a; Zeng, 1998; Zeng et al., 2004). In contrast, an increasing density of live food improved larval survival and development time (Brick, 1974; Nghia et al., 2007a) due to enhanced opportunity for direct encounter and, consequently, a higher probability of the zoea capturing prey (Heasman & Fielder, 1983). However, a high density of live food might be economically unrealistic (Nghia et al., 2007a). In previous studies (Table 2.3), the recommended density of rotifers and *Artemia* in combination diet during zoeal stage of *Scylla* was 10–60 individuals ml^{-1} and 1–10 individuals mL^{-1}, respectively.

Table 2.3: Prey density (individuals mL^{-1}), survival rate and development time of the genus *Scylla* for Z1 to Z5 or megalopae

Species	Prey density (individuals mL^{-1})	Culture vessel	Highest survival rate (%)	Development time (days)	Reference
S. serrata	(20) R + (10) A	Small-scale	60.0 (up to M)	18–19	Chen and Cheng (1985)
S. serrata	(12) R + (5) A	3-L plastic bucket	56.0 (up to M)	15–17	Baylon and Failaman (1999)
S. serrata	(15-20) R at Z1-Z2 & shifted to (15) A at Z3 onwards	300-L tank	15.0 (up to C)	-	Marichamy and Rajapackiam (1991)
S. serrata	(10-15) R + (1-5) A	10,000-L tank	2.6 (up to M)	16–18	Quinitio et al. (2001)
S. serrata	(40) R at Z1-Z2 & shifted to (0.5-4) A at Z3 onwards	1-L plastic beaker	71.7 (highest obtained up to M)	15-16	Suprayudi et al. (2002a)
S. serrata	(40) R at Z1-Z2 & shifted to (10) A at Z3 onwards	2-L plastic jar	58.0 (up to Z5)	15	Davis et al. (2005a)
S. paramamosain	(60) R at Z1-Z2 & shifted to (10) A at Z3 onwards	150 mL	33.0 (up to Z5)	18	Zeng and Li (1999)
S. paramamosain	(30-60) R at Z1-Z2 & shifted to (10-15) A at Z3 onwards	30-100-L fibreglass tank	10.0 (up to Z5)	15	Nghia et al. (2007a)
S. olivacae	(15) R + (5) A	15-L styrofoam box	13.8 (up to Z5)	21	Jantrarotai et al. (2004)

Key: R: rotifers, A: Artemia, the number in (..) is prey density, Z: zoea stage, M: megalopa, C: crablets

d) Enrichment of live food

The live foods have varied nutritious statuses that depend on different strains and culture conditions (Watanabe, Kitajima, & Fujita, 1983). The live foods generally contain only marginal levels of highly unsaturated fatty acids (n3-HUFA) (Leger, Bengtson, Simpson, & Sorgerloos, 1986). Baylon (2009) and Suprayudi, Takeuchi,

and Hamasaki (2004b) reported that crab larvae fed with rotifers or *Artemia* alone with a low n3-HUFA content resulted in high mortality during metamorphosis to megalopa. Enrichment of rotifers and *Artemia* is recommended as a solution for nutritional deficiencies in order to meet the requirements of larvae so as to sustain survival and development up to the megalopa stage (Davis, 2003). Various methods were used to enrich the live food and the effects of enrichment are shown in Table 2.4. It is worth noting that the enrichment of both rotifers and *Artemia* is more effective than enrichment only one type of them in terms of the growth performance of larvae (Davis, 2003). In addition, both excess and/or shortage of dietary n3-HUFA may be a reason for hypermorphogenesis in *Scylla* spp. larvae (Dan, Hamasaki, Kogane, Jinbo, & Ichikawa, 2009) leading to decreased survival (Kobayashi, Takeuchi, Arai, & Sekiya, 2000). Abnormal growth of Z5 and deformities of megalopa were observed when *S. paramamosain* and *S. serrata* crab larvae were fed excess level of docosahexaenoic acid (DHA) (Hamasaki et al., 2002a; Islam, Islam, Yahya, & Hashim, 2017). The level of n3-HUFA required for growth and development of crab larvae depends on different developmental stages and species (Dan et al., 2009; Islam et al., 2017). Kogane, Dan, and Hamasaki (2007) reported that a high DHA and DHA/EPA (eicosapentaenoic acid) ratio was required at first zoeal stage for its development and this value reduced from zoea 2 to the megalopa stage. Previous research also showed that with enriched live food containing n3-HUFA, the optimum DHA/EPA ratio for maintaining a high survival rate and good growth performance in crab larvae was >1.0 for *S. paramamosain* (Nghia, Wille, Vandendriessche, Vinh, & Sorgeloos, 2007b), 2.8–5.3 for *S. tranquebarica* (Kobayashi et al., 2000) and 0.7–1.0 for *S. serrata* (Suprayudi, Takeuchi, & Hamasaki, 2004a).

Table 2.4: Summary of common enrichment methods for live food and their effects on the performance of crab larvae.

Enrichment method	Live food	Species of crab	Effects	Reference
Nannochloropsis, Yugen and EPA28G	Rotifers	*S. serrata*	Improved growth performance	Suprayudi, Takeuchi, Hamasaki, and Hirokawa (2002b)
Tetraselmis algae Super Selco or DHA Selco	Both rotifers & *Artemia*	*S. serrata*	Increased survival, metamorphosis and development time	Davis (2003)
Oleic acid, EPA28G and DHA70G	*Artemia*	*S. serrata*	Larger carapace width obtained and maintained survival of first crablets	Suprayudi et al. (2004a)
Dry Selco and Frippak^{TM} CD2 Ultra	Both live foods	*S. serrata*	Had little effect on survival	Ruscoe et al. (2004b)
Culture Selco emulsion	Both live foods	*S. paramamosain*	High survival rate obtained and improved metamorphosis	Nghia et al. (2007b)
Nannochloropsis sp. and oil Selco	Both live foods	*S. paramamosain*	Increased survival of zoea 5 and megalopa	Islam et al. (2017)
Nannochloropsis sp.	*Artemia*	*S. olivacea*	Improved crablet production	Gunarto et al. (2016)

Key: EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, Dry Selco (INVE's products), Frippak^{TM} CD2 Ultra: larval shrimp food.

2.3.1.2 Fresh food

In normal practice, *Artemia* is usually used successfully for rearing crab larvae until development of the first crablet stage (Holme et al., 2006b). However, increased hatchery production costs due to the expense of *Artemia* cysts and uncertainty concerning their availability are major limiting factors in crab culture (Murthy, Yogeeshababu, Thanuja, Prakash, & Shankar, 2008). Thus, research efforts have focused on replacing *Artemia* by fresh food such as mud worm (*Marphysa* spp.), trash fish, squid and shrimp meat…etc. (Table 2.5). Fresh food is cheaper and more readily available compared to *Artemia*, however, using fresh food alone has resulted in low survival of crablets (Williams et al., 1999). Therefore, a combination of live and fresh food is considered as the optimum feeding regime for the megalopa stage onwards.

Table 2.5: Types of fresh food and survival of *Scylla* spp. crablets

Species	Type of fresh food	Crab larval stage fed	Survival (%)	Reference
S. serrata	*Artemia*	M to C1	38.9 ± 3.5	Williams et al. (1999)
	Dry *Acetes* shrimp		12.8 ± 2.0	
	Mud worm		6.7 ± 1.2	
	Artemia + dry *Acetes* shrimp		40.0 ± 5.6	
	Artemia + mud worm		57.8 ± 3.8	
	Acetes shrimp + mud worm		6.1 ± 1.6	
S. serrata	*Artemia*	M to C1	72.2	Baylon and Failaman (1999)
	Artemia + squid		33.3	
	Artemia + worm		50.0	
	Artemia + shrimp		38.9	
S. serrata	*Artemia* + minced trash fish, green mussel or *Acetes*	M to C	26.2 – 40.0	Quinitio et al. (2001)
S. olivacea	*Artemia*	M to C1	75.0 ± 8.3	Jantrarotai et al. (2004)
	Ground mollusc meat		19.4 ± 9.6	
	Artemia + ground mollusc meat		58.3 ± 0.0	

Key: M: megalopa, C: crablets.

2.3.1.3 Formulated diets

Preliminary studies on replacing unreliable live food with formulated diets in larval culture have been reported since 1971 (Meyers, 1971). A few years later, microencapsulated diets were developed to replace live foods in commercial crustacean hatcheries (Jones, Kurmaly, & Arshard, 1987). Recent research on formulated diets reported that a microbound diet (MBD) was successfully used in a crab hatchery (Genodepa et al., 2004b). Partial or total replacement of live food with MBD has been reported for crab larval stages depending on the species (Table 2.6). In the case of the *Scylla*, although early larvae of *S. serrata* readily ingest MBD, larvae fed 100% MBD had low survival rates due to their limited ability to digest MBD (Holme et al., 2006b). However, from megalopa onwards, complete replacement of live food with MBD is possible without any adverse effects on survival (Genodepa et al., 2004b). Therefore, MBD is recommended for feeding crab larvae from megalopa onwards. In addition, numerous studies on MBD in term of particle size (Genodepa, Southgate, & Zeng, 2004a), binder type (Genodepa et al, 2007b) and addition of nutrients such as lecithin and cholesterol (Holme, Southgate, & Zeng, 2007) have been performed. Although MBD has been reported as a 100%

replacement of live food, the ingredients (dried rotifers and *Artemia* meal) are not easy to find in the local area. Therefore, research on attempts to replace these ingredients with squid meal, fishmeal, krill meal and soybean meal has been conducted (Castine, Southgate, & Zeng, 2008; Holme et al., 2006b). Furthermore, formulated diets are more realistic and cost-effective when they are formulated using globally acceptable or locally available ingredients (Kovalenko et al., 2002; Nguyen, 2014). Hence, there is an urgent need for further studies on the use of cheap and locally available feed ingredients in formulated diets for mud crabs.

Table 2.6: Percentage of MBD as replacement for live food in crustacean hatchery

Species	Percentage of replacement (%)	Fed crab larvae	Survival (%)	Reference
M. rosenbergii	100	Z5 to PL1	73.3	Kovalenko et al. (2002)
S. serrata	100	M to C1	90*	Genodepa et al. (2004b)
	50	Z3 to Z4	66.0	
S. serrata	100	M to C1	46.7–60.0	Holme et al. (2006b)
P. pelagicus	100	M to C1	73.3–93.3	Castine et al. (2008)
Maja brachydactyla	50	Any larval stage	-	Andrés, Rotllant, Sastre, and Estévez (2011)

**Individual culture. Key: Z: zoea, PL: post-larvae; M: megalopa; C: crablets; (-): no data.*

2.3.2 Prophylaxis

The major constrain faced in mud crab hatcheries is high mortality during the larval stages due to bacterial and fungal infections (Lavilla-Pitogo & De la Peña, 2004; Pham et al., 2014). Vibriosis (Liessmann, 2005) and fungi *(Lagenidium callinectes, Haliphthoros milfordensis and Halocrusticida baliensis*) (Hatai, Roza & Nakayama, 2000) are the main pathogens to cause inconsistent survival of crab larvae. In aquatic environments, pathogenic bacteria develop easily and invade the larvae via the ingestion pathway (Defoirdt, Sorgeloos, & Bossier, 2011). Consequently, larvae infected by bacterial and/or fungal diseases have an unpredictable survival rate. Thus, techniques to control fungi and pathogenic bacteria in crab hatcheries are of paramount importance for optimising survival during the larval rearing process.

2.3.2.1 Antibiotics and other chemicals

Prophylaxis can be considered as one of the ideal solutions for controlling the development of fungi and harmful bacteria in the rearing medium. Therefore, the use

of antibiotics in shrimp and crab hatcheries is a very common measure for increasing yield (Azam & Narayan, 2013). Although antibiotics enhance growth and survival, the wide and frequent use of antibiotics results in the development and spread of antibiotic-resistant bacteria (Defoirdt et al., 2011), inducing slow growth rates in *S. serrata* larvae (Azam & Narayan, 2013) and mass mortality in *P. monodon* larvae (Karunasagar, Pai, Malathi, & Karunasagar, 1994). Moreover, extended use of antibiotics can induce morphological deformities in *S. serrata* larvae (bent dorsal, rostral and furcal spines) and juveniles (fused frontal and lateral spines, and asymmetrical and depressed tip of abdominal flap and flap between sternites) (Pates, Quinitio, Quinitio, & Parado-Estepa, 2017), and in giant crabs (*Pseudocarcinus gigas*) (Gardner & Northam, 1997). Excessive use of antibiotics may also result in residues in the aquaculture environment and in products (FAO, 2002) which may change micro-organism communities, cause deterioration of water quality and pose a risk to human health (Gräslund & Bengtsson, 2001).

In addition to antibiotics, other chemicals, such as malachite green, formalin and trifluralin are also used as prophylactic agents in crab larval culture. Although the application of chemicals can lead to higher survival rates and increased growth of crab larvae compared to controls (without chemicals), prolonged usage at high concentration can adversely affect survival and growth of the larvae (De Pedro, Quinitio, & Parado-Estepa, 2007). Thus, there should be judicious use of chemicals in crab hatcheries. Types of antibiotics, chemicals and their dosage for mud crab larvae which have been reported in previous studies are shown in Table 2.7.

Table 2.7: Common antibiotics and chemicals and their dosage in crab hatcheries

Species	Application duration	Antibiotics and other chemicals used	Dose used	Reference
S. serrata	Z1 to M	Penicillin-G Polymycin-B	40 ppm 10 ppm	Brick (1974)
S. serrata	First day of Z1	Streptomycin sulphate	10 ppm	Holme et al. (2006b)
S. serrata	Z1 to Z2	Oxytetracycline	10–25 ppm	Azam and Narayan (2013)
S. serrata	Z1 to M	Oxytetracycline Furazolidone	3–6 ppm 0.5–1.0 ppm	Pates et al. (2017)
S. serrata	Z1 to M	Treflan (trifluralin)	0.1 ppm	Quinitio et al. (2001)
S. serrata	Z1 to M	Formalin Trifluralin	5-15 µg L^{-1} 0.05–0.2 µg L^{-1}	De Pedro et al. (2007)
S. paramamosain	From hatching to 22 days of culture	Formalin Oxytetracycline	20 µg L^{-1} 10 ppm	Nghia et al. (2007a)

Key: Z: zoeal stage; M: megalopa stage; ppm: parts per million.

Application of chemicals and antibiotics is likely to cause a negative impact on the host and environment and also pose a threat to human health (Gräslund & Bengtsson, 2001). Therefore, elimination of the use of these substances by using alternative biocontrol techniques is recommended (De Pedro et al., 2007; Pates et al., 2017). Alternative biocontrol strategies should be focused on maximising protection of the animal and preventing the development of resistant strains (Defoirdt et al., 2011). In particular, application of probiotics in the larval rearing of *Scylla* is suggested as a preferential prophylaxis approach due to their beneficial effect on the crab larvae.

2.3.2.2 Probiotics

Probiotics are defined as any microbial supplement or the components of microbial cells that have beneficial effects on the health of the host (Gram, Melchiorsen, Spanggaard, Huber, & Nielsen, 1999; Salminen, Ouwehand, Benno, & Lee, 1999). Probiotics can be used to substitute antibiotics without any negative impact as they are non-pathogenic and non-toxic microorganisms (Farzanfar, 2006). Previous findings have shown that application of probiotics is beneficial as they improve growth, survival and health status of the host (Elumalai, 2013; Rengpipat, Phianphak, Piyatiratitivorakul, & Menasveta, 1998; Uddin et al., 2013). Probiotics are considered to be a catalytic agent that enhances protease enzyme activity in aquatic

animals to increase their food absorption and growth performance (Irianto & Austin, 2002). The probiotics are also capable of stimulating immune responses against pathogenic microorganisms (Fuller, 1992; Irianto & Austin, 2002). Recent research reported that probiotics play a vital role in maintaining water quality parameters during larviculture (Nimrat, Suksawat, Boonthai, & Vuthiphandchai, 2012; Soundarapandian & Sankar, 2008; Talpur et al., 2013). With regard to application, probiotics are easy to use in aquaculture as they can be administered to the host via live feeds (Gomez-Gil, Herrera-Vega, Abreu-Grobois, & Roque, 1998) and artificial feeds (Rengpipat, Rukpratanporn, Piyatiratitivorakul, & Menasaveta, 2000), and can also be directly applied to the rearing water (Moriarty, 1998). Probiotic products can be stored at room temperature (25–30 °C) and produced largely on an industrial scale (Balcázar et al., 2006).

a) Probiotics in crustacean hatcheries

The use of probiotics as an ideal alternative to antibiotics in crustacean hatcheries is currently recognised to provide consistent and commercially feasible production of seed (Talib et al., 2017). In a previous study, Gatesoupe (1999) reported that probiotics may be applied at an early larval stage, even when the digestive tract and immune system are not fully developed. Types of microbial species, dose and application methods, as well as their effects on the host during larviculture are summarised in Table 2.8.

Table 2.8: Common bacterial probionts employed in crustacean hatcheries

Host species	Microbe	Dose / application method	Effects on host	Reference
Litopenaeus vannamei	*B. fusiformis*	10^6 cfu mL^{-}1 / Via water	Improved survival and accelerated metamorphosis.	Guo et al. (2009)
L. vannamei	*B. coagulan* SC8468	10^5-10^6 cfu mL^{-}1 / Via water	Improved survival and digestive enzyme activities.	Zhou, Wang, and Li (2009)
L. vannamei	*B. sutilis* E20	10^6 cfu mL^{-1} / Via water	Improved survival, development, stress resistance and immune status.	Liu, Chiu, Shiu, Cheng, and Liu (2010)
L. vannamei	B. spp.	10^6 cfu mL^{-1} / Via water or live food	Improved growth, survival and enhanced water quality	Nimrat et al. (2012)
L. vannamei	*Bacillus* licheniformis & *B. subtilis*	10^6 cfu mL^{-1} / Via live food	Improved survival.	Jamali, Imani, Abdollahi, Roozbehfar, and Isari (2015)
L. vannamei & P.monodon	*B.* sp. & *Streptococcus* sp.	5 ppm / Via water	Improved quarter quality.	Soundarapandian and Babu (2010)
P. monodon	*Lactobacillus* spp., *Acidophilus* spp., *B.* spp. & *Saccharomyces cerevisiae*	5 ppm / Via water	Impoved growth, survival and health status.	Uddin et al. (2013)
Fenneropenaeus indicus	*B.* spp.	10^6–10^7 cfu mL^{-1} / Via water or live food	Impoved growth, survival and enhanced digestive enzyme activities.	Ziaei-Nejad et al. (2006)
P. trituberculatus	*Thalassobacter utilis*	10^5–10^6 cfu mL^{-1} / Via water	Improved survival and suppressed growth of *Vibrio anguillarum.*	Nogami, Hamasaki, Maeda, and Hirayama (1997)
P. pelagicus	*Lactobacillus plantarum*	Via water / 5.0 × 10^6 cfu mL^{-1}	Increased digestive enzyme activities and improved water quality.	Talpur et al. (2013)
S. serrata	*Alteromonadaceae* spp.	Via water / 7–8 log cfu mL^{-1}	Suppressed larval necrosis sympton and improved larval survival.	Dan and Hamasaki (2015)
S. paramamosain	*Bacillus* spp.	Via water / 1–2 x 10^4 cfu mL^{-1}	Increased survival and reduced green *Vibrio*	Talib et al. (2017)

Key: Cfu: colony forming unit.

Most of the research was carried in shrimp hatcheries, while a few research was performed in swimming and mud crabs. Dan and Hamasaki (2015) reported that bacteria which have been screened and isolated from water used for rearing *S. serrata* larvae can be used as a probiotic that has potential for suppressing necrosis and improving the survival of mud crab larvae up to Z5. Talib et al. (2017) reported that multispecies of the *Bacillus* genus, when added to rearing water, depressed the growth of green *Vibrio*, leading to increased survival of *S. paramamosain*. However, evidences of the beneficial effects are insufficient to support commercial-scale crab seed production due to various conditions (Waiho et al., 2018). In addition, Waiho et al. (2018) also suggested that administering enriched live food containing a probiotic orally resulted in apparent effects on the host animal. The oral administration of *Bacillus* probiotic via live food increased growth and survival in *L. vannamei* hatcheries (Jamali et al., 2015). Meanwhile, research on using probiotics via oral administration through live food as a prophylaxis method in crab hatcheries is unavailable.

b) Bacillus spp. as probiotics

The most common and successfully used bacteria as probiotics belong to the genera *Bacillus* spp., *Pseudomonas* spp., *Alterromonas* spp., *Thalassobacter* spp. and *Lactobacillus* spp. (Dan & Hamasaki, 2015; Dash et al., 2015; Hai, Buller, & Fotedar, 2009; Nimrat et al., 2012; Nogami et al., 1997; Patra & Mohamed, 2003; Talpur et al., 2013). Among the large number of probiotic products available on the market, *Bacillus* spp. have been most widely used in crustacean culture due to their characteristic advantage (Ambas, Fotedar, & Buller, 2015; Kwong, 2016; Ziaei-Nejad et al., 2006). It has been reported that use of *Bacillus* spp. resulted in enhanced water quality (Nimrat et al., 2012), inhibited pathogens directly by competing for nutrients and surface area (Far, Saad, Daud, Harmin, & Shakibazadeh, 2009; Luis-Villaseñor, Macías-Rodríguez, Gómez-Gil, Ascencio-Valle, & Campa-Córdova, 2011) or indirectly by improving host immunity (Dong et al., 2014; Kumar et al., 2013). They also contribute to increased digestive enzyme activities that lead to improved growth performance (Ziaei-Nejad et al., 2006). In addition, *Bacillus* spp. can also produce polypeptide antibiotics (Malanicheva et al., 2014) and exoenzymes (Moriarty, 1998) against a wide variety of harmful bacteria. It is also known that *Bacillus* spp. has spore forming ability that can be high resistance to terrestrial

environments (Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000) and facilitating reliable production on a commercial scale.

c) Commercially available probiotics for crustacean aquaculture

Numerous commercial probiotics have been successfully applied in crustacean aquaculture (Table 2.9). However, the beneficial effects of commercial probiotics have not been apparent in commercial-scale crab seed production (Dan & Hamasaki, 2015; Waiho et al., 2018). Therefore, there is urgent need for further research to investigate the beneficial effects of probiotics on mud crab hatcheries.

Table 2.9: Commercially available probiotics used for crustacean hatcheries

Commercial product	Probiotic organisms	Species and culture phase	Reference
Protecxin Aquatech	*B. subtilis, B. licheniformis, B. polymyxa, B. laterosporus and B. circulans*	*Fenneropenaeus indicus* hatchery and nursery	Ziaei-Nejad et al. (2006)
Sanolife ® Mic	*Bacillus* spp.	*P. monodon* and *L. vannamei* hatchery	Decamp, Moriarty, and Lavens (2008)
PrimaLac®	*L. acidophilus, L. casei, E faecium* and *B. bifidium*	*L. vannamei* nursery	Soundarapandian and Babu (2010)
Super Biotic and Biodream	*Bacillus sp.* and *Streptococcus sp.*	*P. monodon* hatchery	Miandare, Yarahmadi, and Abbasian (2016)
PondPlus® and Novozymes®	*B. subtilis, B. licheniformis, B. amyloliquefaciens, B. pumilus, B. megaterium, B. velezensis and Brevibacillus parabrevis*	*S. paramamosain* experimental-scale	Talib et al. (2017)

2.3.3 Rearing system

In most mud crab hatcheries, larvae are reared under an open clear flow-through water system with daily water exchange at the rate of 20-50% depending on the larval stage (Genodepa et al., 2004b; Thirunavukkarasu et al., 2014), due to its ease of operation in terms of maintaining water quality and abundant supply of clean seawater in the hatchery (Hirayama, 1974). Aside from a clear water system, a recirculating water system has been successfully used in shrimp hatcheries over the years (Beard & Wickins, 1980; Millamena, Casalmir, & Subosa, 1991), but research of this system in mud crab hatcheries is very limited. Nghia et al. (2007a) reported that a recirculating water system is unsuitable for early mud crab larval culture as larvae are prone to physical damage by the water current. However, it has also been

recommended that recirculating water systems, which are less labour intensive and require low seawater consumption, need to warrant further investigation.

A green water system is also reported to stimulate production of megalopa (Baylon & Failaman, 1999). Several genera of microalgae species, *viz. Chlorella, Nanochloropsis, Chaetoceros, Skeletonema, Tetraselmis* and *Isochrysis*, are supplied into the rearing water during larval stages at a suitable density in the range 5×10^4 to 5×10^5 cells mL^{-1} (Baylon & Failaman, 1999; Mann, Asakawa, Pizzutto, Keenan, & Brock, 2001; Quinitio et al., 2001), which serve as enrichment for live food as well as stabilising water quality (Nghia et al., 2007a). However, sometimes the collapse of additive microalgae occurs and contributes to the fouling of the rearing water (Baylon & Failaman, 1999).

Another promising system in crustacean seed production is the biofloc water system, which has proved to produce post-larvae of *P. vannamei* without adverse effects on survival and growth (De Lorenzo et al., 2015; Manan, Moh, Kasan, & Ikhwanuddin, 2016). Nevertheless, in mud crab hatcheries, preliminary study of the use of biofloc as fresh food to replace *Artemia* has been reported by Nguyen (2014), wherein floc particles can be used as the sole food from megalopa onwards, but not from early zoeal stage. Thus, further research on use of floc particles as a water stabilising agent needs to be conducted as this could contribute significantly towards improvement of larval survival and lead to increased crablet production.

2.4 Biofloc technology

2.4.1 Definition

The system that includes algae, bacteria, zooplankton, feed particles and faecal matter with appropriate mixing and flocculating together in an aerobic water column is called a biofloc system (Browdy, Ray, Leffler, & Avnimelech, 2012). A technique that uses an external carbon source or increased carbon content of feed to enhance the water quality is also referred to as biofloc technology (Crab, 2010).

2.4.2 Biofloc system in crustacean culture

Biofloc technology (BFT) is one of the promising technologies that result in beneficial effects on aquatic animals and environment. Improvement in growth performance due to enhanced digestive enzyme activities in *P. monodon* (Anand et

al., 2014; Arnold, Coman, Jackson, & Groves, 2009) and *L. vannamei* (Xu & Pan, 2012) have been reported as a result of BFT application. Floc particles can also be considered as a natural food source (Emerenciano, Ballester, Cavalli, & Wasielesky, 2012) leading to decreased feed conversion rates and eventually to result in low production costs (Anand et al., 2014; Avnimelech, 1999). BFT is able to improve animals' health by increasing phenoloxidase and respiratory burst activity (Ekasari et al., 2014b). In addition, BFT with limited or zero water exchange can maintain water quality by assimilating ammonia into bacterial biomass (Avnimelech, 1999), leading to low waste water discharge into the environment (De Lorenzo et al., 2016b; Samocha et al., 2007).

In the conventional hatchery, a large quantity of seawater exchange is needed to maintain adequate water quality for different larval culture stages. This also results in a high cost of production, increases the environmental impact and is liable to biosecurity issues (De Lorenzo et al., 2016a). Compared to conventional water treatment technologies, BFT is robust, economical and easy to operate (Crab, Defoirdt, Bossier, & Verstraete, 2012). In shrimp (*L. vannamei*) hatcheries, a biofloc system could save up to 88% of seawater used by the conventional system with daily water exchange without adverse effects on production rates and water quality (De Lorenzo et al., 2015). Furthermore, live biofloc is able to inhibit the growth of *Vibrio harveyi* (Crab, Lambert, Defoirdt, Bossier, & Verstraete, 2010) that can cause mass mortality of *P. monodon* larvae in hatcheries (Karunasagar et al., 1994). In addition, the use of floc particles to replace live food in mud crab, *S. paramamosain* hatcheries has been reported by Nguyen (2014), indicating that fresh biofloc could be used as a food for megalopa culture.

CHAPTER 3: Extension of rotifer (*Brachionus plicatilis*) inclusions in the larval diets of mud crab, *Scylla paramamosain* (Estampodor, 1949): effects on survival, growth, metamorphosis and development time

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3.1 Introduction

Mud crabs belong to the Portunidae family, with the genus *Scylla* including some of the largest species, such as *Scylla serrata*, *S. tranquebarica*, *S. paramamosain* and *S. olivacea* (Keenan, 1999b). *Scylla* spp. are distributed throughout the tropical and subtropical Indo-Pacific, from South Africa to Tahiti, around the North to Okinawa and the South to Port Hacking in Australia (Keenan, 1999a; Keenan et al., 1998). In Vietnam, mud crab cultivation was first introduced to coastal provinces in 1993, mostly in the Mekong Delta (Dat, 1999). *Scylla paramamosain* is the dominant species among the four *Scylla* species (Keenan et al., 1998) and the second most common crustacean species, after the shrimp, reared in the coastal areas of Vietnam (Nghia et al., 2007a). Mud crab aquaculture mainly relies on wild-caught seed (Keenan, 1999a), hatchery-raised seed production is unreliable and inconsistent (Heasman & Fielder, 1983) leading to higher and non-viable prices ($/crablet). Though, lately mud crab hatcheries are using umbrella-stage of *Artemia* incubated for 7 to 9 hours of the cyst (Vinh Chau, Vietnam) to feed zoea 1, yet with inconsistent success.

Recently, the demand for alternative species to shrimp, such as mud crabs, has increased, mainly as a result of the early mortality syndrome (EMS), which adversely affects shrimp production in Vietnam (Vietnam, 2013). The consequent increase in demand has put more pressure on the supply of mud crab seeds. This pressure has further intensified due to the dwindling supply of wild mud crab seed, shifting the emphasis on the production of seed in the hatcheries. However, hatchery production technology has mainly focused on *S. serrata* (Baylon & Failaman, 1999; Davis, 2003; Hassan, Hai, Anil, & Sukumaran, 2011; Mann, Asakawa, & Pizzutto, 1999; Quinitio et al., 1999; Yi et al., 2009), while a limited research is available on the seed production of *S. paramamosain* (Nghia et al., 2007a; Zeng & Li, 1999), especially on feeding regimes. Previous research has confirmed that rotifers and *Artemia* nauplii

are the most acceptable and convenient live feed organisms in larval rearing of various commercial species of the Portunidae family. However, information about the time of withdrawal of rotifers from the larval feeding regime is ambiguous and contradicting. For example, Baylon and Failaman (1999), Faleiro and Narciso (2009) and Redzuari et al. (2012) have stated that rotifers should be fed throughout the zoeal stage to enhance larval survival, whereas Nghia (2004) and Ruscoe et al. (2004b) have suggested that rotifer feeding should be limited to the Z1 stage. The reason for limited rotifer use seems to be based on the complexities associated with the mass production techniques of rotifers, which involves substantial use of floor space, significant costs for microalgae and labour, danger of infections and unpredictable crashes during commercial rotifer production (Nghia, 2004; Ruscoe et al., 2004b).

The rotifer production protocol modified by Lind (2014) and Kostopoulou et al. (2012) is considerably streamlined process, resulting in a high daily rotifer productivity, high growth rates, absence of ciliates, low concentrations of total *Vibrio* (Lind, 2014), prolonged culture time without any crashes and a low production cost, leading to straightforward feeding management protocol (Kostopoulou et al., 2012).

Artemia nauplii have traditionally been used in feeding regimes of most of the mud crab larvae, albeit with mixed outcomes (Baylon, 2009; Dan et al., 2016b). However, due to the advances in practicability of rotifer production techniques and the inconsistent quality and higher costs associated with *Artemia* production in the hatcheries, the increased use of rotifers in *S. paramamosain* hatcheries could be considered as an improvement in larval production techniques and reducing in production costs. To understand clearly a role of rotifer inclusions along with the *Artemia*, the current study aimed to evaluate the extended use of rotifer inclusions in the feeding regimes of mud crab larvae in a commercial megalopa production under the hatchery environment. The evaluation of the feeding regimes was based on the survival improvement, metamorphosis rate, growth and development time of the larvae.

3.2 Materials and methods

3.2.1 Water source

The seawater was pumped into a settling reservoir one day prior to the disinfection with 20 mg L^{-1} calcium hypochlorite to minimise infections from the seawater

supply. After 48 hours of chlorination, the seawater was pumped into a storage tank where no chlorine residue was detected by the colorimetric method. Prior to the experiment, the seawater in the storage tank was pumped into a 60-L plastic bucket and disinfected again with 2 mg L^{-1} Vikor-S (Bayer, Germany); the treated seawater was then used daily in the experiment.

3.2.2 Rotifers and *Artemia* culture

Rotifers (*Brachionus plicatilis*, L-strain, 250 µm (Daintith, 1996)), obtained from the marine fish hatchery in Vung Tau Province, Vietnam ($10^{o}24'51''$N; $107^{o}10'38''$E), were cultured in an indoor 4000-L square concrete tank filled with disinfected seawater (30 g L^{-1}) and supplied with gentle aeration. The rotifers were fed three times (6 am, 12 pm and 6 pm) daily with an equal proportion of baker's yeast and commercially available rotifer diet (S.parkle, INVE Aquaculture, Thailand), following the protocol described by Lind (2014). At six to seven days post-rearing, the rotifers had achieved a density of approximately 500 individuals mL^{-1} and were harvested by using 60-µm filter bags.

Artemia franciscana (Vinh Chau strain, Vietnam), one of the smallest strains of *Artemia*, were soaked in fresh water for one hour to absorb water before being disinfected with sodium hypochlorite at a concentration of 20 mg L^{-1} for 10 minutes under aeration. The cysts were then washed with seawater and placed into an incubation container filled with disinfected seawater (30 g L^{-1}). Strong aeration was continuously provided. The incubation setup was exposed to natural light for 12 hours, a temperature of 28-30^{o}C and a pH of 8.0-8.3. After 15 hours of incubation, newly hatched *Artemia* nauplii were harvested as feed for mud crab larvae.

3.2.3 Source of brood stock and larvae

Mature females of *S. paramamosain* (400 - 500 g) were collected from extensive shrimp farms in the Nam Can district, Ca Mau Province, Vietnam ($8^{o}45'00''$N; $105^{o}01'38''$E), and transported to the hatchery in the same area. Prior to the stocking, the female crabs were washed and disinfected by 100 µL L^{-1} of formalin solution under aeration for 1 hour and finally stocked individually in 30-L plastic buckets to exclude cannibalism. The buckets were filled up to 40% volume of 1 µm filtered and disinfected seawater (30 g L^{-1}). Each bucket was provided with strong aeration. After one day of acclimation, female crabs were subjected to unilateral eyestalk ablation to

stimulate spawning. Each crab was then placed back into the same bucket and fed once in the evening with 10 to 20 g of fresh bivalve (*Anadara granosa*) meat. Daily water change at 200% by volume was conducted in the morning and two hours after each feeding. Ambient temperature and photoperiod were 28-30^{o}C, and 12 light: 12 dark hours, respectively. Just before spawning behaviour set in as indicated by opening and washing of pleopod hair under the abdomen, the female crabs were transferred to a 60-L plastic bucket with a 10-cm layer of sand as substrate. Spawning occurred in these buckets.

Berried females were transferred into plastic boxes (35 x 60 x 40 cm, width, length and height) which were immersed into a 2000-L square fibre-glass tank attached to a sand filtration system to filter and reuse water. The females were reared individually. During the incubation period, the females were starved until their eggs hatched. Faeces and damaged eggs were siphoned out of the tanks; water was exchanged daily at a rate of 20%. On the 11th day post-spawning, the berried females were transferred to another container in which sea water was diluted with 0.1 µL L^{-1} iodine solution for 2 minutes under aeration to prevent fungal, protozoa and bacterial infections to the eggs. Following this, the berried females were released into a 200-L round incubation tank that was filled fresh disinfected seawater up to 80% of the total capacity of the tank for hatching. Temperature, salinity and photoperiod were similar to the previous ambient conditions.

Newly hatched zoeae 1 (Z1) from one spawner were concentrated on the water surface after switching off the aeration. The Z1 were then collected and bathed in 0.1 µL L^{-1} iodine solution for 30 seconds prior to distribution in two 100-L plastic buckets. After 2 hours, the larvae in the first bucket were solely fed newly hatched *Artemia* nauplii at a rate of 5-10 individuals mL^{-1}, termed as regime A (*A: Artemia*) or control, while the larvae in the second bucket were fed purely rotifers at a rate of 20-40 individuals mL^{-1}, subsequently used as regimes 2, 3, 4 and 5. On the following day, one-day-old larvae were collected for the subsequent experiment.

3.2.4 Experimental setup

Fifteen 1.5-L plastic beakers were filled with 1 L of disinfected seawater (30 g L^{-1}). Each beaker was then stocked with 30 healthy and active one-day-old larvae which were collected from the 100-L plastic bucket. All the experimental beakers were

incubated in a 5000-L square concrete tank to maintain a constant temperature of 28 ± 1^{o}C (Fig. 3.1). The beakers were provided with a moderate aeration to prevent settling of larvae and live feed. The light, which attracted crab larvae and live food concentrating on water column and surface, can improve the ability of mud crab larvae for catching the prey, so the light was maintained constantly for 24 hours, including 12 hours of natural light and 12 hours of artificial light provided by a 40-W fluorescent globe. The pH was adjusted approximately to 8.3 ± 0.2 by adding $CaCO_3$ and/or $NaHCO_3$.

Figure 3.1: All the experimental beakers were incubated in a 5000-L square concrete tank.

3.2.5 Feeding regimes

Five feeding regimes based on inclusion of rotifers at different stages of crab larval development were tested in triplicate. The mud crab larvae, from Z1 to megalopa (M) in regime A (control) were exclusively fed *Artemia* nauplii (Table 3.1). From regime 2 onwards (R2-A to R5-A), the rotifer inclusion extended one further stage of larval development from Z1 to the next stage. For example, in R2-A, the rotifer inclusion extended to Z2 stage only followed by *Artemia* to all remaining stages of larval development from Z2. R3-A constituted rotifer inclusions extended to Z3 stages. Similarly, R4-A and R5-A consisted of rotifer inclusions extended to Z4 and

Z5 respectively. From R2-A to R5-A *Artemia* were provided from Z2 to megalopa stage.

Table 3.1: Regimes show extending inclusion of rotifers as a live food in the feeding regimes for *S. paramamosain* larvae.

Regime A (control)	*Artemia* (Z1-M)
Regime 2 (R2-A)	Rotifers (extended to Z2) + *Artemia* (Z2-M)
Regime 3 (R3-A)	Rotifers (extended to Z3) + *Artemia* (Z2-M)
Regime 4 (R4-A)	Rotifers (extended to Z4) + *Artemia* (Z2-M)
Regime 5 (R5-A)	Rotifers (extended to Z5) + *Artemia* (Z2-M)

Key: A: Artemia; R: Rotifers; Z: zoea, M: megalopa; number after the letter represents the larval stage.

3.2.6 Live feed densities in feeding regimes

When mud crab larvae were fed exclusive *Artemia* nauplii and exclusive rotifers, as per the feeding regime described above (section 3.2.5), the density was maintained at 10 and 20 individuals mL^{-1} respectively. If the feeding regime was represented by a mixture of *Artemia* and rotifers, their respective densities was reduced to 5 and 10 individuals mL^{-1}, respectively. This density regime is based on the previous study of Baylon et al. (1999). To obtain the desired densities of rotifers and *Artemia*, every beaker was prepared according to the method described by Baylon (2009). The crab larvae were fed only once in the morning after being transferred into a newly prepared beaker filled with disinfected seawater by using a large bore pipette. At the same time, larval metamorphosis and mortality was recorded for each beaker. All environmental parameters in a newly prepared beaker were maintained similar to those in the replaced beaker. As soon as megalopa stage appeared, they were immediately removed from the rearing beaker in order to avoid cannibalism.

3.2.7 Survival and growth of megalopa

Final survival and rate of metamorphosis were recorded at the end of the experiment (24 days after hatching). The final survival of the larvae, including zoeae and megalopae, was computed using the following equation:

$$\text{Survival rate} = \frac{\sum \text{Final larvae x } 100}{\text{Initial number of zoea 1 stocked}}$$

Percentage of successful metamorphosis of megalopa was computed using the following equation:

$$\text{Metamorphosis rate} = \frac{\sum \text{Final megalopae x } 100}{30 \text{ zoea 1 stocked}}$$

Development time was the duration of the period required for metamorphosis from zoea1 to megalopa stage that was identified when the megalopae started to appear in the beaker.

Five megalopae were then fixed in 10% formalin solution to measure growth performance of the megalopa stage. Their body lengths and carapace widths were measured using a ruler scale inserted into the eyepiece of a microscope (Olympus CX21, USA), according to the method described by Ingle (1997). Wet weight of megalopa was measured using a 0.0001-g precision analytical balance (PA 214-Ohaus, USA).

3.2.8 Statistical analyses

The data of survival, percentage of successfully metamorphosed megalopa and growth of megalopa were presented as mean and standard error (±SE), wherein data presented in percentages were arcsine-square root-transformed (Zar, 2010) prior to analysis. One-way ANOVA was performed to test for significant differences between the regimes; specific differences between means of regimes were detected by Tukey's multiple range test at the 0.05 level of significance. To test a relationship between the survival and days of culture of crab larvae fed according to different feeding regimes, simple linear regression was employed, in which Y represents the survival of larvae and X the day of culture. Development time, body length, carapace width and wet weight of megalopa were compared using Kruskal-Wallis non-parametric test. In the case of significant differences, Mann-Whitney Test was used to compare means of the regimes at the 0.05 level of significance. All statistical analyses were computed using SPSS for Windows, version 24.0.

3.3 Results

3.3.1 Environmental parameters

Water temperature in the concrete tank used as a water bath for all experimental beakers ranged from 27 to 30^{o}C throughout the experiment. Salinity, pH and

alkalinity were constant at 30 g L^{-1}, 8.3 ± 0.2 and 140 mg $CaCO_3$ L^{-1}, respectively. The optimum temperature for zoeal development range from 25-30^{o}C, while optimal salinity values range from 27-35 g L^{-1} for early larval stages (Z1-Z3) and from 23-31 g L^{-1} for late larval stages of *S. paramamosain* (Z4-M) (Li, Zeng, Tang, Wang, & Lin, 1999) and *S. serrata* (Baylon, 2010; Dan & Hamasaki, 2011). Thus, the environmental factors in this experiment were within a normal range for the larval development of mud crabs.

3.3.2 Larval survival

The survival of the larvae progressively decreased in all regimes (Table 3.2). Extending inclusion of rotifers feeding until the Z5 stage resulted in a significantly higher ($P < 0.05$) survival than that of the control from 18 days after hatching onwards. Survival of the larvae showed negative correlation (R^2 from 0.78 to 0.90) with the rearing period; however, different feeding regimes had no significant ($P > 0.05$) influence on this correlation (Table 3.3).

Table 3.2: Mean survival (±SE) of crab larvae fed according to different feeding regimes for 24 days of culture.

Days of culture	Regimes				
	A	R2-A	R3-A	R4-A	R5-A
3	$^{1}70.0 \pm 10.72^{a}$	$^{1}66.7 \pm 0.00^{a}$	$^{1}75.6 \pm 4.84^{a}$	$^{1}72.2 \pm 2.94^{a}$	$^{1}77.8 \pm 1.11^{a}$
6	$^{12}44.4 \pm 7.78^{a}$	$^{12}48.9 \pm 4.01^{a}$	$^{12}57.8 \pm 10.94^{a}$	$^{1}62.2 \pm 2.94^{a}$	$^{12}64.4 \pm 5.88^{a}$
9	$^{23}28.9 \pm 4.01^{a}$	$^{23}32.2 \pm 2.94^{a}$	$^{12}46.7 \pm 11.71^{a}$	$^{12}50.0 \pm 3.33^{a}$	$^{123}54.4 \pm 5.56^{a}$
12	$^{34}18.9 \pm 2.94^{a}$	$^{34}23.3 \pm 5.09^{a}$	$^{12}38.9 \pm 11.28^{a}$	$^{23}35.6 \pm 4.44^{a}$	$^{234}45.6 \pm 6.19^{a}$
15	$^{34}13.3 \pm 1.92^{a}$	$^{34}16.7 \pm 5.77^{a}$	$^{12}34.4 \pm 10.60^{a}$	$^{23}32.2 \pm 4.44^{a}$	$^{234}40.0 \pm 5.09^{a}$
18	$^{34}10.0 \pm 1.92^{a}$	$^{34}13.3 \pm 5.09^{ab}$ (+3.3)	$^{12}30.0 \pm 8.82^{ab}$ (+20.0)	$^{23}30.0 \pm 5.09^{ab}$ (+20.0)	$^{34}35.6 \pm 4.84^{b}$ (+25.6)
21	$^{34}\ 8.9 \pm 1.11^{a}$	$^{4}11.1 \pm 2.94^{ab}$ (+2.2)	$^{12}26.7 \pm 6.94^{ab}$ (+17.8)	$^{23}28.9 \pm 6.19^{ab}$ (+20.0)	$^{34}32.2 \pm 4.84^{b}$ (+23.3)
24	$^{4}7.8 \pm 1.11^{a}$	$^{4}10.0 \pm 1.92^{ab}$ (+2.2)	$^{2}23.3 \pm 8.82^{ab}$ (+15.5)	$^{3}25.6 \pm 4.84^{ab}$ (+17.8)	$^{4}28.9 \pm 4.44^{b}$ (+21.1)

Key: A: Artemia; R: Rotifers; number after the letter represents the larval stage. The number into (.) shows percentage changed in relation to control in each feeding regime. Significant differences were found among all regimes with different superscript letters ($P < 0.05$) in the same row. Significant differences were also found throughout the culture period within the regime with different superscript numbers ($P < 0.05$) in the same column.

Table 3.3: Relationship between the survival and days of culture of crab larvae fed according to different feeding regimes.

Regimes	Equation	Regression (R^2)
A	y = -3.54x + 76.03	0.78 ± 0.04
R2-A	y = -3.41x + 76.69	0.82 ± 0.01
R3-A	y = -2.90x + 82.89	0.86 ± 0.06
R4-A	y = -2.83x + 82.52	0.85 ± 0.01
R5-A	y = -2.74x + 86.10	0.90 ± 0.03

Key: A: Artemia; R: Rotifers; number after the letter represents the larval stage. Y represents the survival of larvae and X the day of culture. Values represent mean ± standard error (SE).

3.3.3 Percentage of successful metamorphosis and development time to megalopa

Extending inclusion of rotifers feeding until the Z3, Z4 and Z5 stages (R3-A, R4-A and R5-A) resulted in a higher ($P < 0.05$) percentage of metamorphosis of megalopa (17.8, 20.0 and 27.8%, respectively) than in the control (2.2%, Fig. 3.2), while development time of the larvae ranged from 17.0 to 22.5 days and was independent ($P > 0.05$) of any feeding regimes (Fig. 3.3).

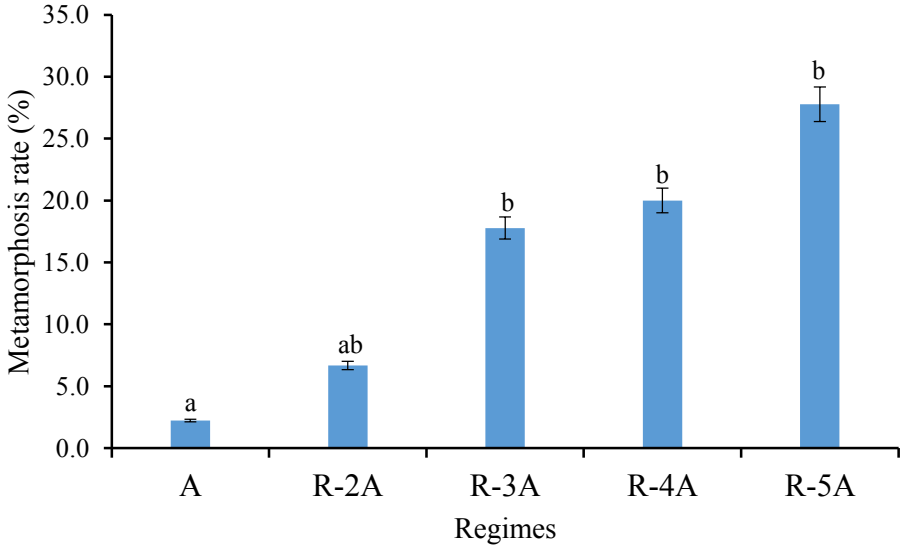

Figure 3.2: Percentages of successful metamorphosis of megalopa fed according to various feeding regimes.

Key: A: Artemia; R: Rotifers; number after the letter represents the larval stage. Significant differences were found among all regimes with different superscript letters (P < 0.05) in the bar chart.

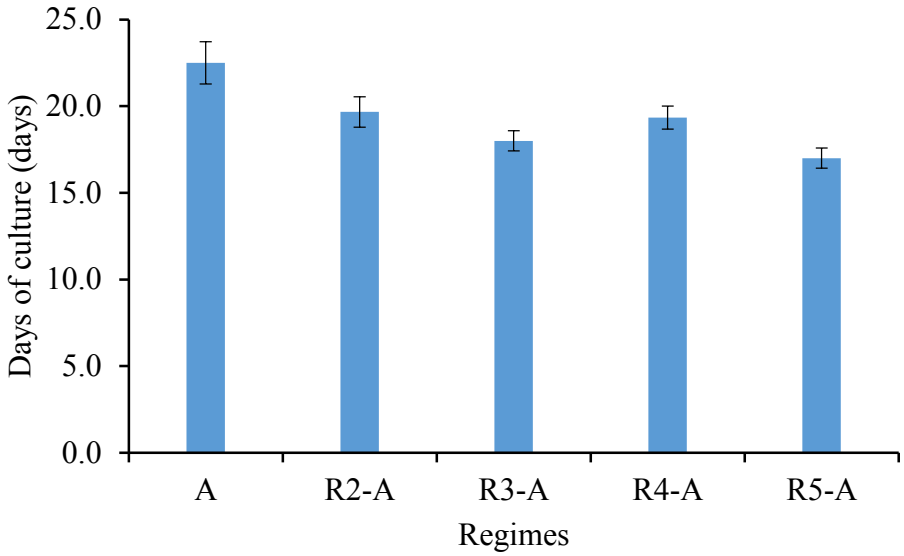

Figure 3.3: Duration of the period required for metamorphosis from zoea1 to the megalopa stage under various feeding regimes.

Key: A: Artemia; R: Rotifers; number after the letter represents the larval stage

3.3.4 Growth performance of megalopa

Growth performance of megalopa resulting from zoeae fed various feeding regimes ranged of 1.65-1.71 mm in carapace width, 4.98-5.11 mm in body length and 3.83-4.05 g in wet weight; however, the growth performance of megalopa did not depend ($P > 0.05$) on any of the feeding regimes (Table 3.4).

Table 3.4: Mean carapace width, body length and wet weight of one-day-old megalopa under different feeding regimes.

Regimes/Parameters	CW (mm)	BL (mm)	WW (mg)
A	1.65 ± 0.04	5.00 ± 0.08	4.05 ± 0.61
R2-A	1.70 ± 0.01	5.03 ± 0.03	4.02 ± 0.09
R3-A	1.71 ± 0.02	5.02 ± 0.06	3.83 ± 0.03
R4-A	1.68 ± 0.01	5.11 ± 0.03	3.93 ± 0.04
R5-A	1.69 ± 0.02	4.98 ± 0.04	3.94 ± 0.05

Key: A: Artemia; R: Rotifers; number after the letter represents the larval stage. CW: Carapace width; BL: Body length; WW: Wet weight. Values represent mean ± SE.

3.4 Discussion

There were five zoeal instars which do not always show synchronised moulting. They usually extend and overlap approximately 1 day, when moulting to the next stage (Davis, Wille, Hecht, & Sorgeloos, 2005b). Hence, no larval stage can be

designated by a single day due to their asynchronous moulting, it is relatively clear to depict survival by 'days of culture' rather than by individual development stage as in Table 3.2. In our study, except the megalopa stage, all larval developing stages took approximately 3 days to complete, whereas megalopa stage took 6-7 days to complete.

A diet composed of rotifers and *Artemia* has the ability to improve larval survival in *S. serrata* (Baylon et al., 1999; Nghia, 2004; Ruscoe et al., 2004b), *S. paramamosain* (Zeng & Li, 1999) and *Thalamita crenata* (Godfred et al., 1997). In general, these studies did not show any potential benefits when rotifer feeding was extended until the late larval stages in portunid crabs, and until the late zoeal stages in *S. paramamosain*. Under laboratory conditions, our study confirmed the benefits of extending feeding of rotifers until the late zoeal stages in *S. paramamosain* seed production.

In the present study, rotifers and *Artemia* could be considered as first food for early crab larvae, as reflected by successfully metamorphosed to next stage, whereas it has been reported that 100% of unfed larvae could not moult and died by day 7 (Holme et al., 2006b). Similar our results have been reported in mud crabs (Baylon, 2009; Baylon et al., 2004; Davis, 2003; Hassan et al., 2011; Nghia, 2004; Ruscoe et al., 2004b; Zeng & Li, 1999), swimming crabs (*Portunus* spp.) (Dan, Ashidate, & Hamasaki, 2016a; Ikhwanuddin et al., 2012; Redzuari et al., 2012) and giant freshwater prawn (*M. rosenbergii*) (Barros & Valenti, 2003).

The crab larvae from Z1 to Z2 achieved a higher survival when rotifers were employed as a first food (Baylon, 2009; Ruscoe, Shelley, & Williams, 2004a; Ruscoe et al., 2004b; Zeng & Li, 1999). This could be due to their small size, low moving speed and contain desired nutrients (Dhert, 1996), however, feeding only rotifers until the late zoeal stage (Z5) led to a complete mortality at Z5 (Baylon, 2009), delayed larval development (Nghia, 2004) or possible extension to one extra zoeal stage (Z6), as shown by Zeng et al. (2004). Due to this reason, our study did not include 'feeding rotifers only' as one of the treatments.

Like rotifers, *Artemia* nauplii are also acceptable as a first food and the larvae fed *Artemia* nauplii alone survived and metamorphosed to the megalopa stage in the present study. This result is also in line with the previous studies of Baylon et al.

(1999), Brick (1974), Davis et al. (2005a) and Heasman and Fielder (1983). We observed that only healthy and active crab larvae could catch and handle *Artemia* nauplii under the controlled feeding regime, while the weak ones were only able to ingest a few residual parts of the dead *Artemia* from the bottom of the beaker. As a result, the weak larvae either died or their development time to the megalopa stage was prolonged, induced by on-time failure to metamorphose to the next stage. Thus, demonstrating that the survival of the larvae fed exclusively *Artemia* dropped rapidly as the study progressed. The significant difference in survival of larvae in this treatment was detected at day 9th while the treatments that involved the extension of rotifer inclusions after Z2 were significant from day 12th onwards. Baylon (2009) also stated that the early larval stages are passive predators, so they are unable to pursue and catch larger and fast moving prey including *Artemia* nauplii (Baylon, 2009; Nghia, 2004; Zeng & Li, 1999); sometimes, only certain parts, such as the head and appendages of the *Artemia* nauplii, are ingested (Baylon, 2009; Zeng & Li, 1999). As a result, a low survival rate (7.8%) and delayed development time (22.5 days) was observed in mud crab larvae that were exclusively fed *Artemia* (control) in the present study. Similarly, low survival and prolonged Z6 stage in the ontogenetic development of mud crabs was detected by Zeng et al. (2004); in some cases, the animals even failed to metamorphose beyond the Z5 stage (Nghia, 2004; Suprayudi et al., 2002a) when only *Artemia* nauplii were used as a feed. The limited catchability of early zoeal stages implied that *Artemia* were not a suitable food for early crab larvae.

Additionally, various zoeal instars in larval development of decapod crustaceans (Criales & Anger, 1986; Pestana & Ostrensky, 1995) and immature megalopa (Dan et al., 2013) were observed with feeding exclusively on *Artemia* nauplii. Morphological variation and formation of immature megalopa adversely affected the survival during transition from the Z5 to the megalopa stage, as evidenced by 2.2% of successful megalopa metamorphosis in this study, consistent with a previous study of Dan et al. (2013) in swimming crabs. Low EPA levels in both newly hatched and starved *Artemia* could result in low survival and prolonged intermoult periods of *S. serrata* larvae (Suprayudi et al., 2004a) and *P. trituberculatus* larvae (Dan et al., 2016b). Therefore, it is also believed that feeding exclusive *Artemia* throughout the

larval development cannot supply enough essential nutrients which are necessary for further larval development.

Extending inclusion of rotifers in the diets of mud crab larvae until Z5 stage significantly improved the survival and metamorphosis than when larvae were fed exclusive *Artemia* (control). Baylon et al. (2004) observed that the crab larvae increased ingestion of *Artemia* on the day before moulting, whereas ingestion of rotifers was increased one day after the moulting. Prior to moulting, the larvae with a hard exoskeleton had higher nutrient requirements as they had to anabolise their tissues and should be strong enough to catch and handle *Artemia* nauplii. On the other hand, immediately after the moulting, the larvae were weaker with a softer exoskeleton, leading to limit the ability to catch *Artemia.* Therefore, the larvae switched their dietary preference towards rotifers to accumulate more nutrient necessary for maintaining and recovering energy essential for the survival (Baylon et al., 2004). It is speculated that continuous accumulation of nutrients derived from both rotifers and *Artemia* nauplii during the larval development period resulted in increased survival of the crab larvae.

In addition, the co-existence of rotifers and *Artemia* in the rearing water could overcome the problem of *Artemia* starvation (Dan et al., 2016b). The *Artemia* starvation is prevented as *Artemia* can feed on rotifer excrement and/or on the proliferating bacterial populations established due to the presence of rotifer excrements (Dan et al., 2016b). This study also reported that the feeding of starved *Artemia* nauplii to the late zoeal stages can adversely affect the survival and morphology of the successive stages of mud crab larvae. Even, in the case of presence of *Artemia,* if rotifers are excluded at the Z1 or Z2 stages as suggested by Nghia (2004) and Ruscoe et al. (2004b), starvation of *Artemia* may appear after the withdrawal of rotifers. This possibly induces morphological variation and immature megalopa, both causing high megalopa mortalities.

Apart from *Artemia* starvation, excessive *Artemia* can result in a higher percentage failure rate of megalopa moulting (Suprayudi et al., 2002a). In the current study, excessive *Artemia* could occur in the feeding regimes where exclusively *Artemia* (control regime) were used or extension of rotifer inclusions was stopped and replaced to *Artemia* at the early stages. Due to the density of *Artemia* in these

treatments was increased similar to the control. Whereas, the feeding rotifer inclusions mixed with *Artemia* until the late zoeal stages could not happen *Artemia* excess due to limiting the density of *Artemia* in these feeding regimes.

The early zoeal stages can easily catch and ingest rotifers (Nghia, 2004) at a rate four times higher than that for *Artemia*, followed by the gradual reduction in the quantity of ingested rotifers to almost the quantity of ingested *Artemia* when the larvae reach the Z5 stage (Baylon et al., 2004). These authors also stated that the larvae at Z1, Z2 and Z3 stages had lower *Artemia* ingestion rates in the presence of rotifers. By including rotifers in the feeding regimes of mud crab larvae, the *Artemia* usage was reduced by 50% without compromising survival and metamorphosis until Z4 stages. When rotifer inclusion was extended until Z5 stage, in the presence of *Artemia*, the survival and metamorphosis of megalopa was further improved.

Artemia cysts contribute more than 50% of the variable costs in any crab hatchery (Quinitio et al., 2001). Extending the period of rotifer inclusions in the dietary regime for crab seed production could significantly improve the economic efficiency of the hatchery by making significant reduction in the usage of *Artemia*. In addition, mass and continuous rotifer production procedures (Kostopoulou et al., 2012; Lind, 2014) can be managed easily with a significantly reduced production cost.

Therefore, the prolonged inclusion of rotifers in combination with *Artemia* until the late zoeal stage can improve the production of megalopa in the hatchery. However, research is recommended to determine whether enrichment of live feed in this feeding regime can further improve the larval performance of crab.

CHAPTER 4: Effects of extended-rotifers inclusion and live food-enrichment with probiotics on the survival, metamorphosis, development time and growth of mud crab, *Scylla paramamosain* (Estampador) larvae

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4.1 Introduction

Mud crab, *Scylla paramamosain,* is a commercially important source of income for coastal fishers in the Asia-Pacific region (Keenan, 1999a). In Vietnam, *S. paramamosain* is the most prevalent cultured species in coastal areas (Le Vay et al., 2001; Lindner, 2005) and the second most common crustacean species after the shrimp (*P. monodon and L. vannamei*) (Nghia et al., 2007a). The pond-based crab culture system totally relies on wild-caught seed (Keenan, 1999a), which has become limited by over-exploitation and diminishing mangrove habitats (Le Vay et al., 2001). Although mud crab seed production has been studied extensively (Baylon & Failaman, 1999; Brick, 1974; Davis, 2003; Heasman & Fielder, 1983; Mann et al., 1999; Marichamy, 1996; Nghia et al., 2007a; Noorbaiduri & Ikhwanuddin, 2015; Quinitio et al., 1999; Yi et al., 2009), the survival of hatchery-produced crablets is still unreliable and inconsistent (Baylon & Failaman, 1999; Hamasaki et al., 2011), mainly due to mass mortality during the zoeal (Z) and megalopa stages, especially during metamorphosis from Z5 to megalopa (Hamasaki et al., 2002b). Mass mortalities are mainly linked to poor larval nutrition (Hamasaki et al., 2002b; Suprayudi et al., 2004b) due to inappropriate feeding regimes.

Nutritional deficiencies and inappropriate feeding regime, for example, feeding either rotifers (Baylon, 2009; Nghia, 2004), or *Artemia* alone (Ruscoe et al., 2004b; Zeng et al., 2004), or excessive or starved *Artemia* (Dan et al., 2016b; Suprayudi et al., 2002a) to mud crab larvae resulted in low survival and prolonged larval development time. On the contrary, *Artemia* enriched with lecithin and cholesterol (Holme et al., 2007) as well as feeding a mixture of rotifers and *Artemia* to crab larvae (Nghia, 2004; Ruscoe et al., 2004b; Zeng & Li, 1999) improves survival. Nghia (2004) and Zeng and Li (1999) have recommended that the inclusion of rotifers should be extended until Z2 or Z3 stages to increase the survival of crab larvae. Similarly, our previous study (Quy, Fotedar, & Thy, 2018b) showed that

including rotifers until the late zoeal stages (Z3 to Z5), mixed with *Artemia*, increased in a higher metamorphosis of megalopa. Thus, this confirms that including the extension of rotifers in the feeding regime of mud crab larvae can contribute additional nutrients essential for moulting of most of the larval stages, as *Artemia* alone are incapable of providing these additional nutrients to the larvae (Baylon, 2009).

Similar to larval mal-nutrition, fungal, parasites and bacterial infections contribute to poor survival; however, enriching live feeds with probiotics increases survival rates (Dan & Hamasaki, 2015). Probiotics are defined as bio-friendly agents that can control and compete with pathogenic bacteria as well as stimulate the growth of aquatic animals without undesirable side effects (Farzanfar, 2006). The beneficial effects of dietary inclusion of probiotics are displayed through competition for habitat and nutrients to displace harmful bacteria (Moriarty, 1998), provision of nutrients and enhanced digestive enzyme activities (Gupta, Verma, & Gupta, 2016; Wang, 2007; Zhou et al., 2009; Ziaei-Nejad et al., 2006), improvement of water quality (Nimrat et al., 2012), enhancing immune responses (Ambas, 2015; Kumar et al., 2013; Rengpipat et al., 2000; Tseng et al., 2009) and producing antiviral substances (Lakshmi, Viswanath, & Sai Gopal, 2013; Pandiyan et al., 2013). Although the beneficial uses of probiotics, including bacteria of the genus *Bacillus*, have been reported in larval, post-larval and juvenile stages of shrimp (Balcázar & Rojas-Luna, 2007; Dong et al., 2014; Guo et al., 2009; Jamali et al., 2015; Liu et al., 2010; Nimrat et al., 2012; Rengpipat et al., 1998; Vinoj, Vaseeharan, DavidJayaseelan, Rajakumaran, & Ravi, 2013; Yu, Li, Lin, Wen, & Ma, 2008; Ziaei-Nejad et al., 2006) and in mud crab larvae and juveniles (Dan & Hamasaki, 2015; Talib et al., 2017; Wu et al., 2014), no information is available on the effects of supplementing probiotics and extending the inclusion of rotifers on the survival, metamorphosis, development time and growth of mud crab larvae. Thus, the aim of this study was to evaluate the effects of enriching live feeds (*Artemia* and rotifers) with commercially available *Bacillus* spp. as probiotics and extending the inclusion of rotifers until late zoeal stages of *S. paramamosain.*

4.2 Materials and methods

4.2.1 Water source

Seawater (29 ± 1 mg L^{-1}) was pumped from the river and aged for one night in a reservoir prior to disinfection with 20 mg L^{-1} calcium hypochlorite. The disinfected seawater was transferred into the storage tank after two days when no chlorine residues had been detected. The seawater was treated again with 2 mg L^{-1} Vikor-S (Bayer, Germany) for further use in the experiment.

4.2.2 Live food culture

According to the method described by Lind (2014), L-strain rotifers (*Brachionus plicatilis*) originated from a marine fish hatchery were stocked into indoor 4,000-L square concrete tank. The tank was filled with 60% of disinfected seawater (30 g L^{-1}) and provided with moderate aeration using airstones. Rotifers were fed a mixture diet of commercially available rotifer feed (S.parkle, INVE Aquaculture, Thailand) and baker's yeast (50:50) at a rate of 0.4 g for one million rotifers as recommended by Lind (2014) at 6 am, 12 pm and 6 pm daily. When density of rotifers (about 500 individuals $mL^{-}1$) was achieved, they were partially harvested to feed to mud crab larvae.

Before incubation, the *Artemia* cyst were disinfected with 20 mg $L^{-}1$ sodium hypochlorite solution for 10 minutes to avoid any infection of harmful bacteria and fungi and then washed with seawater. The salinity and pH of disinfected seawater for *Artemia* incubation were 30 g L^{-1} and 8.3, respectively. Strong aeration and light intensity (2,000 lux) were also maintained by air pump and lamp, respectively. The newly hatched *Artemia* nauplii appeared after 15 hours of incubation at 29°C. The *Artemia* were harvested for enrichment or feeding directly to mud crab larvae.

4.2.3 Method of enriching the live foods

Bacillus spp. is known as an effective probiotic bacteria in shrimp culture (Ambas, Suriawan, & Fotedar, 2013), so a commercial probiotic (Lymnozyme FT-2B, Cisbay, USA) including *Bacillus subtilis, B. licheniformis, B. amyloliquefaciens and B. pumilus* was used to enrich rotifers and *Artemia* in the current study. The rotifers, partially harvested from mass production concrete tank (4,000-L) were washed with fresh seawater and stocked in a 10-L plastic bucket containing 50% volume of

disinfected seawater with gentle aeration. Probiotics and S.parkle at a rate of 1 mg g^{-1} of rotifer feed were placed together into a blender (HR2195/00, Philips, China) and blended with 100 mL of deionized water. This blended mixture was then poured into the bucket containing rotifers. The feeding rate of this mixture was adjusted following the rotifer stocking densities in the rearing medium which was 0.4 g of rotifer commercial feed meant to feed one million rotifers (for rotifer density of 1,000-1,500 rotifers mL^{-1}). This was equivalent to 40 mL of the mixture of probiotics and rotifer commercial feed. This dose was based on the recommendation of feed company. After 6 hours of enrichment, rotifers were harvested and fed to crab larvae.

In contrast, newly hatched *Artemia* from 1 g cyst (280.000-300.000 nauplius g^{-1}) were only soaked in the commercial probiotic suspension and disinfected seawater at a rate of 1 mg L^{-1}, with strong aeration. This enrichment process lasted only for 1 hour in order to avoid nutrient losses in *Artemia* due to their quick assimilation efficiencies. The enrichment protocol was strictly followed and the supplemented weight of the probiotic powder was measured by an analytical balance (PA 214-Ohaus, USA) with 0.0001 g precision. The newly hatched *Artemia* (instar 1) is not fully developed and thus are unable to ingest the food from water, though, they can absorb water containing *Bacillus*. As a result, the *Bacillus* could be resident of both gut and the external body of *Artemia*. To verify the total *Bacillus* spp. in enriched rotifers and *Artemia*, homogenised sample taken from whole enriched rotifers or *Artemia* was used to determine total *Bacillus* spp. on tryptic soy agar (TSA) plates. Due to limiting facilities at commercial hatchery, total *Bacillus* spp. concentration in enriched rotifers and *Artemia* were counted at the beginning and also at the end of the trial in plates containing TSA medium, according to the method described by Tomasiewicz, Hotchkiss, Reinbold, Read, and Hartman (1980).

4.2.4 Mud crab brood stock and larval source

Mature and berried females of *S. paramamosain* were fattened and managed as described by Quy et al. (2018b). During the fattening period, the salinity and water temperature ranged from 28 to 30 g L^{-1} and 28 to 29°C, respectively, while photoperiod was 12: 12 of light: dark cycle following natural light. One day before hatching, the berried female was transferred and individually reared in a 200-L round tank for hatching.

Newly hatched zoea 1 (Z1), from single spawners, were collected and bathed in 0.1 µL L^{-1} iodine solution for 30 seconds and they were then temporarily reared into a 100-L plastic bucket. The larvae were fed purely with the rotifers without enrichment with probiotics at a rate of 40-50 individuals mL^{-1}. On the next day, the larvae were used for the experiment.

4.2.5 Experimental design

Based on the results of previous study on the inclusion of rotifers in the feeding regime of mud crab larvae (Quy et al., 2018b), two feeding regimes *viz.* extending rotifers inclusion mixed with *Artemia* until Z4 and Z5 stages, were selected. In the current study, rotifers and *Artemia* in these feeding regimes (extending rotifers until Z4 and Z5) were enriched with a commercial probiotic. The enriched/un-enriched live food was defined as a first factor and extension of rotifers inclusion as a second factor. A 4 x 2 factorial experimental design with eight various combinations of enriched or un-enriched or a mixture of enriched and un-enriched live food (factor 1) in each of two feeding regimes (factor 2) in triplicate was tested (Table 4.1). Enriched or un-enriched rotifers were fed from Z1 crab larvae and extended until the next day of the moulting to Z4 and Z5 stages, depending upon the selection of the extension of rotifers inclusion, whereas enriched or un-enriched *Artemia* nauplii were offered to these treatments from Z2 stage until the megalopa stage.

Table 4.1: Experimental feeding treatments (design) showing combination of enriched/un-enriched live food and extension of rotifer inclusions for *S. paramamosain* larvae.

Extension of rotifer inclusion	Enrichment			
	un-enriched *Artemia* and rotifers	En-Rotifers only	En-*Artemia* only	Both *Artemia* and rotifers enriched
Until zoea 4 stage	R & A	En-R & A	R & En-A	En-R & En-A
Until zoea 5 stage	R & A	En-R & A	R & En-A	En-R & En-A

Key: R: Un-enriched rotifers; A: Un-enriched Artemia; En-R: Enriched rotifers; En-A: Enriched Artemia.

Twenty-four 1.5-L plastic beakers in which each beaker was filled with 1 L of disinfected seawater (30 g L^{-1}) and stocked with 30 mud crab Z1 collected from the 100-L plastic bucket. The beakers were moderately aerated to prevent settlement of live food and larvae. All the experimental beakers were maintained at a constant

temperature (28 ± 1°C) by immersing in a 5,000-L square concrete tank as a water bath (Fig. 4.1). To improve the ability to catch the prey and possibly reduce any mortality by cannibalism or infection, the photoperiod was maintained constantly for 24 hours by a 40-W fluorescent lamp. The pH of disinfected seawater was maintained at 8.3 ± 0.2 by adding $CaCO_3$ and/or NaHCO3 before distribution to beakers.

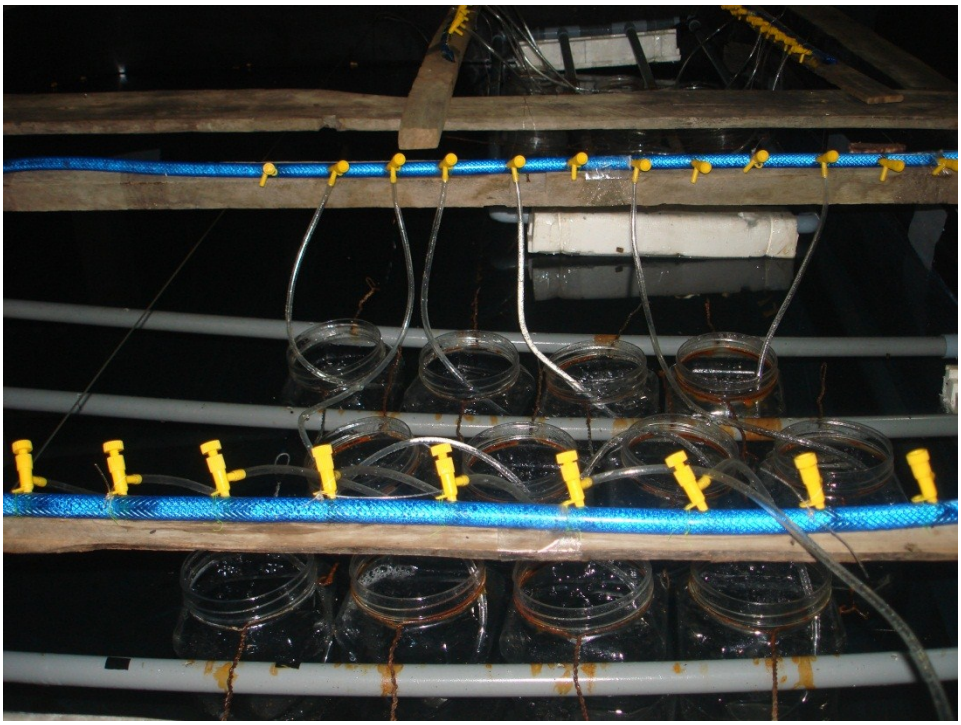

Figure 4.1: All the experimental beakers were incubated in a 5000-L square concrete tank as a water bath.

Mud crab larvae were fed only rotifers at a rate of 20 individuals mL^{-1} from Z1 to Z2 and then fed a mixture of rotifers and *Artemia* at a rate of 10 and 5 individuals mL^{-1}, respectively, until Z4 and Z5, depending upon the treatment in factor 2. After rotifer inclusions were stopped, the density of *Artemia* in the feeding regime was increased from 5 to 10 individuals mL^{-1} until the end of the experiment. The desired densities of rotifers and *Artemia* nauplii in every beaker were prepared following the method described by Baylon (2009). Feeding was given once a day in the morning after all mud crab larvae were transferred into a newly prepared beaker. The newly prepared beakers were filled with disinfected seawater of the same water parameters of the replaced beakers and the larvae were carefully and quickly transferred using a bore pipette. Water quality parameters such as salinity, pH and Alkalinity were measured by refraction meter, pH 2 meter and kH test kit (Sera, Germany), respectively at

storage tank prior to filling into newly prepared beakers. The temperature in the water bath tank was measured by thermometer every day. At the point of the time replacing beaker, larval metamorphosis and mortality were recorded for each beaker. When megalopa stage appeared, they were immediately moved out to avoid cannibalism. Five megalopae were collected and preserved in 10% formalin solution for further measurements of carapace widths, body lengths and wet weights. The experiment was ended at 24 days after hatching. The survival of the larvae, consisting of zoeae and megalopae were computed using the following equation:

$$\text{Survival rate} = \frac{\sum \text{Final larvae x } 100}{30 \text{ zoea 1 stocked}}$$

Percentage of successful metamorphosis of megalopa was computed using the following equation:

$$\text{Metamorphosis rate} = \frac{\sum \text{Final megalopae x } 100}{30 \text{ zoea 1 stocked}}$$

Development time was identified when the megalopae started to appear in the beaker. Carapace width and body length of megalopa were measured using a scale inserted into the eyepiece of a microscope (Olympus CX21, USA), then they were dried on tissue paper for wet weight. The wet weight of megalopa was weighted by an analytical balance (PA 214-Ohaus, USA) with 0.0001g precision.

4.2.6 Statistical analysis

All data in the treatments presented in percentages, such as survival of larvae and percentage of successfully metamorphosed megalopa, were arcsine-square root transformed (Zar, 2010) before analysis. Two-way ANOVA was used to test for significant differences among treatments. When no significant interactive effects between enriched/un-enriched live food and extension of rotifer inclusions, two-way ANOVA was continuously employed to test for various enrichment/non-enrichment and two levels of extensions of rotifers inclusion. If there were significant interactive effects between enriched/un-enriched live food and extension of rotifer inclusions, one-way ANOVA was employed to test for various effects of enrichment/non-enrichment, extension of rotifers inclusion and interaction between enrichment/non-enrichment and extension of rotifers inclusion. Significant differences between the treatments were detected by Tukey's multiple range tests at the 0.05 level of

significance. To test a relationship between the survival and day of culture of crab larvae fed with different enrichments and extension of rotifer inclusion mixed with *Artemia*, simple linear regression was employed, in which Y represents the survival of larvae and X the day of culture. For growth performance, Kruskal-Wallis non-parametric tests was used to compare megalopa carapace width, body length and wet weight because of unequal sample size (amount of megalopa of some replicate in treatments were not enough); significant differences between the treatments were detected by Mann-Whitney Test at the 0.05 level of significance. All statistical analyses were performed using IBM SPSS Statistics 24.0.

4.3 Results

4.3.1 Environmental parameters and concentration of total *Bacillus* spp. of enriched live food

During the experiment, the salinity and pH in all beakers were stable at 30 g L^{-1} and 8.6, respectively. Alkalinity was constant at 140 mg $CaCO_3$ L^{-1}. Water temperature was 27.9 ± 0.5 °C throughout the experimental period.

After enriching live food, the total *Bacillu*s spp. counted in enriched rotifers and *Artemia* were shown in Table 4.2.

Table 4.2: Total *Bacillus spp*. concentration in enriched rotifers and *Artemia*

	Total *Bacillu*s spp. concentration (x 10^6 cfu g^{-1})		
	At the beginning of the trial	At the end of the trial	Average
Rotifers	2.6 ± 0.56	2.0 ± 0.70	2.3 ± 0.62
Artemia	4.5 ± 0.63	3.3 ± 0.07	3.9 ± 0.78

Key: Cfu: colony forming unit.

4.3.2 Larval survival

There were no significant ($P > 0.05$) interactive effects between enriched/un-enriched live food with probiotics (factor 1) and extension of rotifers inclusion (factor 2) on mud crab larval survival; the main effect was examined independently for two factors (Table 4.3). Larval survival in all treatments (in both factors) decreased gradually ($P < 0.05$) during the culture period. The survival of larvae fed both enriched rotifers and *Artemia* was higher ($P < 0.05$) than that of larvae fed a diet without enrichment (R & A) from 15 days after hatching (DAH) onwards and a

diet enriched with rotifers only from 15 to 18 DAH. However, the larval survival at the end of the feeding experiment was not altered ($P > 0.05$) by enrichment when enrichment was carried out individually either to rotifers or *Artemia*. Similarly, extension of rotifer inclusions influenced ($P < 0.05$) the survival of larvae from 18 DAH onwards, in which the larvae fed rotifers mixed with *Artemia* until Z5 showed significantly higher survival than those fed rotifer inclusions until Z4.

Table 4.3: Mean survival ± standard error (SE) of crab larvae fed according to different enrichments and extending rotifer inclusions mixed with *Artemia* for 24 days of culture

Days	Factors					
	Enriched/un-enrichment live food				Extension of rotifer inclusions	
	R & A	En-R & A	R & En-A	En-R & En-A	Until Z4	Until Z5
3	$^{1}95.0 \pm 1.67^{a}$	$^{1}93.3 \pm 2.11^{a}$	$^{1}96.7 \pm 1.49^{a}$	$^{1}97.2 \pm 0.56^{a}$	$^{1}96.9 \pm 0.76^{a}$	$^{1}94.2 \pm 1.31^{a}$
6	$^{2}72.2 \pm 2.94^{a}$	$^{12}78.3 \pm 4.01^{a}$	$^{2}79.4 \pm 2.91^{a}$	$^{12}86.1 \pm 2.78^{a}$	$^{2}79.7 \pm 2.61^{a}$	$^{2}78.3 \pm 2.61^{a}$
9	$^{23}57.2 \pm 7.95^{a}$	$^{23}69.4 \pm 3.98^{a}$	$^{23}70.0 \pm 4.39^{a}$	$^{23}76.7 \pm 1.92^{a}$	$^{23}65.8 \pm 4.48^{a}$	$^{23}70.8 \pm 2.76^{a}$
12	$^{234}45.0 \pm 9.26^{a}$	$^{34}48.3 \pm 7.33^{a}$	$^{34}57.8 \pm 6.48^{a}$	$^{234}71.1 \pm 3.51^{a}$	$^{34}51.1 \pm 6.03^{a}$	$^{34}60.0 \pm 4.91^{a}$
15	$^{345}31.7 \pm 7.54^{a}$	$^{45}37.2 \pm 7.62^{a}$	$^{4}47.8 \pm 4.99^{ab}$	$^{345}61.7 \pm 4.77^{b}$	$^{45}38.9 \pm 5.32^{a}$	$^{45}50.3 \pm 5.15^{a}$
18	$^{45}23.3 \pm 5.84^{a}$	$^{45}29.4 \pm 6.96^{a}$	$^{45}37.2 \pm 4.16^{ab}$	$^{456}54.4 \pm 5.62^{b}$	$^{56}30.0 \pm 4.85^{a}$	$^{456}42.2 \pm 4.93^{b}$
21	$^{5}16.1 \pm 4.67^{a}$	$^{45}22.2 \pm 6.19^{ab}$	$^{5}24.2 \pm 3.06^{ab}$	$^{56}40.6 \pm 6.74^{b}$	$^{6}20.0 \pm 3.74^{a}$	$^{56}31.7 \pm 4.56^{b}$
24	$^{5}13.9 \pm 3.59^{a}$	$^{5}20.0 \pm 5.84^{ab}$	$^{5}21.7 \pm 3.19^{ab}$	$^{6}38.9 \pm 6.70^{b}$	$^{6}18.1 \pm 3.75^{a}$	$^{6}29.2 \pm 4.38^{b}$

Key: R: Un-enriched rotifers; A: Un-enriched Artemia; En-R: Enriched rotifers; En-A: Enriched Artemia. Significant differences were found among all treatments with different superscript letters ($P < 0.05$) in the same row in the each factor. Significant differences were also found throughout the culture period within each treatment with different superscript numbers ($P < 0.05$) in the same column.

There was a negative correlation between larval survival and culture period (Table 4.4), with regression indices (R^2) ranging from 0.96 to 0.99. These indices were, however, independent (*P > 0.05*) of enrichments and extension of rotifers inclusion in diets with *Artemia*.

Table 4.4: The relationship between the survival and day of culture of crab larvae fed with different enrichments and extension of rotifer inclusions mixed with *Artemia*

Treatment	Equation	Regression (R^2)
R & A	y = -3.91x + 97.46	0.96 ± 0.02
En-R & A	y = -3.69x + 99.60	0.97 ± 0.01
R & En-A	y = -3.54x + 101.90	0.99 ± 0.01
En-R & En-A	y = -2.74x + 102.50	0.99 ± 0.00
R extended until zoae 4	y = -3.81x + 101.28	0.98 ± 0.01
R extended until zoae 5	y = -3.13x + 99.43	0.99 ± 0.01

Key: R: Un-enriched rotifers; A: Un-enriched Artemia; En-R: Enriched rotifers; En-A: Enriched Artemia. Y represents the survival of larvae and X the day of culture. Values are mean ± SE.

4.3.3 Rate of metamorphosis and development time of megalopa

Mud crab larvae fed both enriched rotifers and *Artemia* (En-R & En-A) showed a higher (*P < 0.05*) rate of metamorphosis of megalopa (37.2%) than those fed a diet without enrichment (R & A) (12.8%) and a diet enriched with rotifers only (En-R & A) (17.2%). Similarly, the larvae fed rotifer inclusions mixed with *Artemia* until Z5 showed a significantly higher (*P < 0.05*) metamorphosis rates (27.5%) than larvae fed rotifer inclusions until Z4 (15.8%) (Fig. 4.2). Morphological variation was also less seen in the feeding treatments that had high successful metamorphosis of megalopa. However, the duration to metamorphose from Z1 to the megalopa stage (development time) did not completely depend (*P > 0.05*) on enrichments and feeding duration with rotifers (Fig. 4.3).

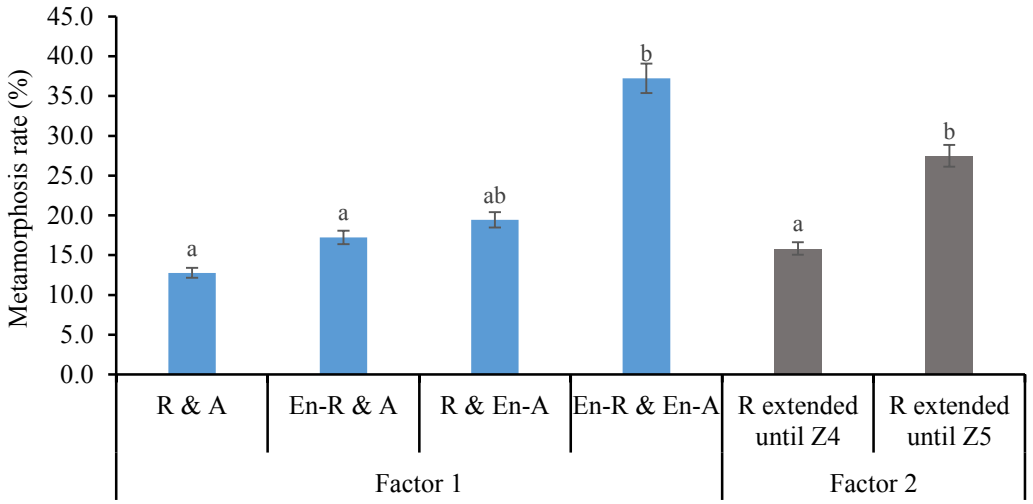

Figure 4.2: Percentage of successful metamorphosis of megalopa fed according to different enrichments and extending rotifer inclusions mixed with *Artemia*.

Key: R: Un-enriched rotifers; A: Un-enriched Artemia; En-R: Enriched rotifers; En-A: Enriched Artemia. Significant differences were found among all treatments with different superscripts letters (P < 0.05) in the same colour bars.

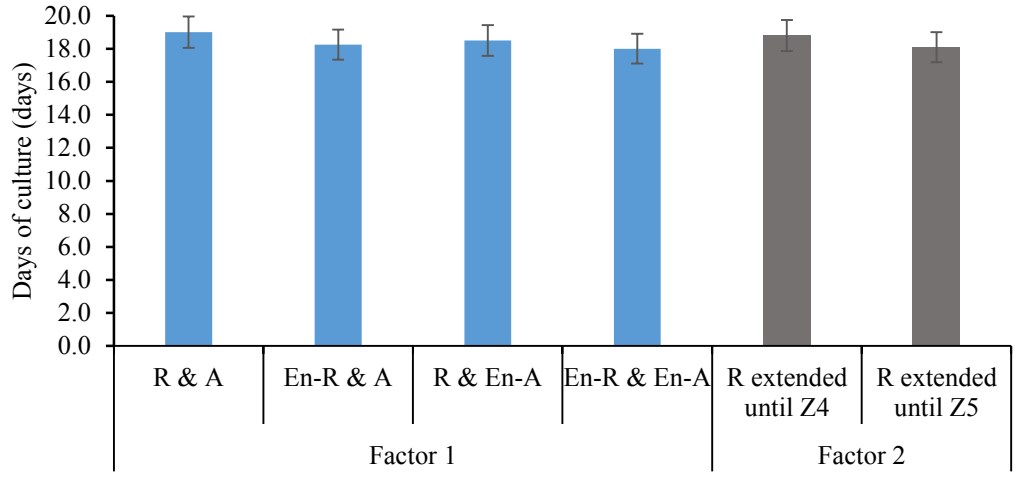

Figure 4.3: Duration of rearing period needed to be metamorphosed from zoea 1 to megalopa stage under various feeding regimes.

Key: R: Un-enriched rotifers; A: Un-enriched Artemia; En-R: Enriched rotifers; En-A: Enriched Artemia.

4.3.4 Growth performance of megalopa

Enrichment of live food did not significantly ($P > 0.05$) influence carapace width, body length and wet weight of megalopa, but extension of rotifer inclusions mixed with *Artemia* until Z5 improved ($P < 0.05$) carapace width and wet weight of megalopa (1.66 mm and 4.02 mg, respectively) compared to the treatment in which rotifers were included until Z4 (1.62 mm and 3.77 mg, respectively; Table 4.5).

Table 4.5: Mean carapace width, body length and wet weight of one-day-old megalopa fed according to different enrichments and extension of rotifer inclusions mixed with *Artemia*

Parameters	Factors					
	Enriched/un-enriched live food				Extension of rotifers	
	R&A	En-R&A	R&En-A	En-R&En-A	Until Z4	Until Z5
CW (mm)	1.63 ± 0.01	1.64 ± 0.01	1.63 ± 0.02	1.66 ± 0.01	1.62 ± 0.01[a]	1.66 ± 0.01[b]
BL (mm)	4.91 ± 0.05	4.97 ± 0.03	4.96 ± 0.01	4.98 ± 0.03	4.93 ± 0.03[a]	4.98 ± 0.02[a]
WW (mg)	3.84 ± 0.11	3.90 ± 0.12	3.92 ± 0.12	3.94 ± 0.07	3.77 ± 0.07[a]	4.02 ± 0.05[b]

Key: R: Un-enriched rotifers; A: Un-enriched Artemia; En-R: Enriched rotifers; En-A: Enriched Artemia; CW: Carapace width; BL: Body length; WW: Wet weight. Values are mean ± SE. Significant differences were found among all treatments with different superscript letters (P<0.05) in the same row in the each factor.

4.4 Discussion

Previous research has shown that *Bacillus* spp. can have significant effects on crustaceans (Table 4.6), mediated by improved water quality (Nimrat et al., 2012; Soundarapandian & Sankar, 2008), enhanced digestive enzyme activities (Ziaei-Nejad et al., 2006) and increased health status (Uddin et al., 2013) of the host. Likewise, a feeding regime including rotifers and *Artemia* improved the survival of mud crab larvae (Baylon & Failaman, 1999; Zeng & Li, 1999).

Table 4.6: Uses of probiotics *Bacillus* species in crustacean aquaculture

Probiotics	target host species	Doses and administration duration	Response	References
B. subtilis UTM 126	*L. vannamei*	10^5 cfu g^{-1} feed for 28 days	-Resistance of *Vibrio* species	Balcázar & Rojas-Luna (2007)
B. subtilis	*L. vannamei*	10^{10} cfu g^{-1} feed for 30 days	-Increased survival and yield and reduced *Vibrio*	Far et al. (2009)
B. subtilis	*L. vannamei*	10^{10} cfu g^{-1} feed for 20 days	-Against white spot syndrome virus	Fu, Wang, Wu, & Li (2011)
B. subtilis E20	*L. vannamei*	10^9 cfu L^{-1} for 14 days	-Increased survival and immune response of larvae	Liu et al. (2010)
B. subtilis E20	*L. vannamei*	10^8 cfu g^{-1} feed for 98 days	-Increased survival and disease resistance	Tseng et al. (2009)
B. subtilis	*P. monodon*	10^{10} cfu g^{-1} feed for 90 days	-Disease protection	Rengpipat et al. (2000)
B. S11	*P. monodon*	0.4 mg g^{-1} feed for 90 days	-Increased growth and immunity	NavinChandran et al. (2014)
B cereus	*M. rosenbergii*	10^9 cfu g^{-1} diet for 60 days	-Increased survival, growth, feed use, digestive enzyme activity and innate immune response	Gupta et al. (2016)
B. coagulans	*M. rosenbergii*	10^9 cfu g^{-1}diet for 60 days	-Increased growth, feed use and immune parameters	Kumar et al. (2013)
B. licheniformis	*Marsupanaeus japonicus*	10^8 cfu g^{-1} diet for 60 days	-Increased survival, growth, immune response and reduced *Vibrio*	Dong et al. (2014)
Bacillus ssp.	*L. vannamei*	10^5 cfu mL^{-1}	-Increased survival and resistance of *Vibrio* species,	Luis-Villaseñor et al. (2011)
Bacillus ssp.	*L. vannamei*	10^9 cfu g^{-1} diet for 60 days	-Improved growth and survival of larvae	Nimrat et al. (2012)
Bacillus ssp.	*Fenneropenaeus indicus*	10^6 cfu mL^{-1}	-Improved growth, survival and digestive enzyme activity of larvae	Ziaei-Nejad et al. (2006)

To our knowledge, this is the first study investigating the effects of extending rotifer feeding in an *Artemia*-based diet with enriched live foods, both *Artemia* and rotifers, with a commercial probiotic based on *Bacillus* spp. in mud crab (*S. paramamosain*) larvae. In the current study, a diet that included both enriched rotifers and *Artemia* with probiotics increased survival and metamorphosis rates of megalopa compared to a diet without enriched live food. The same results were obtained in *L. vannamei* fed both enriched rotifer and *Artemia* (Jamali et al., 2015). Total bacteria and probiotic *Bacillus* increased in the host (Jamali et al., 2015) that resulted in increasing activity of digestive enzymes (Ziaei-Nejad et al., 2006) and inhibiting the growth of pathogens (Decamp et al., 2008), which in turn improved larval survival. In addition, Nogami et al. (1997) reported that the bacterial strain PM-4 (*Thalassobacter utilis*), which was added daily into rearing water increased the survival of swimming crab (*Portunus trituberculatus*) larvae by repressing the growth of harmful bacteria and fungi. Dan and Hamasaki (2015) also found that larval necrosis symptoms were suppressed and survival of early *S. serrata* larvae was improved when probiotics was inoculated in the larval rearing water. Although the mode of probiotic administration differed between our study and the previously mentioned studies, the beneficial outcomes of probiotics were not significantly different (Ziaei-Nejad et al., 2006). Similar to the factorial 1 outcome, the inclusion of rotifers mixed with *Artemia* until Z5 improved megalopa survival, metamorphosis rate and growth compared to the treatment where rotifers were included only until Z4.

Previous studies have also shown that a mixed diet of rotifers and *Artemia* until the late zoeal stages improved larval survival of *S. serrata* (Baylon & Failaman, 1999), *P. pelagicus* (Redzuari et al., 2012), *Upogebia pusilla* (Faleiro & Narciso, 2009) and *Panopeus herbstii* (Harvey & Epifanio, 1997), but these studies failed to mention the benefits of extending inclusion of rotifers. In the present study, morphological variation and formation of immature megalopa, resulting from accumulation of insufficient nutrients (Dan et al., 2013) were less seen in the crab larvae fed rotifer inclusions mixed with *Artemia* until Z5 than in those fed rotifer inclusions until Z4. This could be due to the starvation of *Artemia* that can be reduced to a certain extent by feeding them rotifer excrements (Dan et al., 2016b). Crab larvae can be exposed to less starved *Artemia* when rotifers are present until Z5 in a mixed diet with *Artemia.*

Additionally, morphological variation and formation of immature megalopa, which are caused by quantity of available food (Pestana & Ostrensky, 1995), may be less appearance as rotifers and *Artemia*, both are available in rearing water until Z5. According to Genodepa et al. (2004a), the feeding behaviour of mud crab larvae is raptorial. The late zoeal stages showed a strong preference for *Artemia*, which supplied the majority of energy intake required for moulting to the next stage (Harvey & Epifanio, 1997); however, after moulting, they preferred rotifers, which provided energy for survival (Baylon et al., 2004). This indicates that extending rotifers inclusion, mixed with *Artemia*, until Z5 can provide additional benefits in reducing the morphological variation and the formation of immature megalopa, which would result in mass mortality of crab larvae (Dan et al., 2013).

For cost-effective aspect, Ruscoe et al. (2004b) and Baylon (2009) found that extending rotifers inclusion until late zoeal stages with *Artemia* does not provide economic benefits due to the use of expensive resources in terms of floor space, microalgae and labour (Nghia, 2004; Ruscoe et al., 2004b). On the contrary, rotifers used in the present study were produced according to the procedures described by Lind (2014) that could harvest high daily productivity with low production cost. Furthermore, extending the period of rotifer inclusion in the feeding regime could reduce the use of *Artemia* (Quy et al., 2018b). Therefore, extending rotifers inclusion mixed with *Artemia* until Z5 is possible to reduce cost production for mud crab hatchery.

Probiotics may rapidly alter the intestinal microbiota of the larvae. Gatesoupe (1999) showed that the intestinal microbiota of early larval stages was easily altered by the invasion of the microorganisms from water and food, even when their digestive tract was not fully developed and feeding had not yet commenced. In the present study, a mixture of *Bacillus* spp. was inoculated in live food at the beginning of the experiment; therefore, any harmful bacteria that could invade the cultured species through the food chain (Van Stappen, 1996) may have been reduced or eliminated. Enriched live food can also become a vector for bringing desirable microbes into the digestive system of the host (Lavilla-Pitogo, Catedral, Pedrajas, & De la Peña, 2002), thereby potentially improving the properties of the indigenous microflora of the larvae as well as interfering with the development of harmful bacteria. For example, when the probiotic *Bacillus* spp. was used, *Vibrio* numbers were reduced and no

luminous *Vibrio* responsible for losses in shrimp hatcheries existed (Moriarty, 1998, 1999). Similarly, Far et al. (2009) and Rengpipat et al. (1998) have reported that *B. subtilis* proliferated and replaced *Vibrio* spp. in the digestive tract of shrimp (*P. monodon*) treated with probiotics that increased in survival and growth of the shrimp. In agreement with these results, our study showed that the survival and metamorphosis rates of the larvae fed both enriched rotifers and *Artemia* with probiotics were higher than those fed a diet without enrichment. The reduction in the number of probiotic bacteria (Dan & Hamasaki, 2015) or low concentrations of probiotics in the diet (Tseng et al., 2009) induced individual differences among the replicated dietary treatments in any feeding trial. This may explain the fact that in the current study, enrichment of only one type of live feed could not significantly improve survival and rate of metamorphosis in crab larvae.

A study by Ruscoe et al. (2004b) on *S. serrata*, as well as our earlier study (Quy et al., 2018b) on *S. parasamosain*, showed that feeding a mixture of un-enriched rotifers and *Artemia* until Z4 and Z5 stages obtained similar megalopa survival. On the contrary, feeding a mixture of rotifers and *Artemia* until Z5 rather than Z4, irrespective of enrichment, improved megalopa survival, metamorphosis rates, and growth in this study. It is apparent that enrichment can be considered as a factor contributing to these differences. However, there were no significant interactive effects between enrichment/non-enrichment with probiotics and extension of rotifers inclusion on mud crab larval survival and metamorphosis rates, probably because the beneficial effect of the two factors studied was more of an additive affect rather than a synergistic affect.

4.5 Conclusion

In conclusion, inclusion of enriched rotifers mixed with enriched *Artemia* until the stage Z5 improved survival, metamorphosis rate and growth of megalopa. Further, the enrichment of both rotifers and *Artemia* as a live feed is crucial to achieve beneficial outcomes in crab hatcheries. However, this result needs to be validated under commercial farming operations, wherein other natural and/or anthropogenic factors interplay. Nevertheless, the current study can serve as guideline on the use of appropriate feeding regimes to improve the survival of mud crab larvae. There is also a need to compare the bio economics between the use of exclusive formulated feed and the feeding regime used in the current study.

CHAPTER 5: Impact of different rearing systems on survival, growth and quality of mud crab (*Scylla paramamosain*) megalopa reared from early zoeae

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5.1 Introduction

The mud crab (*Scylla paramamosain*) is one of the most important marine resources in the South China Sea (Keenan et al., 1998), and dominates among the *Scylla* genus in the Mekong Delta, Vietnam (Lindner, 2005; Petersen et al., 2013). Various mud crab seed production techniques have been reported (Davis, 2003; Nghia et al., 2007a) and majority of research has been carried out using an open water flow-through system due to its ease in operation and abundant supply of seawater in the hatchery site (Hirayama, 1974). However, the system is known to create environmental degradation by discharge of waste water from the hatcheries (Abeysinghe, Shanableh, & Rigden, 1996). It is prone to disease out-breaks due to the common source of water (Primavera, 2006; Samocha et al., 2007). Likewise, it has higher operational costs due to large volume of seawater transported or pumped to the hatchery (Mallasen & Valenti, 1998; Piamsak & Somkiate, 1980).

Conversely, closed water systems such as recirculating water, green water and biofloc water systems are more cost-effective, biosecure and environmentally friendly due to reduced dependency on new fresh seawater. The removal of nitrogenous metabolites from the closed water system by microorganisms (Avnimelech, 1999; Hirayama, 1974) is considered as the major concern. The reduced amount of water and employing reused water for the hatchery operation leads to decrease environmental impacts, improve biosecurity (De Lorenzo et al., 2015) and guarantee year-round seed supply (Ebeling & Timmons, 2012). In addition, the green water and biofloc water systems could supplement nutrients (Izquierdo et al., 2006; Lober & Zeng, 2009; Manan, Moh, Kasan, Suratman, & Ikhwanuddin, 2017) and inhibit harmful bacterial development (Huervana, De La Cruz, & Caipang, 2006).

An extreme change in water quality under different types of rearing systems may affect the quality of megalopa by reducing the immunity of the animal. Although different methods are used to determine the quality of larvae and post-larvae of

crustaceans, stress tests, which are rapid, cheap and simple (Samocha et al., 1998), have been used as a tool to evaluate megalopa quality in the present study. The quality of megalopa was assessed by evaluating a survival response of the larvae to the selected stressors such as ammonia exposure and simulated transport conditions. Survival rate after exposing to the stressors can be considered as an indicator to assess the post-larval quality (Samocha et al., 1998). The ammonia stress test is sensitive and it can use a single concentration of ammonia to evaluate larval quality (Cavalli, Lavens, & Sorgeloos, 1999). However, this concentration has to be based on previous knowledge of the LC_{50} dose for each specific larval stage. In addition, the transport stress test is a sensitive indicator used to determine the quality of megalopa, as morbidity and mortality are inevitable phenomena caused by stressors such as air exposure, handling and physical disturbance (Fotedar & Evans, 2011). Many studies on recirculating water (Mallasen & Valenti, 1998), green water (Izquierdo et al., 2006; Tendencia et al., 2015) and biofloc water systems (De Lorenzo et al., 2016b; Khanjani et al., 2017) are well documented for shrimp hatcheries and nurseries, but information on the effect of the closed systems in terms of survival, growth and quality of megalopa are limited to date. The aim of the present study was to compare the effects of four rearing systems *viz.* clear water, green water, recirculating water and biofloc water systems on survival, growth and quality of newly metamorphosed megalopa of mud crab.

5.2 Materials and methods

5.2.1 Source of crab larvae

The mature females of mud crab (*S. paramamosain*) were transported from an extensive shrimp farm to a shrimp hatchery in Nam Can district ($8^0 45' 00'' N$; $105^0 01' 38'' E$), Ca Mau Province, Vietnam. The females were then reared until appearing newly hatched zoea, according to the previous procedure (Quy et al., 2018b). The newly hatched zoea 1 (Z1), from a single spawner, were collected and bathed in 0.1 µL L^{-1} iodine solution for 30 seconds prior to stocking in a 6,000-L concrete tank at 100 larvae L^{-1}. After 2 hours of stocking, the larvae were fed L-strain of rotifers at a rate of 40–50 individuals mL^{-1}. On the following day, Z1 were collected for the trial.

5.2.2 Preparation of various rearing systems

5.2.2.1 Green water system

Green water was prepared in a 6 m^3 square fibreglass tank of 2.0 m x 2.0 m x 1.5 m (width x length x height). The tank was filled with treated seawater (28 g L^{-1}) and stocked with small tilapia, *Oreochromis niloticus*, (10 g- average body weight) at 30 fish m^{-3}. The fish were fed with commercial feed (30% CP, GreenFeed Co Ltd, Vietnam) twice daily at 3% of their body weight (54 g = 30 fish x 10 g x 6 m^3 x 3%) between 9 am and 5 pm. After five days, the water colour appeared green and the most abundant species of phytoplankton in the green water is mainly *Chlorella sp.* (>80% in abundance) (Huervana et al., 2006). This green water was used for the experiment when the algae concentration was measured to be 2–4 million mL^{-1} using a haemocytometer.

5.2.2.2 Biofloc water system

Biofloc water was also prepared in a 6 m^3 square fibreglass tank that was filled with disinfected seawater (28 g L^{-1}). To establish biofloc formation in the fibreglass tank, commercial feed and molasses were added every day. Amount of commercial feed was calculated based on the amount of ammonia-nitrogen excreted by tilapia in the green water tank when assuming that tilapia excreted 70% as ammonium. Consequently, 37.8 g (70% x 54 g) of commercial feed was added into the fibreglass tank. This amount of feed produces 1.63 g ammonia-nitrogen into the water based on the formula described by (Ebeling, Timmons, & Bisogni, 2006) and 18.9 g of carbon based on the theory of Avnimelech (1999). The carbon/nitrogen (C/N) ratio was adjusted to 15/1 to promote biofloc formation in the tank; therefore 24.5 g of carbon was added to maintain the C/N ratio. As 18.9 g of carbon was available in the feed, only 5.6 g (24.5 g-18.9 g) of carbon was needed to maintain the C/N ratio. Molasses, used to supplement carbon in the present study, contained 23.4% of carbon with specific gravity of 1.3, so 5.6 g carbon was attained from 23.9 g of molasses. Overall, 23.9 g of molasses were supplemented together with 37.8 g of commercial feed into the fibreglass tank on a daily basis to establish biofloc in the tank. Strong aeration was provided to agitate the water and prevent the settling of feed and floc particles. This biofloc build up was used for the trial.

5.2.2.3 Recirculating water system

A closed recirculating water system consists of three components, one 60-L plastic larvae rearing bucket, one 60-L plastic biofilter bucket, and one 10-L plastic physical filter bucket. In the biofilter bucket, 25% of the bucket volume was filled with oyster shell (*Crassostrea belcheri*) as a substrate for the growth of beneficial nitrifying bacteria (*Nitrosomonas* spp. and *Nitrobacter* spp.), while cotton was placed inside the physical filter bucket. Water from the biofilter bucket was pumped into the rearing bucket by a submersible pump (8W, aquarium pump, China). The water in the rearing bucket flows through the physical filter bucket, where water gets filtered through the cotton before flowing back into the biofilter bucket by gravity. The water was pumped from the biofilter bucket to the rearing bucket with the flow rate adjusted to approximately 2 L min^{-1}. On the first day, 25 mg L^{-1} ammonium chloride (NH_4Cl) and 10 mg L^{-1} sodium nitrite ($NaNO_2$) were added into the recirculating system to build up nitrifying bacteria. The concentration of ammonia was monitored regularly until it was close to zero, indicating that the nitrogen processing ability of the biofilter had been established. The closed recirculating water system was set up for two weeks prior to commencement of the trial.

5.2.3 Experimental design

The experimental design consisted of four different rearing systems with three replicates each using twelve 60-L plastic buckets. The experimental setup was maintained indoors and each bucket was filled up to 90% of its volume with disinfected seawater (28 g L^{-1}). Active and healthy Z1 from the 6,000-L concrete stank were stocked in each bucket at 30 Z1 L^{-1}. The clear water system was considered as a control system.

In the green water bucket, the concentration of algae was maintained at 10^5 cells mL^{-1} by supplementing a high concentration of algae from the green water tank. This concentration was based on a previous study by Simon (1978) for marine shrimp (*Penaeus stylirostris* and *Litopenaeus vannamei*). The desired concentration of algae was prepared according to the method described by Lober and Zeng (2009). The dominant algae, *Chlorella* spp., often get reduced to approximately 50% of the initial density after 3 days of inoculation (Maddox & Manzi, 1976). To maintain the concentration of algae during the experimental period, a high concentration of algae

from the green water tank was added into the buckets every three days. Before addition of algae, the concentration of algae in the rearing water bucket and the green water tank were counted using a hemocytometer under a microscope (Olympus CX21, USA).

Meanwhile, the biofloc system was prepared by adding 10 L of water of matured biofloc from the 6 m^3 biofloc tank into each plastic bucket and then mixed with 40 L of rearing seawater. Molasses was added into biofloc water buckets at the beginning and when the total ammonia in the rearing water was higher than 1.0 mg L^{-1}. The molasses dose was calculated according to methods of Avnimelech (1999) and Ebeling et al. (2006) who found that approximately 6 g of carbon can convert 1 g of total ammonia nitrogen from the water to a bacterial biomass.

Aside from these systems above, it pays attention for the recirculating water system where the outlet pipe in the rearing bucket has to cover by screening net with the mesh size of 125 μm to prevent rotifer or *Artemia* washing out from the rearing bucket due to water flows through the physical filter bucket.

Water in the green, biofloc and recirculating systems remained unexchanged during the trial period, but these systems were supplemented with fresh water in order to compensate for water loss due to evaporation. In contrast, the water in the clear water system was exchanged at a rate of 40% by disinfected seawater every four days to avoid coincidence of the moulting period. The moulting period of each zoeal stage took place on two or three days (Baylon, 2009). Thus, utmost care was taken due to the moulting larvae are very sensitive and prone to stress with slight alterations in water quality parameters. Mud crab larvae in all the treatments were fed L-strain rotifers (*Brachionus plicatilis*) enriched with commercial probiotics (Lymnozyme FT-2B, Cisbay, USA) at a rate of 10 individuals mL^{-1} from Z1 until Z5 stage and enriched *Artemia* (*Artemia franciscana*) with probiotics at a rate of 2 individuals mL^{-1} from Z2 until megalopa stage. The density of *Artemia* was increased from 2 to 4 individuals mL^{-1} when feeding with rotifers was stopped at Z5. The desired density of rotifers and *Artemia* nauplii was prepared and supplied into every bucket based on the method of Baylon (2009). The rotifers were fed to the larvae only once at 12 pm, while newly hatched *Artemia* nauplii were fed twice daily at 6 am and 6 pm. Intense aeration with air stones was provided to prevent settlement of larvae and live food.

During the culture period, the photoperiod was maintained at 12 hours of natural light during day time and 12 hours of artificial light by a 40-W fluorescent lamp at night time. The temperature was 28 ± 1^{o}C. Dissolved oxygen (DO) was maintained over 7.0 mg L^{-1} by aeration. The pH was adjusted to approximately 8.3 ± 0.2 by adding $CaCO_3$ and/or NaHCO3. The total ammonia nitrogen (TAN) concentration was measured with an ammonia meter, model HI 96715 (Hanna, Romania), while nitrite was measured using a nitrite test kit (Sera, Germany). The analyses of total ammonia and nitrite were performed every four days before water was exchanged in the clear water system.

5.2.4 Data collection

5.2.4.1 Survival and growth of megalopa

The final survival and growth of megalopa was computed as described previously (section 3.2.7).

5.2.4.2 Quality of megalopa

At the termination of the experiment, 20 newly metamorphosed megalopae from each rearing bucket were collected for the evaluation of megalopa quality. Ten megalopae were used for the ammonia toxicity test and the other ten were used for the simulated transport stress test. Both short term stress tests were used as tools to determine quality of megalopa reared under the four different water systems.

a) Ammonia stress test

In the ammonia-N bioassays, megalopae were exposed to ammonia-N concentration of 48.31 mg L^{-1} at 48 h LC_{50} as determined previously for *S. serrata* megalopae, which is a closely related species (Neil, Fotedar, & Shelley, 2005). The ammonia solution was prepared according to the protocol described by Chen, Ting, Lin, and Lin (1990); 2.75 g nitrogen in the form of ammonium chloride (NH_4Cl) (Merck, Germany) was diluted with 1 L distilled water and then the volume was made up to 11 L using disinfected seawater in a 40-L plastic bucket. The solution in the bucket was stirred until homogenous mixing and then distributed into 12 plastic trays (20 cm x25 cm x 4 cm: width x length x height). These trays were filled up with 1 L of ammonia-N solution and then stocked with 10 newly metamorphosed megalopae to perform acute ammonia-N stress test for 48 hours. This setup was placed at about 28

^{o}C room temperature and was unexposed to direct sunlight. During the exposure period, the megalopae were starved and no water exchange was done. Survival of megalopa was recorded at four different time points of 0, 12, 24 and 48 hours, and total ammonia concentration, pH and temperature were recorded at 0, 12, 24 and 48 hours.

b) Simulated transport stress test

For the simulated transport bioassays, 12 plastic trays (20 cm x25 cm x4 cm), each with wet thin nylon nets (mesh size < 2 mm) placed at the bottom, were used to maintain moist conditions. Ten megalopae were released into the wet nets of all trays and were kept in the place where the ammonia stress test was performed earlier. During the transport period, the trays were unshaken and megalopae were starved. Survival of megalopa was recorded at 0, 12, 24 and 48 hours. If the megalopae changed from transparent to opaque or a lack of response to gentle pressure with a probe was observed, they were considered dead.

5.2.5 Statistical analyses

Data are shown as the mean ± standard error (SE). The data presented in percentages were arcsine-square root-transformed (Zar, 2010) prior to the analysis. Survival and growth parameters of megalopa reared under four different rearing systems and results of ammonia and transport stress tests were compared using one-way ANOVA analysis of variance (SPSS for Windows, version 24.0). Specific differences among rearing systems were detected using Tukey's multiple range test at 0.05 level of significance.

5.3 Results

5.3.1 Environmental parameters

During the experiment period, dissolved oxygen, temperature and pH were maintained within the standard levels (Table 5.1). Meanwhile, a significant impact of different rearing systems was observed on the levels of TAN and nitrite (Table 5.2) on different days. At the end of the experiment, TAN was high in the green water system (2.3 mg L^{-1}) and significantly different ($P < 0.05$) to the clear water, recirculating water and biofloc water systems. On the other hand, the lowest TAN was obtained in the recirculating water system (0.1 mg L^{-1}), which was significantly

different ($P < 0.05$) compared to clear and biofloc water systems. The nitrite level in the clear water system increased at the end of the experiment; it reached 5.0 mg L^{-1} and differed ($P < 0.05$) from the rest of the systems. The lowest nitrite was also obtained in the recirculating water system and differed ($P < 0.05$) from the green water and biofloc water system at the end of the experiment.

Table 5.1: Dissolved oxygen, temperature and pH in different rearing systems during the experimental period

Systems	CW	GW	RW	BW
Days				
DO (mg L^{-1})				
1st	8.8 ± 0.03	8.7 ± 0.06	8.4 ± 0.09	8.6 ± 0.09
4th	9.0 ± 0.03	8.9 ± 0.06	9.0 ± 0.03	9.0 ± 0.03
8th	8.8 ± 0.03	8.8 ± 0.07	8.9 ± 0.06	9.0 ± 0.03
12th	8.6 ± 0.09	8.6 ± 0.07	8.6 ± 0.07	8.5 ± 0.09
16th	8.6 ± 0.06	8.5 ± 0.06	8.7 ± 0.03	8.7 ± 0.03
20th	8.4 ± 0.06	8.3 ± 0.06	8.6 ± 0.06	8.6 ± 0.03
Temperature (°C)				
1st	27.6 ± 0.03	27.8 ± 0.15	27.6 ± 0.09	27.6 ± 0.07
4th	28.0 ± 0.03	28.1 ± 0.03	28.1 ± 0.03	28.1 ± 0.01
8th	27.8 ± 0.06	27.8 ± 0.03	27.8 ± 0.03	27.8 ± 0.06
12th	29.3 ± 0.00	29.4 ± 0.23	29.6 ± 0.03	29.3 ± 0.03
16th	28.0 ± 0.03	28.1 ± 0.06	28.0 ± 0.00	28.0 ± 0.00
20th	28.1 ± 0.03	28.1 ± 0.00	28.0 ± 0.0	28.0 ± 0.03
pH				
1st	8.5 ± 0.00	8.5 ± 0.00	8.5 ± 0.00	8.5 ± 0.00
4th	8.5 ± 0.00	8.5 ± 0.00	8.5 ± 0.00	8.5 ± 0.00
8th	8.4 ± 0.00	8.4 ± 0.00	8.4 ± 0.00	8.4 ± 0.00
12th	8.4 ± 0.00	8.4 ± 0.00	8.3 ± 0.03	8.4 ± 0.03
16th	8.4 ± 0.00	8.4 ± 0.00	8.2 ± 0.06	8.3 ± 0.03
20th	8.4 ± 0.00	8.4 ± 0.03	8.1 ± 0.03	8.2 ± 0.03

Data represented as mean ± SE; n=3. Key: CW: Clear water; GW: Green water, RW: Recirculating water, and BW: Biofloc water systems.

Table 5.2: Total ammonia and nitrite in different rearing systems during the experimental period

Systems	CW	GW	RW	BW
Days				
TAN (mg L^{-1})				
1st	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4th	0.02 ± 0.00 [a]	0.02 ± 0.00 [a]	0.06 ± 0.01 [a]	0.19 ± 0.02 [a]
8th	0.28 ± 0.04[a]	0.56 ± 0.07[b]	0.13 ± 0.03[a]	1.17 ± 0.05[c]
12th	0.67 ± 0.05[b]	0.77 ± 0.09[b]	0.20 ± 0.03[a]	1.72 ± 0.02[c]
16th	1.19 ± 0.02[b]	1.27 ± 0.06[b]	0.16 ± 0.04[a]	1.61 ± 0.06[c]
20th	1.71 ± 0.11[b]	2.27 ± 0.05[c]	0.07 ± 0.01[a]	1.70 ± 0.12[b]
Nitrite (mg L^{-1})				
1st	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4th	0.50 ± 0.00 [a]	0.50 ± 0.00 [a]	0.50 ± 0.00 [a]	0.50 ± 0.00 [a]
8th	1.01 ± 0.00[b]	1.02 ± 0.00[b]	0.67 ± 0.17[ab]	0.51 ± 0.00[a]
12th	2.02 ± 0.00[c]	1.67 ± 0.33[bc]	1.00 ± 0.00[ab]	0.53 ± 0.00[a]
16th	4.99 ± 0.00[b]	0.67 ± 0.17[a]	0.67 ± 0.17[a]	0.83 ± 0.17[a]
20th	5.01 ± 0.01[c]	1.05 ± 0.02[b]	0.51 ± 0.01[a]	0.99 ± 0.02[b]

Data represented as mean ± SE; n=3. Key: CW: Clear water; GW: Green water, RW: Recirculating water, and BW: Biofloc water systems. Significant differences were found among treatments with different superscript letters (P < 0.05) in the same row.

5.3.2 Total ammonia and un-ionised ammonia concentration during ammonia stress test

Initial total ammonia in the present study measured was higher than the desired ammonia concentration of 48.31 mg L^{-1}. However, this concentration reduced followed the testing time. The total ammonia and un-ionised ammonia during the ammonia stress test period are shown in Table 5.3. Un-ionised ammonia was calculated based on temperature, pH and salinity values according to method described by Whitfield (1974).

Table 5.3: Temperature, pH, salinity, total ammonia and un-ionised ammonia concentrations at various time intervals in the ammonia test

Parameters\Time (hours)	0	12	24	48
Temperature (^oC)	28	28	28	28
pH	8.1	8.0	7.9	7.8
Salinity (g L^{-1})	28	28	28	28
Ammonia-N (mg L^{-1})	57.0	51.1	45.1	42.5
Un-ionised ammonia (mg L^{-1})	4.10	2.97	2.11	1.59

5.3.3 Survival and growth of megalopa

At the end of the trial, the highest survival of megalopa was achieved in the green water system and was significantly higher ($P < 0.05$) from the rest of the systems. The survival of megalopa reared under the biofloc water system was not significantly different ($P > 0.05$) from the clear water system, but both these systems reached higher ($P < 0.05$) survival than the recirculating water system, which yielded the lowest amount of megalopa (Fig. 5.1). Based on visual observation, uneaten *Artemia* that had grown to adult size were seen in the biofloc water system. This was not observed in other treatments. Body length, carapace width and wet weight of megalopa were not affected ($P > 0.05$) by different rearing systems (Table 5.4).

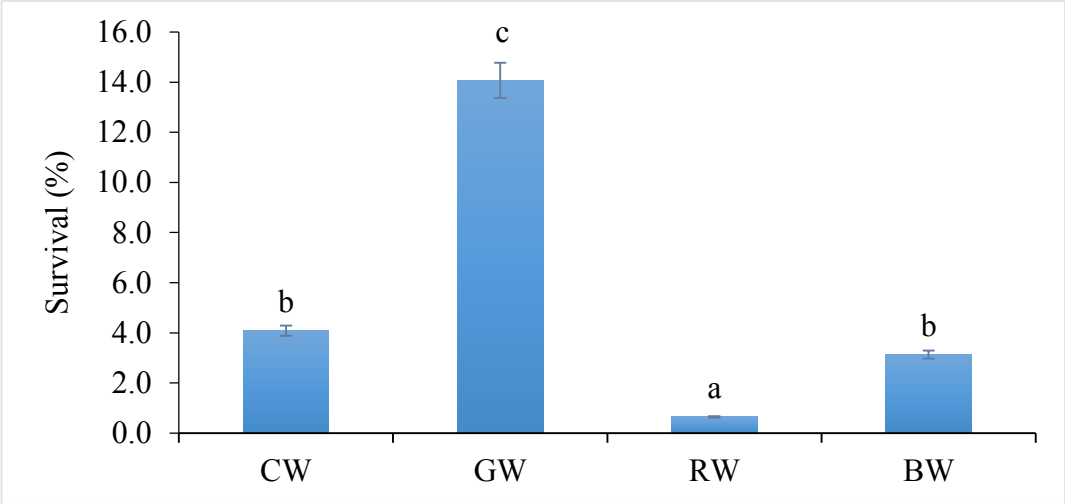


Figure 5.1: Mean survival of megalopa reared under four different rearing systems.

Key: CW: Clear water, GW: Green water, RW: Recirculating water, and BW: Biofloc water systems. Significant differences were found among the treatments with different superscript letters (P < 0.05) in the bar chart.

Table 5.4: Mean body length, carapace width and wet weight of megalopa reared under different rearing systems

Treatment/Parameters	Body length (mm)	Carapace width (mm)	Wet weight (mg)
CW	4.76 ± 0.02	1.66 ± 0.01	3.80 ± 0.18
GW	4.76 ± 0.04	1.67 ± 0.02	3.85 ± 0.20
RW	4.79 ± 0.03	1.66 ± 0.02	3.99 ± 0.15
BW	4.71 ± 0.01	1.63 ± 0.00	3.37 ± 0.15

Data represented as mean ± SE; n=*3. Key: CW: Clear water; GW: Green water; RW: Recirculating water; and BW: Biofloc water systems.*

5.3.4 Survival of megalopa after stress tests

The survival of megalopa reared under the recirculating water system was low, so there were not enough megalopae for the stress tests. As a result, the ammonia and simulated transport stress tests were performed in the clear, green and biofloc water systems only. The survival of megalopa exposed to the LC_{50} value of ammonia-N is shown in Fig. 5.2. The survival of megalopa exposed to acute ammonia was reduced to 50% at the end of the test, but it was not significantly different ($P > 0.05$) among the various rearing systems. Conversely, the results of the simulated transport stress test were significantly different ($P < 0.05$) in terms of survival of megalopa from the different rearing systems (Fig. 5.3). After exposure to air during 24 hours of transport, survival of megalopa reared under the green water system was the highest but similar ($P > 0.05$) to the clear water system. The lowest survival was obtained in the biofloc water system at 24 hours. However, at the end of 48 hours, the survival of megalopa reared under the clear water system considerably dropped and was similar ($P > 0.05$) to the biofloc water system. The significantly ($P < 0.05$) highest survival of megalopa reared under the green water system was still obtained at the end of the test.

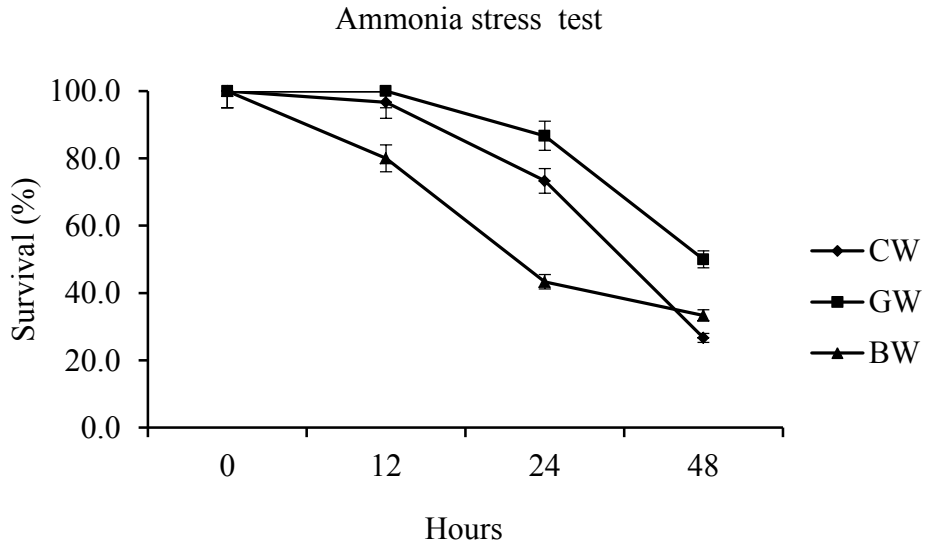


Figure 5.2: Mean survival of megalopa exposed to 48h LC50 value of total ammonia.

Key: CW: Clear water; GW: Green water and BW: Biofloc water systems.

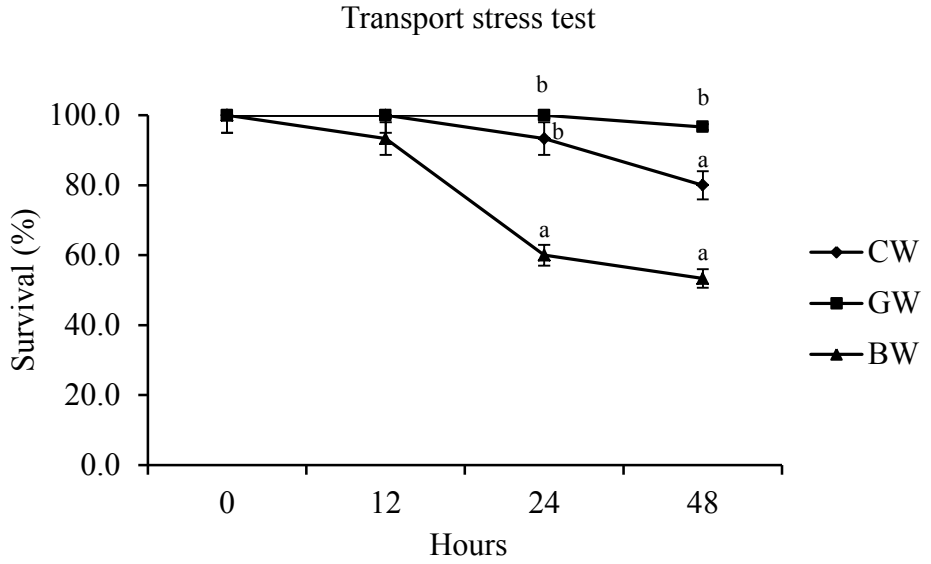


Figure 5.3: Mean survival of megalopa exposed to air during 48 hours of simulated transport.

Key: CW: Clear water; GW: Green water and BW: Biofloc water systems. Significant differences (P < 0.05) were found among the treatments with different superscript letters at the same time.

5.4 Discussion

Total ammonia nitrogen (TAN) in green water and nitrite in clear water systems increased (2.3 mg L^{-1} TAN and 5 mg L^{-1} nitrite, respectively) at the end of the trial period. Degradation of water quality could be due to the decay of *Chlorella* spp., which often reduces their concentration after 3 days of inoculation (Maddox & Manzi, 1976), resulting in high ammonia. Nitrite concentration was established by the nitrification process and increased by the culture period (Chand & Sahoo, 2006; Tacon et al., 2002). Based on a previous report of a 48h LC_{50} value of 45.0–52.0 mg L^{-1} TAN (equivalent to 3.3–3.8 mg L^{-1} NH_3-N) for zoeal stages (Neil et al., 2005), and a report of a 96h LC_{50} value of nitrite-N (25.5–69.9 mg L^{-1}) for mud crab (*S. serrata*) larvae (Seneriches-Abiera, Parado-Estepa, & Gonzales, 2007), and then application factor of 0.1 suggested by Sprague (1971) to predict a safe level of TAN and nitrite for rearing crab larvae. The safe levels were calculated by multiplying the LC_{50} with an empirical factor of 0.1, so it was 4.5-5.2 mg L^{-1} TAN –equivalent to 0.33–0.38 mg L^{-1} NH_3-N for zoeal stages and 2.5–6.9 mg L^{-1} nitrite for all larval stages. Thus, TAN (2.3 mg L^{-1} TAN - equivalent to 0.31 mg L^{-1} NH_3-N) in green water and nitrite (5 mg L^{-1} nitrite) in clear water systems was within the safe levels for crab larval development.

Our previous study (Quy et al., 2018a) showed that rotifers and *Artemia* enriched with probiotics improved survival and metamorphosis rate of megalopa. The finding of this study also confirms that survival of megalopa fed enriched rotifers and *Artemia* with probiotics could improve higher when they reared under green water system. Consistent with our study, an increased survival of post-larvae of giant freshwater prawn (*Macrobrachium rosenbergii*) was observed in a green water system (Lober & Zeng, 2009).

A higher survival of megalopa in the green water system could be due to the contribution of a beneficial nutrient from algae supplementation directly or indirectly through rotifers and *Artemia* (Lober & Zeng, 2009; Manzi, Maddox, & Sandifer, 1977). However, it is known that mud crab larvae (*S. serrata*) are carnivorous in nature (Genodepa et al., 2004a) and are thus unable to ingest and digest phytoplankton directly (Hassan et al., 2011). This indicates that algae from the green water system could indirectly contribute to the enrichment of nutrients through live food, leading to improved survival of megalopa. Our results are consistent with a previous study performed in the same species (Nghia et al., 2007a). The present study is also in agreement with Dan et al. (2016b) who found that *Artemia* fed digestible *Nannochloropsis* (broken cell walls) containing high levels of EPA improved survival of megalopa stage onwards in *Portunus tritubercalatus*.

Furthermore, it is established that discontinuation of supplementing phytoplankton (*Chlorella* or *Nannochloropsis*) resulted in the starvation of rotifers and *Artemia* (Dan et al., 2016c); alternatively, a combination of phytoplankton and *Artemia* in the larval culture tank could also induce starvation in *Artemia,* as *Artemia* cannot digest these phytoplankton (Sick, 1976) due to rigid cell walls (Gerken, Donohoe, & Knoshaug, 2013). Based on the above findings, phytoplankton supplementation could result in low survival of larvae as observed in *P. tritubercalatus* (Dan et al., 2016c). Contrary to this, Dan et al. (2016b) reported that when *Artemia*, rotifers and algae (*Chlorella* and *Nannochloropsis*) were supplied simultaneously in rearing water of *P. tritubercalatus* larvae, the digestion ability of *Artemia* improved significantly as they may have fed on these algae crushed by rotifers, leading to overcoming *Artemia* starvation. As result of this, brachyuran crab larval survival increased, and production was ultimately improved. This finding was in accordance with the results of the present study and was also supported by result of Hassan et al.

(2011) who found that the mud crab larvae reared under green water, having rotifer and *Artemia* as larval food, could obtain better survival.

The other beneficial effects of algae addition in green water is to contribute to improving megalopa survival. Algae as a substrate facilitate in creating a green background that reduces stress and increases feeding efficiency (Lober & Zeng, 2009). The grown algae also helps to maintain water quality (Tendencia et al., 2015) by removing nitrogenous metabolites out of the rearing water (Cheah & Ang, 1979; Cohen, Finkel, & Sussman, 1976). In addition, previous studies confirmed that algae in the green water system can secrete unknown bioactive chemicals that inhibit luminous bacteria, leading to increased survival (Huervana et al., 2006; Lio-Po et al., 2005).

Like the green water system, the biofloc water system can also provide more natural food through the floc particles such as bacteria, protozoa, phytoplankton, zooplankton (ciliates, rotifers, etc.) and nematodes (Manan et al., 2017; Ray et al., 2010). Previous studies showed that biofloc acts as potential food for mussels (*Perna viridis*), white shrimp (*L. vannamei*) (Ekasari et al., 2014a) and pink shrimp (*Farfantepenaeus paulensis*) (Emerenciano, Ballester, Cavalli, & Wasielesky, 2011). According to an estimate by Burford, Thompson, McIntosh, Bauman, and Pearson (2004), 18–28% of nitrogen retention of the *L. vannamei* was contributed by microbial flocs, but in the present study, it is unknown what extent of floc particles contributed to daily food intake of crab larvae. It is speculated that leftover *Artemia* have grown up to adult stage in the biofloc water system. This phenomenon was not seen in the rest of the systems, though application of the feeding regime was similar in all systems. Additionally, poor quality of biofloc in terms of nutritional composition, depending upon supplementing carbon sources (Crab, Chielens, Wille, Bossier, & Verstraete, 2010a) as well as floc size (Ekasari et al., 2014a), might have induced low survival in this study. Moreover, high presence of adult *Artemia* and microorganisms in biofloc water might have induced stress to the larvae and degraded rearing water quality (Ballester et al., 2017), leading to lower survival of megalopa compared to the green water system.

The recirculating water system has been successfully employed in marine fish (Gelfand et al., 2003) and in shrimp culture (Beard & Wickins, 1980; Mishra et al.,

2008; Reid & Arnold, 1992) in terms of maintaining good water quality (Wickins, 1976) and increasing production (Esparza-Leal, Cardozo, & Wasielesky, 2015; Piamsak & Somkiate, 1980). However, the recirculating water system showed the lowest survival of megalopa in the present study, which might be due to low availability of suspended solid wastes (rotifer excrements) resulting to poor nutrient availability in the rearing water that might have induced starvation of *Artemia*. Likewise, rotifers may have been trapped in the physical filter making them unavailable to zoeae. Feeding starved *Artemia* or only *Artemia* led to lower survival of *S. paramamosain* (Zeng et al., 2004), *S. serrata* (Ruscoe et al., 2004b) and *P. tritubercalatus* larvae (Dan et al., 2016b). In addition, the presence of three long spines on the carapace of early crab larval stages make them vulnerable to physical damage, as they might be entrapped on the mesh screen in the recirculating system (Nghia et al., 2007a). Furthermore, reused water in the recirculating water system might have low concentration of trace elements or deposited metabolites, pheromones and toxic substances that resulted in prolonged larval development and low survival (Mallasen & Valenti, 1998). Based on these, it is speculated that either a decrease of trace element or deposition of waste products in the rearing water could have induced a delay in metamorphosis and thus increased mortality of mud crab megalopa.

To evaluate the quality and resistance of megalopa to environmental stressors, the ammonia stress test was performed, which is a sensitive indicator to determine larval quality based on survival as early as Z4 (Cavalli et al., 1999). However, this test showed no significant difference in the survival of megalopa reared under different rearing systems in the present study. Significantly differences among the replicates within the treatment indicated that cannibalism affected the cumulative mortality of megalopa rather than ammonia toxicity. Therefore, the ammonia stress test in the present study failed to determine the quality of megalopa reared under different rearing systems.

In contrast, the results of the simulated transport stress test revealed that megalopae reared under the green water system had higher quality than those reared under the clear and biofloc water systems as reflected by higher resistance to the air exposure until the end of the 48 hours of transport. During the transport period, a starved animal might use the accumulated nutrients, including protein and lipid, to supply the

metabolic energy demand to keep them alive (Anger, 1986; Dawirs, 1983; Ikeda, 1971). This finding is consistent with our result, wherein high nutrients accumulated in previous larval stages in the green water system could increase survival of megalopa during the transport period. In addition, Fotedar, Tsvetnenko, and Evans (2001) found that low energy production could compromise the immune system of the rock lobster (*Panulirus cygnus*) when they were exposed to air. Thus, low nutrient accumulation during larval stages in clear water and biofloc water systems may also result in low resistance of megalopa to air exposure during transport, resulting in a low survival rate.

5.5 Conclusion

To summarise the above findings, the results demonstrated that the green water system obtained beneficial outcomes in mud crab larval rearing from zoea 1 to the megalopa stage, as reflected by improved survival and quality of megalopa. The study also revealed that the recirculating water system had adverse effects on live food and megalopa survival. These novel findings have important implications for selecting a rearing water system for successful operation of a mud crab hatchery.

Section II

Production of Crablets

CHAPTER 6: Selection of locally available diets for rearing *Scylla paramamosain* megalopa to crablet stage

6.1 Introduction

Mud crab (*Scylla* spp.) aquaculture has been practiced for many years in Southeast Asia (Keenan, 1999a; Petersen et al., 2013). *Scylla paramamosain*, one of four species of the *Scylla* genus (Keenan, 1999b), is the dominant species currently cultured in Vietnam (Lindner, 2005; Petersen et al., 2013). Although the seed production technique is well documented for mud crabs (Davis, 2003; Nghia et al., 2007a; Thirunavukkarasu et al., 2014), hatchery production is entirely dependent on the provision of expensive live food including *Artemia* and rotifers (Southgate & Partridge, 1998). Due to the cost and variable nutrient content of rotifers and *Artemia* (Kovalenko et al., 2002; Sorgeloos, 1986), numerous studies have attempted to replace them with cheaper and more readily available food sources.

Previous studies have investigated the partial substitution of *Artemia* with dried *Acetes* spp. (Quinitio et al., 2001; Quinitio et al., 2002; Williams et al., 1999), macerated shrimp and clam meat (Marichamy, 1996), trash fish (Shelley & Lovatelli, 2011), and chopped mussels (Jantrarotai et al., 2004; Rodriguez et al., 2001) for rearing mud crab megalopa to crablets.

Similarly, partial or a total substitution of live food with formulated feed has been explored in crab hatcheries to reduce the cost of production and supplement essential nutrients that are deficient in live food. Microbound diets (MBD) have been used to replace *Artemia* in larval feed for megalopa of *S. serrata* (Genodepa et al., 2004b; Holme et al., 2006b), swimmer crab (*Portunus pelagicus*) (Castine et al., 2008), and spider crabs (*Maja brachydactyla*) (Andrés et al., 2011). Therefore, formulated feed is more reliable and cost-effective when it is formulated with either globally acceptable or locally available ingredients (Kovalenko et al., 2002; Nguyen et al., 2014). However, the ingredients of MBD such as squid meal and dried rotifer meal are not locally available.

The egg custard diet, first developed by Ling (1969), is prepared with a simple mixture of readily available yolk and white of chicken eggs, yeast powder, and vitamins. The hand-prepared egg custard diet was used as an alternative to expensive live food or commercial feed (Boonyaratpalin & New, 1995; Hien, Hai, Phuong,

Ogata, & Wilder, 2005). The nutritional status of this diet has been significantly improved by the inclusion of lipid and lecithin sources (Alam, Ang, & Begum, 1995; Hien et al., 2005), shrimp, clam and fish meat (Murthy et al., 2008), squid, bivalve and poultry byproduct meal (Sin, Nurhusna, & Shapawi, 2016), and various binder sources (Kovalenko, 2001). The egg custard diet can be produced with specific particle sizes for each larval stage (Hien et al., 2005), and has been shown to successfully increase larval survival and metamorphosis of the giant freshwater prawn (*Macrobrachium rosenbergii*) (Hien et al., 2005; Murthy et al., 2008).

Acetes spp. are an important live food that are cheap and readily available in local hatcheries of Vietnam. However, information regarding the use of *Acetes* spp. in mud crab hatcheries is scarce, despite the success that has been achieved raising megalopa to crablets on this food source. In addition, the availability of *Acetes* spp. is seasonal, leading to an inconsistent supply to hatcheries. Thus, mixing *Acetes* with other dietary ingredients such as fresh shrimp meat, egg custard, and/or commercial feed may be considered.

Cannibalism accounts for up to 50% of the total mortality in crab nurseries (Davis, 2003), which may consequently increase the growth performance of surviving crabs (Møller, Lee, Paterson, & Mann, 2008). Therefore, the present study aimed to evaluate the efficacy of seven different diets including live *Acetes* spp. (LA); minced shrimp meat (MSM); locally formulated feed (LFF); commercial feed (CF); and combination of three diets of LA with MSM, LFF, and CF (LA + MSM, LA + LFF, and LA + CF). The efficacy of the culture was measured by evaluating the survival, growth, and duration from megalopa to first crablet stage. All the diets were tested under two different culture conditions; namely, individual and communal culture.

6.2 Materials and methods

6.2.1 Source of brood stock and megalopa*

Mature females of *S. paramamosain* weighing 460 ± 45 g were collected from extensive shrimp farms in the Nam Can district, Ca Mau Province, Vietnam

[*] *Though it is recommended that megalopa used in this trial should come from the green water culture system as previous experiments have indicated that green water culture results in higher survival, however, due the mass requirements of megalopa, rainy season and facilities available at the experiment site did not permit me to use green water culture system.*

($8^0 45' 00'' N$; $105^0 01' 38'' E$) and transported to nearby hatchery. Fattening of mature and berried females was performed according to our previously published protocol (Quy et al., 2018b).

Newly hatched zoeae 1, from a single spawner, were collected and bathed in 0.1 µL L^{-1} iodine solution for 30 seconds prior to distribution in a 6,000-L concrete tank at 100 larvae L^{-1} for the mass megalopa production. The larvae were fed enriched L-strain rotifers with commercial probiotics (Lymnozyme FT-2B, Cisbay, USA) at a density of 20–40 individuals mL^{-1} from the first day until Z2 appeared. From Z2 to Z5, they were continuously fed a mixture of probiotic-enriched rotifers and *Artemia* at 10 and 2 individuals mL^{-1}, respectively. From Z5 onwards, enriched rotifers were replaced by enriched *Artemia* at 4 individuals mL^{-1}. The rotifers and *Artemia* were both enriched with probiotics according to our previously published protocol (Quy, Fotedar, & Thy, 2018a). Enriched *Artemia* were fed to crab larvae twice daily at 6 am and 6 pm, while enriched rotifers were fed once daily at 12 pm. The tank was strongly aerated to prevent settlement of larvae and live food. During the culture period, 40% of the water in the mass culture tank was exchanged by disinfected seawater on 8, 11, and 14 days after hatching (DAH). The photoperiod was maintained at 12 hours of natural light at day time and 12 hours of artificial light by a 40 W fluorescent lamp at night time. The pH of culture seawater was adjusted to approximately 8.3 ± 0.2 by adding $CaCO_3$ and/or $NaHCO_3$. Normally, megalopae appeared at 15 DAH under the culture conditions described above. Newly moulted megalopae were collected the next day for the trial.

6.2.2 Preparation and proximate composition of diets

The composition of LFF was based on the formula of egg custard diet used for rearing *M. rosenbergii* described by Hien et al. (2005) and modified to use local ingredients. The basic LFF consisted of fresh shrimp meat (30.0% total weight of the diet), eggs yolk (41.7%), milk powder (Anlene Gold milk, New Zealand; 23.8%), soybean oil (Truong An company, Vietnam; 3.0%), lecithin (Tan Sao A company, Vietnam; 1.5%) and vitamin C (ELCO C-100k, Germany, 200 mg kg^{-1} of diet). Dry ingredients (milk powder and vitamin C) and moist ingredients (fresh shrimp meat, eggs yolk, soybean oil, and lecithin) were thoroughly mixed in separate containers. All ingredients were then combined and thoroughly mixed with a blender (HR2195/00, Philips, China) until a smooth consistency was achieved. The

homogenized ingredients were placed into a container and steamed for 20 minutes, then allowed to cool to room temperature and stored at 4 °C until use. Prior to feeding, LFF was manually crushed through a sieve to produce the desired particle size of 400–800 μm, which has been identified as the optimal size for megalopa (Genodepa et al., 2004a). Feed particles were collected using a net with a smaller mesh size (400 μm). The commercial feed used in the experiment was shrimp larval feed (Lansy-Post) that is made in INVE, Thailand.

Minced shrimp (*Metapenaeus ensis*) meat was prepared from fresh shrimp meat which had been freshly collected from an extensive shrimp farm, and placed in boiling water for 5 minutes then immediately cooled by adding fresh water. Shrimp meat was exposed to sunlight for 1 hour to reduce its moisture content (to a final content of 48.2 ± 0.2%) and then ground with a blender. These particles were pushed through a sieve to obtain 400–800 μm particles, and stored in a freezer until further use.

Live *Acetes* (*Acetes* spp.) with body length of 10 ± 0.5 mm were collected daily using a scoop net from an extensive shrimp pond and stored in a 1,000-L fibre-glass tank under aeration until use.

Proximate analysis of all the feeds used for the experiments were determined according to standards methods (AOAC, 1990) as shown in Table 6.1.

Table 6.1: Proximate compositions of local formulated feed, commercial feed, minced shrimp meat and live *Acetes*

Proximate composition (%) (based on dry weight)	Diets			
	CF (Lansy-Post)	LFF	MSM	LA
Crude protein	46.5	32.9	59.9	58.6
Crude lipid	13.5	23.0	3.0	7.7
Crude fibre	0.6	0.2	0.5	3.2
Ash	9.1	5.4	4.4	11.9
Moisture	3.3	45.7	48.2	83.4

Key: CF: Commercial feed; LFF: Locally formulated feed; MSM: Minced shrimp meat; LA: Live Acetes.

6.2.3 Experimental setup

6.2.3.1 Communal culture

For seven test diets, under communal rearing conditions, 21 earthen dugout holes of 60 × 60 × 20 cm (width × length × depth) were lined with thin plastic at sides and bottom and filled with 80% of the total volume with disinfected seawater. Each hole was stocked with 250 one-day-old megalopae carefully transferred from the mass megalopa culture tank. The holes were set up with static clear seawater that was exchanged with fresh disinfected seawater at a rate of 50% every three days. For the treatments feeding single diet, megalopae were fed LA at approximately 20 individuals L^{-1}; while MSM, LFF, and CF were provided in excess of 2 mg L^{-1} as recommend by Genodepa, Southgate & Zeng (2004) to ensure that feed was always available at the bottom of earthen dugout holes. For the treatments feeding mixed diets, the density of LA fed to megalopa was reduced from 20 to 10 individuals L^{-1} and the quantity of MSM, LFF, and CF from 2 to 1 mg L^{-1}. All diets were fed twice daily at 9 am and 6 pm. Leftover static feed was siphoned out before commencement of the next feed, and the supply of LA to all holes was visually adjusted to achieve the density described above. All the earthen dugout holes were provided with moderate aeration by air pump. The dugout holes were created in a greenhouse to avoid high temperatures by reducing direct exposure to sunlight. Natural conditions of photoperiod and temperature were maintained. Dissolved oxygen and total ammonia were measured using an oxygen meter (model HI 9147; Hanna, Romania) and ammonia meter (model HI 96715; Hanna) every three days prior to water exchange. The pH of disinfected seawater was adjusted to approximately 8.3 ± 0.2 by adding $CaCO_3$ and/or $NaHCO_3$ before being supplied to the earthen holes.

6.2.3.2 Individual culture

For individual culture, 20 plastic beakers (0.5 L) were randomly arranged for each diet. Thus, 140 plastic beakers were filled with 250 mL of disinfected seawater and submerged in a 6,000 L concrete tank maintained at a constant temperature of 28 ± 1 ^{o}C. Individual megalopa was transferred from mass megalopa production tank and stocked into each beaker. Every morning, the water in each beaker was completely exchanged with disinfected seawater of similar temperature and salinity. At the time of water exchange, dead megalopa or newly moulted first crablets in each beaker

were recorded. Megalopae were fed once daily up to satiation after water exchange with the same diets used for communal culture. The experiment was terminated when all megalopae had either moulted into the first crablet stage or died.

6.2.4 Survival and growth parameters

The survival of crablets was computed using the following equation:

$$\text{Survival (\%)} = \frac{\sum \text{First crablets} \times 100}{\text{Initial number of megalopae stocked}}$$

At the end of the communal culture experiment, five newly metamorphosed crablets from each replicate were collected and preserved in 10% formalin to measure their carapace lengths, widths, and wet weights. Carapace length and width were measured using a ruler scale in the eyepiece of a microscope (Olympus CX21, USA). Crablets were then dried on tissue paper for wet weight measurement using a 0.0001g precision analytical balance (PA 214-Ohaus, USA).

6.2.5 Statistical analyses

All data were subjected to one-way analysis of variance (ANOVA) to test for significant differences between the dietary treatments. The data were then subjected to Tukey's multiple range test at the 0.05 level of significance. One way ANOVA and Tukey's multiple range test were also used to test the differences in survival gap of crablets between individual and commune culture within the different diets. Data of survival presented in percentages were arcsine-square root-transformed using the table described by Zar (2010) prior to analysis. All statistical analyses were computed using SPSS for Windows, version 24.0.

6.3 Results

6.3.1 Environmental parameters

Water temperature in the earthen dugout holes ranged from 29.0 to 30.5°C throughout the culture period; while salinity, DO, and pH ranged from 20 to 25 g L^{-1}, 6.9 to 8.3 mg L^{-1} and 8.3 to 9.0, respectively. Total ammonia during the experimental period was maintained below 1.5 mg L^{-1} (Table 6.2).

Table 6.2: Total ammonia in various diets treatments during the experimental period

Parameter	TAN (mg L^{-1})		
Treatments/days	1st	4th	7th
CF	0.0 ± 0.00	0.7 ± 0.02	1.2 ± 0.11
LFF	0.0 ± 0.00	1.0 ± 0.01	1.1 ± 0.07
MSM	0.0 ± 0.00	1.2 ± 0.04	0.7 ± 0.07
LA	0.0 ± 0.00	0.3 ± 0.03	0.5 ± 0.01
LA + CF	0.0 ± 0.00	0.7 ± 0.03	1.2 ± 0.03
LA + LFF	0.0 ± 0.00	0.8 ± 0.08	0.8 ± 0.06
LA + MSM	0.0 ± 0.00	0.7 ± 0.06	1.0 ± 0.07

Key: CF: Commercial feed; LFF: Locally formulated feed; MSM: Minced shrimp meat; LA: Live Acetes; LA + CF: Live Acetes and commercial feed; LA + LFF: Live Acetes and locally formulated feed; LA + MSM: Live Acetes and minced shrimp meat.

6.3.2 Survival of crablets

Survival rates from megalopa to crablet stage fed different diets under individual and commune culture conditions are illustrated in Fig. 6.1. The survival rate was high (85.0–100.0%) when reared individually, but survival of the crablets in commune culture ranged from 47.9 to 87.5%. In communal culture, the highest survival rate observed for megalopae fed LA. This treatment diet was significantly higher ($P < 0.05$) than those of MSM, LFF, or CF; but it was not significantly different to those of LA + MSM, LA + LFF, or LA + CF ($P > 0.05$). The differences in survival rates of crablets on the same diets between individual and commune culture are also illustrated in Fig 6.1. The smallest difference was observed for megalopa that were fed with LA, followed by LA + MSM, LA + LFF, and LA + CF; and the largest difference was observed for megalopa that were fed with MSM, LFF, or CF. However, the differences in the survival gap between individual and commune culture within the different diets were not significant ($P = 0.058$).

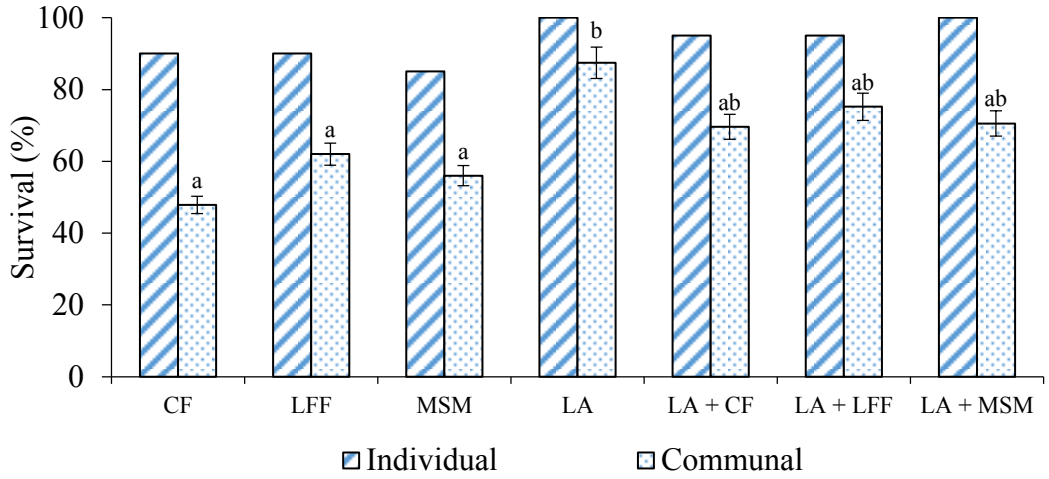


Figure 6.1: Survival of crablets fed various diets under individual and communal culture.

Key: CF: Commercial feed; LFF: Locally formulated feed; MSM: Minced shrimp meat; LA: Live Acetes; LA + CF: Live Acetes and commercial feed; LA + LFF: Live Acetes and locally formulated feed; LA + MSM: Live Acetes and minced shrimp meat. Different superscript letters indicate significant differences ($P < 0.05$) in the bars with the same design.

6.3.3 Growth performance of crablets

Crablets resulting from megalopae fed LA were measured to have the largest carapace length, which was significantly different ($P < 0.05$) from the carapace length of crablets resulting from megalopae fed MSM, LFF, LA + MSM or LA + CF (Table 6.3). Meanwhile, the carapace widths and wet weights of the crablets were not significantly different ($P > 0.05$) between any of the diets (Table 6.3).

Table 6.3: Carapace length and width, and wet weight of the first crablets to emerge from megalopae fed various diets

Diet	Carapace length (mm)	Carapace width (mm)	Wet weight (mg)
CF	2.71 ± 0.01^{ab}	3.37 ± 0.08	7.87 ± 0.28
LFF	2.70 ± 0.02^{a}	3.35 ± 0.01	7.72 ± 0.03
MSM	2.69 ± 0.02^{a}	3.32 ± 0.04	7.56 ± 0.21
LA	2.77 ± 0.02^{b}	3.49 ± 0.06	8.11 ± 0.22
LA + CF	2.65 ± 0.01^{a}	3.30 ± 0.03	7.65 ± 0.09
LA + LFF	2.71 ± 0.01^{ab}	3.31 ± 0.03	7.61 ± 0.06
LA + MSM	2.70 ± 0.01^{a}	3.30 ± 0.02	7.85 ± 0.08

Key: CF: Commercial feed; LFF: Locally formulated feed; MSM: Minced shrimp meat; LA: Live Acetes; LA + CF: Live Acetes and commercial feed; LA + LFF: Live Acetes and locally formulated feed; LA + MSM: Live Acetes and minced shrimp meat. Different superscript letters in the same column indicate significant differences ($P < 0.05$).

6.3.4 Proportions of megalopa and newly moulted crablets

The proportions of megalopa and newly moulted crablets recorded for different dietary treatments from day 6–10 are shown in Fig. 6.2. In all treatment groups, megalopae started moulting into the first crablet stage on day 7. In the LA and LA + CF-fed groups, all megalopae synchronously moulted into the first crablet stage one day earlier than was observed for the other dietary treatments. Megalopae in all treatment groups moulted to the first crablet stage by day 10.

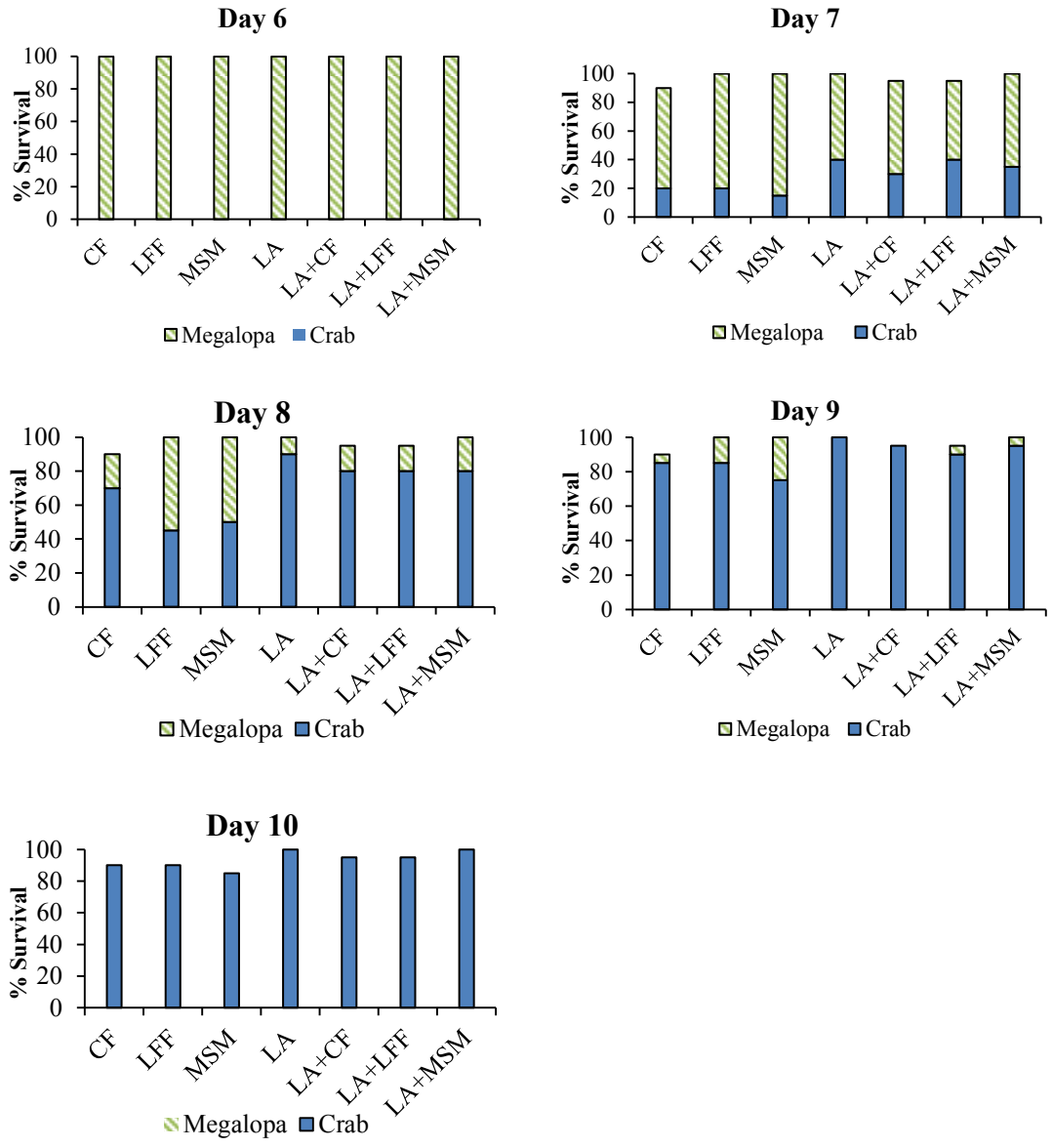


Figure 6.2: Survival and proportions of megalopa and first crablet stage from day 6 to 10 for megalopae fed with various diets.

Key: CF: Commercial feed; LFF: Locally formulated feed; MSM: Minced shrimp meat; LA: Live Acetes; LA + CF: Live Acetes and commercial feed; LA + LFF: Live Acetes and locally formulated feed; LA + MSM: Live Acetes and minced shrimp meat.

6.4 Discussion

Total replacement of *Artemia* with MBD (Genodepa et al., 2004b; Holme et al., 2006b) or partial replacement of live food with dried polychaeta mud worm (*Marphysa* spp.), dried *Acetes* shrimp (*Acetes* spp.) (Williams et al., 1999), or macerated prawn flesh (Heasman & Fielder, 1983) have been investigated for *S. serrata* megalopa. The results of these studies suggested that these feeds fed either alone or as part of a combination diets could be used to successfully raise megalopa to the crablet stage. In the present study, crablets resulting from megalopae fed with LA, MSM, LFF, CF, or a combination diet of LA with MSM, LFF, or CF in either individual or communal culture conditions resulted in higher or similar survival than previously reported (Table 6.4). Thus, we have shown that all the test diets in this study are valid alternatives to MBD or *Artemia* for raising megalopa to the crablet stage. Furthermore, our observations have confirmed that the feeding behavior of *S. paramamosain* is more raptorial from the megalopa stage onwards, highlighting their ability to accept a wide variety of food types.

Table 6.4: Comparison of survival of newly moulted first crablet stage fed different diets from others studies

Species	Age of megalopa*	Diets	Rearing system	Survival (%)	References
S. serrata	2nd megalopa	*Artemia*	Communal	38.9	Williams et al. (1999)
		Artemia and dried *Acetes* shrimp	Communal	40.0	
		Artemia and dried mud worm	Communal	57.8	
S. serrata	2nd megalopa	*Artemia*	Communal	53.0	Heasman and Fielder (1983)
		Artemia and macerated prawn	Communal	87.0	
S. serrata	2nd megalopa	*Artemia*	Communal	<20.0	Genodepa et al. (2004b)
		MBD	Communal	<20.0	
		Artemia	Individual	90.0	
		MBD	Individual	90.0	
S. serrata	2nd megalopa	MBD	Individual	46.7–60.0	Holme et al. (2006b)
S. paramamosain	1st megalopa	CF, LFF, MSM, and LA as individual or combination diets of LA + CF, LFF, or MSM	Individual	85.0–100.0	Present study
			Communal	47.9–87.5	

*Key: *Age of megalopa measured from moulting day; CF: Commercial feed; LFF: Locally formulated feed; MSM: Minced shrimp meat; LA: Live Acetes; MBD: Microbound diet.*

In our study, survival of crablets reared under commune culture was lower than those reared under individual culture. This difference could be associated with

cannibalism, which increased greatly from the megalopa stage onwards (Quinitio et al., 2001). The low survival of *S. serrata* crablets due to cannibalism has been reported in communal culture, while survival of the crablets reared under individual culture with similar diets were up to 90% (Genodepa et al., 2004b). However, in the present study, the survival gap between individual and communal culture within the different diets were not significantly different indicating that the effect of cannibalism did not affect our evaluation of different diets.

Genodepa et al. (2004b) and Holme et al. (2006b) reported that mud crab megalopae undergo a weaning process during the first few days following a complete change in feeding regime from live food to MBD. During the weaning period, cannibalism may increase due to starvation, leading high mortality rates (Genodepa et al., 2004b). However, in the present study, the weaning process does not appear to affect the survival of megalopa fed MSM, LFF, or CF as reflected by the similar levels of cannibalism between the groups. The megalopa used in the present study were reared on enriched rotifers and *Artemia* with probiotics until the late zoeal stage. Thus, a continuous accumulation of nutrients derived from both live foods during the larval development period (Quy et al., 2018b) may exert a beneficial carry-over effect on the megalopa stage. This energy reserve may support them during the weaning period, resulting to less cannibalism and effective weaning to MSM, LFF, and CF.

In decapod larvae, the survival and moulting success from megalopa to crablet stage are considered primary parameter to assess the efficacy of diets (Genodepa et al., 2004b). In the present study, LA-fed megalopa showed higher survival of newly moulted crablets than those fed MSM, LFF, or CF. We could observe that the inactive diets sank to the bottom of the container, which is less attractive to the megalopae than live food did. Further, MSM, LFF, and CF could increase particle sedimentation leading to degradation of water quality and augmentation of bacterial load, both of which may result in low survival of crablets (Quinitio et al., 1999; Williams et al., 1999). Crustacean larvae can capture food guided by their both chemo- and mechanoreceptors (Cox & Johnston, 2003). In the present study, similar to a carnivorous marine copepod (Yen, 1982) and to megalopa of *P. pelagicus* (Castine et al., 2008), movement of LA, more so than any other inactive food, can elicit and supersede the capture response of megalopa. This indicates that vibrations

caused by the swimming of live *Acetes* stimulated hunting behavior in mud crab megalopae.

The early mud crab larvae have limited abilities to digest formulated feed (Andrés et al., 2011; Genodepa et al., 2004b), which may have resulted in less synchronous moulting into first crablets. Asynchronous moulting often exacerbates cannibalism (Quinitio et al., 2001), and consequent low larval survival.

Artemia is the most important live food that results in synchronized moulting (Genodepa et al., 2004b) and the highest overall survival rares of megalopa (Holme et al., 2006b). Our study also showed that as increased survival of first crablets from megalopa that were fed LA exhibited more synchronous moulting and shorter development time to crablets than those fed MSM, LFF, CF, LA + MSM, or LA + LFF. This demonstrates that LA is one of the most preferable diets for nursery rearing of megalopae into crablets.

A combination of different types of foods may be beneficial in mud crab rearing. For example, a combination of shrimp commercial feed and live food (rotifers and *Artemia*) has been suggested to improve the growth and survival of mud crab larvae (Quinitio et al., 1999). In this combination diet, the commercial feed may provide essential fatty acid (n-3 HUFA) to mud crab larvae directly or indirectly serve as enrichment for rotifers and *Artemia*. Similarly, Williams et al. (1999) also found that feeding a combination of either dried mud worms and *Artemia* or *Acetes* shrimp and *Artemia* to megalopa resulted in higher survival of first crablets than individual feeding of dried mud worms or *Acetes*. In addition, a mixture of live food and MBD has been seen to reduce the stresses imposed on *S. serrata* during the weaning process, resulting in consistently high survival of megalopa (Genodepa et al., 2004b; Holme et al., 2006b). However, in our study, the combination of LA with MSM, LFF, or CF did not show any additional benefits to LA alone. This could be because LA fulfills the nutritional requirements for megalopa development.

There is no published information indicating that live feed can increase the growth performance of *S. paramamosain*. However, studies on other species including *S. serrata* (Holme, Zeng, & Southgate, 2006a) and *P. pelagicus* (Castine et al., 2008) have produced contradictory results, indicating that live food increases growth performance. Whilst our results found carapace length to increase when a diet of LA

was provided, however, carapace length cannot be considered as a potential growth indicator (Lober & Zeng, 2009).

6.5 Conclusion

In conclusion, all the diets that were tested in this study are appropriate for the nursing phase of megalopa, as reflected by successful moults and high survival of first crablets. However, LA resulted in enhanced survival, synchronous moulting, and decreased duration of metamorphosis time leading into the first crablets, and was therefore the most suitable diet for megalopae. The outcomes of the present study contribute insight to the current practices of using locally available and economical feed material.

CHAPTER 7: Evaluating different water rearing systems to raise crablets of mud crab (*Scylla paramamosain*) from megalopae

7.1 Introduction

Mud crab (*Scylla paramamosain*) aquaculture has been practised for many years in Southeast Asia (Petersen et al., 2013). In South Vietnam, preliminary studies on rearing *S. paramamosain* larvae, a dominant species of the *Scylla* (Le Vay et al., 2001), has been reported since 1993 (Dat, 1999). Mud crab culture, as a promising alternative to shrimp culture, contributes a large proportion of income and fresh food for coastal communities (Petersen et al., 2013). For a sustainable production, a mud crab farmers rely on wild and hatchery-raised seeds (Keenan, 1999a). However, the requirement for seedstocks has not been met due to high demand in recent years. Further, the natural collection of wild seeds has declined significantly due to over-exploitation, diminishing mangrove habitats (Le Vay et al., 2001) and pollution (Ma et al., 2014), whereas production of hatchery-produced crablets is unstable due to effluent water pollution and salinity drop during the rainy season.

In aquaculture, the type of rearing system plays a vital role, as it may cause stress and affect the health of the culture species (Ardiansyah & Fotedar, 2016). Adverse impacts of stress related to extreme variations beyond acceptable ranges for water parameters may influence the quality of crablets by reducing immunity of the animal. A promising rearing practice with reduced water exchange that can minimize the introduction of pathogens with the incoming water and increase biosecurity for aquaculture operation (Krummenauer, Samocha, Poersch, Lara, & Wasielesky, 2014) has emerged. This offers less dependence on the natural water source (Mongirdas, Žibienė, & Žibas, 2017). The zero or minimal seawater exchange management practice has contributed to reducing the operational costs of larval culture (Mallasen & Valenti, 1998; Piamsak & Somkiate, 1980) and improved the sustainability and environmental compatibility (Izquierdo et al., 2006). The beneficial impacts of zero or minimal water exchange has been documented for the culture of *Macrobrachium rosenbergii* (Ballester et al., 2017), *Litopenaeus vannamei* (Krummenauer et al., 2014; Ray, Seaborn, Vinatea, Browdy, & Leffler, 2012; Xu, Morris, & Samocha, 2016), *Penaeus monodon* (Arnold et al., 2009), *Farfantepenaeus brasilliensis* (Emerenciano et al., 2012) and *S. paramamosain* (Nghia et al., 2007a).

Stress tests, that are rapid, inexpensive and simple, have been commonly used to evaluate the larval and the post larval quality in crustacean hatcheries (Samocha et al., 1998). Air exposure during simulated transport conditions and ammonia exposure can be used as stressors to evaluate crustacean quality (Fotedar & Evans, 2011; Samocha et al., 1998) by evaluating the survival response.

In our previous study (unpublished), impact of different rearing systems was carried out in the first phase from zoea to megalopa stage. Additionally, a study related to a rearing system for zoeal stages (hatchery phase) was reported by Nghia et al. (2007a), but information on the impact of rearing system on the mud crab nursing phase, from megalopa to crablet stage, is not available. Therefore, the present study was aimed to assess the water quality, survival, growth and quality of crablets using different rearing systems with zero water exchanges to culture megalopa to crablet stage.

7.2 Materials and methods

7.2.1 Source of brood stock and megalopa

Source of brood stock and megalopa were prepared as described in Section 6.2.1.

7.2.2 Preparation of green water, biofloc water and recirculating water system

Green and biofloc tanks were prepared as described in Section 5.2.2.1 and 5.2.2.2. When the algae concentration in green water tank reached 2–4 million mL^{-1} as well as floc particles in the biofloc tank were built up, green and biofloc water in these tanks were used to set up the experiment.

The closed recirculating water system consisted of three components, one earthen dugout hole of 60 cm x 60 cm x 20 cm (width x length x depth) defined as the rearing tank, one 60-L plastic biofilter bucket and one 10-L plastic physical filter bucket. In the rearing tanks, all sides along the bottom were placed with thin plastic sheets to avoid seepage of water. In the biofilter bucket, oyster shell (*Crassostrea belcheri*) substrates were filled up to 25% of bucket volume for growth of beneficial nitrifying bacteria (*Nitrosomonas* and *Nitrobacter)*, while in the physical filter bucket, cotton was placed inside for physical filtration. Water from the physical filter bucket was pumped into the biofilter bucket with a submersible pump (8W, aquarium pump, China) and then the water flowed through the rearing tank and finally returned

to the physical filter bucket by gravity. The water was filtered by cotton in physical bucket before being pumped into biofilter bucket again. The water was pumped from the physical filter bucket into the biofilter bucket with the flow rate adjusted to approximately 2 L minute^{-1}. Two weeks prior to the commencement of the trial, nitrifying bacteria in the recirculating water system was built up as per the established procedure performed described in Section 5.2.2.3.

7.2.3 Experimental design

For all the experiments, 12 earthen dugout holes of 60 cm x 60 cm x 20 cm (width x length x depth) were set up in a greenhouse. Each earthen dugout hole was lined with thin plastic at their sides, and the bottom was filled with 50 L of rearing water. In the green water system, algae were maintained at a density of 10^5 cell mL^{-1} as suggested by Simon (1978) for marine shrimp (*P. stylirostris* and *L. vannamei*). The algae were added only once at the commencement of the experiment, and the concentration was calculated using the method suggested by Lober and Zeng (2009).

The biofloc water system was prepared by mixing 10 L of matured biofloc collected from a biofloc tank and 40 L of disinfected seawater into each earthen dugout hole. Molasses was added into the biofloc water system at the commencement of the experiment and once the total ammonia in the rearing water was more than 1.0 mg L^{-1}. The molasses dose to convert total ammonia from the water into bacterial biomass was calculated based on previous reports by Avnimelech (1999) and Ebeling et al. (2006). The recirculating water system was prepared as described in Section 7.2.2.

Two hundred fifty active one-day-old megalopae were transferred from the mass culture tank to each earthen dugout hole, and were fed mixed diet of locally formulated feed (LFF) at a rate of 1 mg L^{-1} and live *Acetes* (LA) at 10 indviduals L^{-1}, twice daily at 9 am and 6 pm. All the earthen dugout holes were provided with moderate aeration by air pump. The water from the green, recirculating and biofloc systems was not exchanged during the experimental period; however, these systems were compensated for water loss by evaporation. The water in the clear water system (as a control system) was exchanged at a rate of 50% every three days with disinfected seawater. During the culture period, the temperature and photoperiod were maintained similar to natural conditions. The DO was maintained at more than 7.0 mg L^{-1} by aeration and pH was maintained between 8.3 and 8.7 by adding

agricultural lime. Total ammonia nitrogen (TAN) was measured using an ammonia meter, model HI 96715 (Hanna, Romania), while nitrite was measured using a nitrite test kit (Sera, Germany). These parameters were measured every three days and before water were exchanged in the clear water system.

7.2.4 Survival and growth parameters

The survival and growth of crablets were computed as described in Section 6.2.4.

7.2.5 Crablet quality parameters

To determine the quality of crablets reared under different water systems, 20 newly moulted crablets from each earthen dugout hole were collected and divided into two groups. Ten of the first group was exposed to air for 48 hours of a simulated transport stress test, whereas another ten was exposed to 96 hours of an ammonia stress test.

7.2.5.1 Simulated transport stress test

In the bottom of twelve plastic trays (20 cm x 25cm x 4 cm), wet thin nylon nets of mesh size < 2 mm, were placed as followed during transport for the purpose of the sale of crablets. Each tray was stocked with ten newly moulted crablets. All trays were placed indoors with stable temperature of 28 ± 1 ^{o}C and they were not fed. Survival of crablets was recorded at four different time points of 0, 12, 24 and 48 hours after testing. If the crablets color changed from transparent to opaque or showed no response when gently touch with tweezers, they were considered dead.

7.2.5.2 Ammonia stress test

One hundred twenty 0.5-L plastic beakers filled with 0.2 L of total ammonia solution were used. The concentration of the total ammonia solution used in stress test was based on 96-h LC_{50} value of 95.35 mg L^{-1} of total ammonia as reported for the fifth crablet stage of *S. serrata*, a closely related species (Romano & Zeng, 2007a). Ammonia solution was prepared according to procedure described by Chen et al. (1990). Briefly, 14.87 g nitrogen in the form of ammonium chloride (NH_4Cl) (Merck, Germany) was dissolved in1 L distilled water and then continuously diluted with 23 L of disinfected seawater of the same water quality as used in larval rearing. The ammonia solution was stirred vigorously until complete dissolution and then distributed into 0.5-L plastic beakers. One newly moulted crablet was released into

each plastic beaker. All beakers were placed in the same area where the transport stress test was performed earlier. During the stress test, no feeding and water exchanges were done. Survival of the crablets, total ammonia concentration, pH and temperature were recorded at 0, 6, 12, 24, 48 and 96 hours of exposure.

7.2.6 Statistical analyses

All data were stored in Microsoft Excel and presented as means ± standard error (SE) (n=3). The survival and growth data were subjected to one-way analysis of variance (ANOVA). The data presented in percentages were transformed arcsine-square root before analysis (Zar, 2010). Specific differences between the treatment means were detected by Tukey's multiple range tests at 0.05 level of significance. All statistical analyses were computed using SPSS for Windows, version 24.0.

7.3 Results

7.3.1 Environmental parameters

The temperature, pH and DO did not show significant ($P > 0.05$) effects in the four different rearing systems in the present study (Table 7.1). However, there was a significant difference ($P < 0.05$) in TAN and nitrite among the systems (Fig. 7.1). TAN in the biofloc water system increased as the time of culture progressed and was higher ($P < 0.05$) than that in the recirculating water system at the end of the experiment, but it did not differ ($P > 0.05$) from that in the green and clear water system. A fluctuation of TAN was observed in the clear water system but was not significantly different ($P > 0.05$) from that in the recirculating water system at the end of the experiment. Nitrite was lower in the green and biofloc water systems ($P < 0.05$) than in the clear water system during the experimental period but did not differ ($P > 0.05$) from that in the recirculating water system at the end of the experiment.

Table 7.1: Dissolved oxygen, temperature and pH in different rearing systems during the experimental period

Systems	CW	GW	RW	BW
Days				
DO (mg L^{-1})				
1st	8.1 ± 0.06	7.9 ± 0.06	8.1 ± 0.03	8.0 ± 0.06
4th	8.2 ± 0.06	8.3 ± 0.03	8.1 ± 0.03	7.9 ± 0.03
7th	8.0 ± 0.06	8.1 ± 0.06	8.0 ± 0.03	7.9 ± 0.07
Temperature (^{o}C)				
1st	27.6 ± 0.03	28.1 ± 0.03	27.6 ± 0.03	28.0 ± 0.00
4th	28.0 ± 0.00	28.1 ± 0.03	28.1 ± 0.03	28.1 ± 0.01
7th	27.9 ± 0.06	27.9 ± 0.03	28.0 ± 0.03	28.0 ± 0.00
pH				
1st	8.6 ± 0.00	8.6 ± 0.00	8.6 ± 0.00	8.6 ± 0.00
4th	8.6 ± 0.03	8.5 ± 0.03	8.5 ± 0.03	8.6 ± 0.03
7th	8.6 ± 0.06	8.4 ± 0.03	8.6 ± 0.17	8.8 ± 0.15

Data represented as mean ± SE; n=3. Key: CW: Clear water; GW: Green water, RW: Recirculating water, and BW: Biofloc water systems.

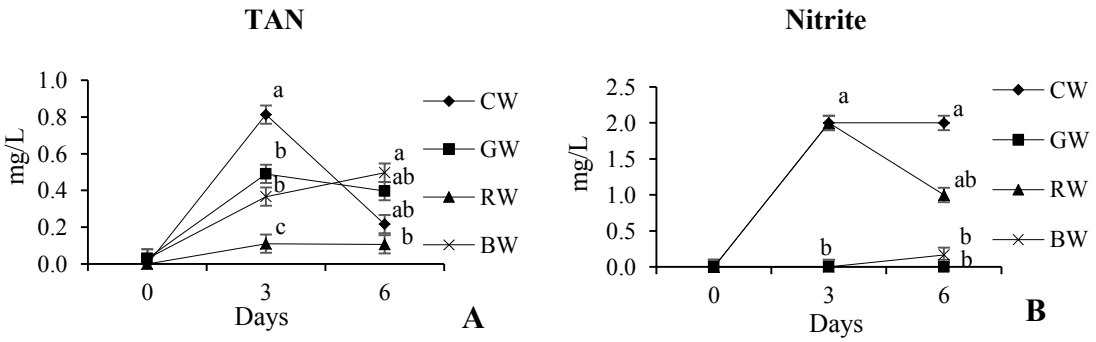


Figure 7.1: Total ammonia nitrogen (TAN) (A) and nitrite (B) concentrations in different rearing systems during the experimental period.

Key: CW: Clear water; GW: Green water; RW: Recirculating water and BW: Biofloc water systems. Significant differences were found among all treatments with different (P < 0.05) superscript letters presented on top of error bars on the same day.

7.3.2 Survival and growth of first crablets

Survival of first crablets reared under four different rearing systems is shown in Fig. 7.2. Survival of crablets reared under the recirculating water and biofloc water systems were similar (*P > 0.05)* to those crablets reared under the clear water system (control), and these systems obtained higher (*P < 0.05*) survival of crablets than the

green water system. In the green water system, dead megalopae floating on the water surface and abnormal megalopae attached with full algae filaments in their abdomen were observed. These abnormal megalopae were unable to moult into crablets and died a few days later. This phenomenon was not observed in the other systems. The carapace width of crablets reared under the recirculating water system was larger ($P < 0.05$) than those reared under the green water system, but similar ($P > 0.05$) to those reared under the control and biofloc water systems (Table 7.2). However, there was no significant difference ($P > 0.05$) in carapace length and wet weight of crablets among the systems (Table 7.2).

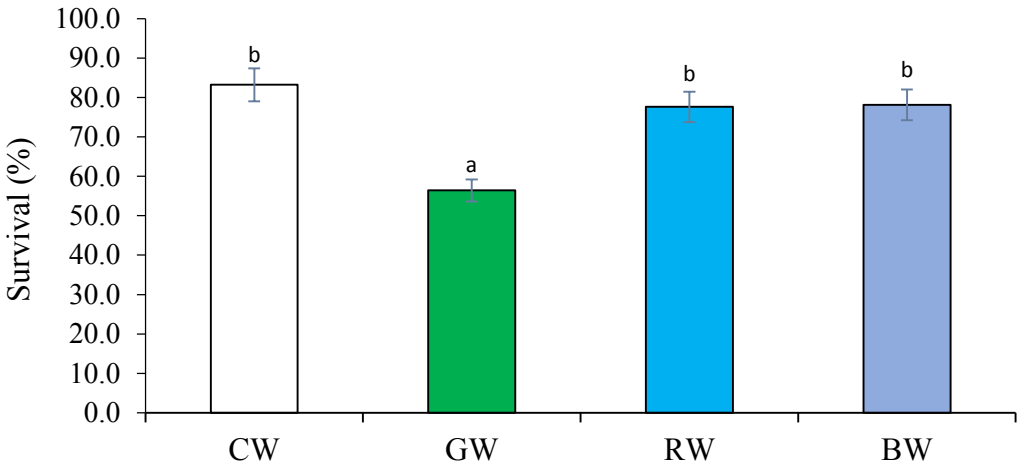


Figure 7.2: Mean survival of first crablets cultured under four different water rearing systems.

Key: CW: Clear water; GW: Green water; RW: Recirculating water and BW: Biofloc water systems. Significant differences were found among treatments with different superscript letters (P < 0.05) in the bar chart.

Table 7.2: Mean carapace length and width, and wet weight of first crablets

Treatment\Parameters	Length (mm)	Width (mm)	Weight (mg)
CW	2.92 ± 0.02	3.52 ± 0.02^{ab}	8.60 ± 0.18
GW	2.90 ± 0.02	3.45 ± 0.01^{a}	7.93 ± 0.20
RW	2.95 ± 0.03	3.56 ± 0.01^{b}	8.65 ± 0.15
BW	2.92 ± 0.02	3.52 ± 0.02^{ab}	8.31 ± 0.15

Data are presented as means ± standard error (SE), n=3. Key: CW: Clear water; GW: Green water; RW: Recirculating water and BW: Biofloc water systems. Significant differences were found among treatments with different superscript letters (P < 0.05) in the same column.

7.3.3 Total ammonia and un-ionized ammonia concentration during ammonia stress test

The ammonia stress test was stopped at 24 hours after exposure as the survival of the tested crablets was close to zero. Therefore, the total ammonia concentration, pH and temperature recorded until 24 hours is shown in Table 7.3. The conversion into un-ionized ammonia was calculated according to a formula described by Whitfield (1974).

Table 7 3: Temperature, pH, total ammonia and un-ionized ammonia concentration at 0, 6, 12 and 24 hours of the ammonia test

Parameters\Time (hours)	0	6	12	24
Temperature (^{o}C)	28 ± 0.00	28 ± 0.00	29 ± 0.00	29 ± 0.00
pH	8.3 ± 0.00	8.2 ± 0.00	8.1 ± 0.00	8.1 ± 0.00
Total ammonia (mg L^{-1})	93.0 ± 3.00	85.0 ± 1.41	78.0 ± 2.00	67.0 ± 1.00
Un-ionized ammonia (mg L^{-1})	10.3 ± 0.33	7.6 ± 0.09	6.1 ± 0.16	5.2 ± 0.08

7.3.4 Survival of first crablets after simulated transport and ammonia stress tests

No significant difference ($P > 0.05$) in the survival of crablets among different rearing systems was observed during exposure to the air in the 48-hour simulated transport stress test (Fig. 7.3), while the ammonia stress test showed that the survival of crablets exposed to the 96-h LC_{50} value of total ammonia was different ($P < 0.05$) among various rearing systems (Fig. 7.4). Survival of crablets reared under the recirculating water system was higher ($P < 0.05$) than the control at 24 hours after exposure to acute ammonia toxicity. The survival of crablets reared under the biofloc water system was similar ($P > 0.05$) to the control and the survival of crablets in these systems dropped close to zero 24 hours after exposure. Survival of crablets reared under the green water system was the lowest when exposed to acute ammonia toxicity; then survival dropped to zero 24 hours after exposure.

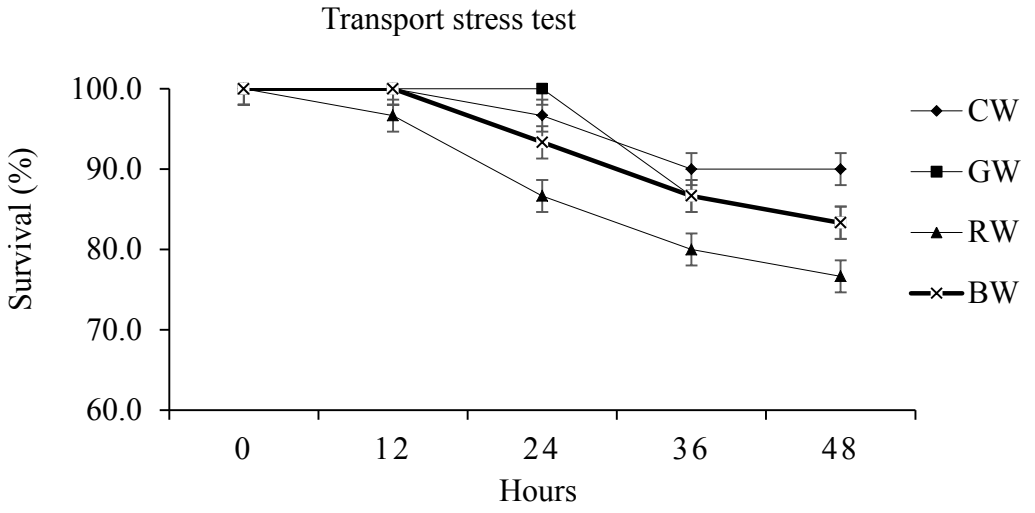


Figure 7.3: Mean survival of first crablets exposed to air during 48 hours of the transport stress test.

Key: CW: Clear water; GW: Green water; RW: Recirculating water and BW: Biofloc water systems.

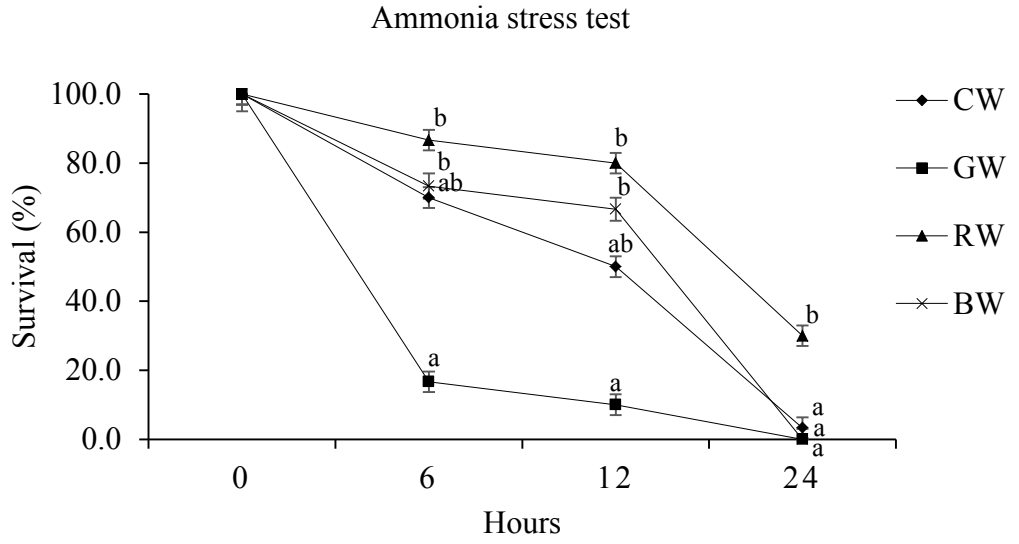


Figure 7.4: Mean survival of first crablets exposed to LC50 value of total ammonia for 24 hours.

Key: CW: Clear water; GW: Green water; RW: Recirculating water and BW: Biofloc water systems. Significant differences were found among treatments with different superscript letters (P < 0.05) at the same time.

7.4 Discussion

In water rearing systems, TAN gradually accumulates with the culture time (Chang, Chiang, Cheng, & Chang, 2015) and eventually becomes toxic to the cultured species (Emerenciano et al., 2011; Romano & Zeng, 2013).). Toxic effects of elevated

ammonia leads to adverse effects on the development and growth of crustaceans, altered gas exchange and acid/base balance, induced osmoregulatory disruptions and causes histological damage (Romano & Zeng, 2013). Previous study (Romano & Zeng, 2007b) reported that exposure to sub lethal total ammonia levels induced anterior gill damage in *S. serrata* juveniles, decreased development of the swimming crab (*Portunus pelagicus*) (Liao, Wang, & Lin, 2011), and reduced immunity of the swimming crab (*P. trituberculatus*) (Yue, Pan, Xie, Zheng, & Li, 2010), leading to increased susceptibility to pathogens (Chang et al., 2015).

In the present study, TAN was stable in the green water and recirculating water systems, whereas fluctuated in the clear water system. The drop of TAN could be due to the exchange of water as it increases by mineralization of organic detritus (Lin & Chen, 2003) prior to the water exchange and then decreases after the water exchange (Deb, 1998). In the biofloc water system, the reduction in conversion of heterotrophic bacteria to available biomass results in a higher TAN at the end of the experiment (Ekasari, Crab, & Verstraete, 2010). However, the highest level of TAN observed in this system (0.5 mg L^{-1}) was still lower than the safe levels of 9.53 mg L^{-1} as reported for early *S. serrata* juveniles (Romano & Zeng, 2007a).

Survival is considered primary responses during the nursing phase (Arnold, Sellars, Crocos, & Coman, 2006) that can be used to compare the efficiency among different rearing systems. In the present study, survival of first crablets reared under the green water system was lower than reared under the clear water system. On the contrary, the green water system is reported that it could be improved survival of larvae of *M. rosenbergii* (Lober & Zeng, 2009; Maddox & Manzi, 1976; Manzi et al., 1977). Similarly, our previous study (unpublished) showed that the green water system had increased zoeal survival. The difference in survival between the present and previous studies could be due to either different species or behavioural changes according to larval stage development. To substantiate this, it can be speculated that the megalopa that tends to settle down on the bottom (Genodepa, 2004; Holme et al., 2006b) can easily be infected by bacteria and algae filaments, and thus could result in high mortality in the present study. While, larvae of prawn or zoea of mud crab are less in immediate vicinity of harmful bacteria and filamentous algae from sediment accumulation due to their swimming behavior in the water column. Similarly, Arnold, Sellars, Crocos, and Coman (2005) found that sediment accumulating in

areas of low water levels can become anaerobic that also resulted in high shrimp (*P. esculentus*) mortalities as they actively buried in the sediments (Arnold et al., 2005, 2006). This demonstrated that sediment accumulated from algae supplementation can have adverse effect on survival of megalopa.

The growth performance of post-larvae (PL) of shrimp can significantly be affected by the type of rearing system. A recirculating water system improved PL growth performance of Pacific white shrimp (*L. vannamei*) more than a biofloc system (Esparza-Leal et al., 2015), whereas Emerenciano, Cuzon, Paredes, and Gaxiola (2013) and Izquierdo et al. (2006) reported better growth performance of PL of *Farfantepenaeus duorarum* and *L. vannamei* in the biofloc water system than in the clear water system. However, the present result showed that the growth performance of crablets was not affected by the type of rearing systems. In fact, mud crab larvae are carnivorous and their feeding behaviour, in terms of selecting food particle size, changes according to the larval development (Genodepa et al., 2004b). The particle size acceptance identified for mud crab megalopa was larger than 400 µm (Genodepa et al., 2004b), thus, algae and floc particles from the green water and biofloc water systems were not an ideal food for megalopa due to their small size ranging from 100 to 200 µm (Azim & Little, 2008; Azim, Little, & Bron, 2008). Further, the growth dependence of crustacean PL on the rearing system can be attributed to species and food source variations.

The stress tests can be employed as important tools to determine PL quality of crustacean (Cavalli et al., 2000; Samocha et al., 1998). In the present study, the simulated transport stress test did not show any significant effects of the various rearing systems on the quality of crablets, whereas our earlier study reported significant changes in megalopa quality in different rearing systems (unpublished data). This could be due to an increase in resistance to the stressor with increasing age (Briggs, 1992; Samocha et al., 1998). Thus, transport stress duration up to 48 hours in the present study may not be enough to reflect in significant differences.

Conversely, high tolerance to ammonia was observed for crablets reared under the recirculating water system, indicating that the crablets obtained were of good quality. Mishra et al. (2008) reported that good health conditions of *L. vannamei*, reflected by less external fouling and lower intestinal bacterial load, could be obtained in a raceway system with limited water exchange. It can be postulated that good water

quality maintained by the recirculating water system could result in better crablet quality, which is consistent with the result of a previous study in *L. vannamei* (Esparza-Leal et al., 2015).

The ammonia stress test is a very sensitive indicator that determines the quality of larvae as early as larval stage Z4 within 24 hours (Cavalli et al., 1999). In the present study, the quality of crablets reared under various rearing systems was distinguished at 24 hours in terms of survival response, but the survival was much lower than what was expected at this time. The 96-h LC_{50} dose of ammonia used in this study was applied to fifth crablets of *S. serrata* with average weight of 373 ± 24 mg (Romano & Zeng, 2007a). However, first crablets with an average weight of 8.36 ± 0.15 mg were exposed to this dose and this might have resulted in inducing higher toxicity and ultimately the higher mortality within 24 hrs of the exposure.

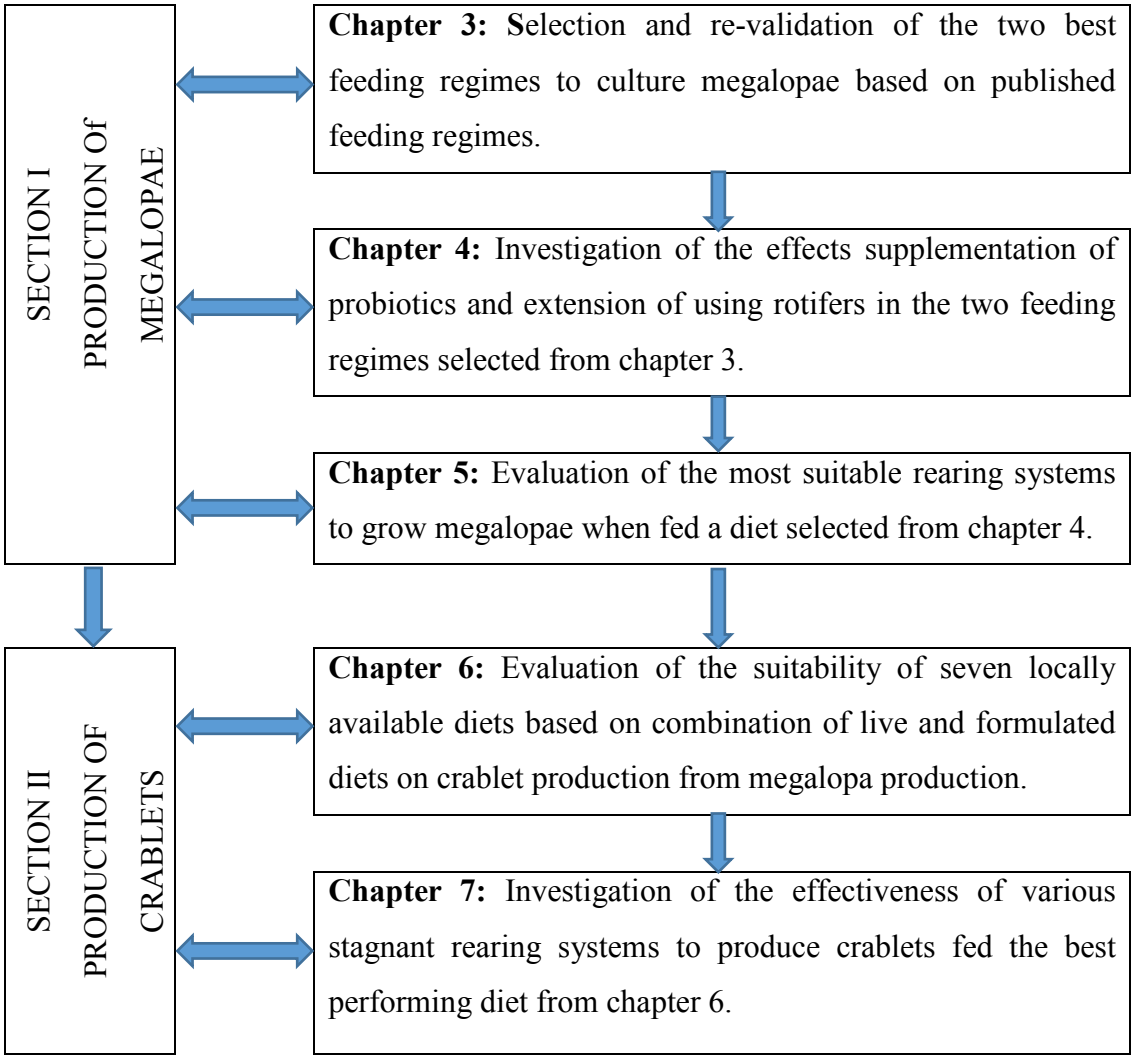
The recirculating and biofloc water systems could save approximately 50% of the seawater than the clear water system by not conducting two times water exchanges during the nursery cycle, thus, reducing the cost of production associated with disinfection and pumping of water (De Lorenzo et al., 2015), and also allowing easy establishment of hatcheries in areas distant from the seawater source (Piamsak & Somkiate, 1980). In addition, these system could also increase in biosecurity in order to avoid the incidence of disease, thus facilitate in the year-round production of quality seeds (Mallasen & Valenti, 1998).

7.5 Conclusion

In conclusion, the present outcomes have demonstrated that crablets can be produced under recirculating water and biofloc water systems without any adverse effects on their survival and growth performance.

CHAPTER 8: General discussion, conclusions and recommendations

This chapter is aimed to summarise and discuss the entire set of conclusions drawn from previous research chapters. The ultimate aim of this research is to find the most suitable feeding regimes and practical rearing systems for the production of crablets to improve the current survival rate achieved by various mud crab hatcheries in South Vietnam. A summary of the series of experiments conducted and the corresponding results is shown as follows:



Selection of an appropriate feeding regime

Among the four *Scylla* species, the eggs and newly moulted larvae of *S. paramamosain* are very small (Nghia, 2004), which creates considerable difficulties for seed production. Small eggs often contain very little yolk reserve, and so newly hatched Z1 larvae require sooner feeding after the hatching to supplement the nutrition requirements to complete the development to the next stage (Davis, 2003). There are further disadvantages of rearing small crab larvae as their feeding gets restricted to smaller food particles and may be preyed upon by a wider variety of larger predators (Davis, 2003). The smaller larvae need longer development time to metamorphose from Z1 into the first crablet stage that creates additional difficulties in captivity (Davis, 2003). In addition, mud crab larvae are highly susceptible to disease (Nghia et al., 2007a) and also exhibit cannibalistic behavious (Quinitio et al., 2001) resulting in an unpredictable survival rate in captivity.

The physical and nutritional characteristics of various prey organisms (Ruscoe et al., 2004b) as well as the quality of larvae (Nghia, 2004) must be taken into account when determining their feeding regimes. Repeatedly, research has shown that rotifers and *Artemia* are two important components of live feed in raising crab larvae (Dat, 1999; Davis et al., 2005a; Ikhwanuddin et al., 2012; Zeng & Li, 1999).

Rotifers are optimal first food for Z1 of *S. paramamosain* due to their small size (Nghia, 2004; Zeng & Li, 1999). Apart from their size, other factors such as behaviour, ease of digestion, or the presence of some specific nutrients when compared with *Artemia* may be responsible for the observed improvement in larval performance (Davis, 2003). Therefore, they can also be considered a necessary component of the mud crab larval diet.

Similarly, *Artemia* is a larger prey and represents a higher nutritional value than rotifers (Nghia, 2004), and accounts for the majority of the energy intake for the crab larvae (Harvey & Epifanio, 1997). However, *S. paramamosain* larvae are only able to catch newly hatched *Artemia* from Z2 onwards, and the ability of Z2 to catch *Artemia* depends on the quality of the larvae, which can vary between the batches of *S. paramamosain* (Nghia, 2004). Although some studies advocate the use of smaller strain of *A.* nauplii (± 457 μm) or decapsulated *A.* cysts (Davis et al., 2005a) or live umbrella-stage *Artemia* (Nghia, 2004) for Z1, all these above research reported

lower survival of larvae compared to treatment where rotifers were fed. Thus, it is advisable that *Artemia* can be introduced as a feed source at the Z2 or Z3 stage of *S. paramamosain* in order to achieve the best possible overall zoeal survival (Nghia, 2004; Zeng & Li, 1999).

Past research has confirmed that in order to achieve acceptable larval production, both rotifers and *Artemia* need to be in a mixture of live feeds to be fed to larvae (Baylon et al., 1999; Zeng & Li, 1999). However, their time of introduction and withdrawal, particularly rotifers is still a target of research. The timing of the introduction of rotifers and *Artemia* is well documented (Davis, 2003; Nghia, 2004; Zeng & Li, 1999), while data regarding the timing of withdrawal of rotifers from the rearing system is inconsistent. Nghia (2004) and Ruscoe et al. (2004b) have reported that inclusion of rotifers alongside *Artemia* in diets that are offered beyond the Z2 stage does not improve megalopa survival or metamorphosis duration. In contrast to this, our results indicate that a diet of rotifers mixed with *Artemia* until the last zoeal stage had positive effects on the duration of metamorphosis and survival rate of mud crab megalopa.

Unfavourable feeding conditions during the culture period, including inadequate quantities of food supply and/or poor quality of diets, may affect the survival and development of the mud crab larvae. Our study confirms that discontinuous accumulation of nutrients can occur when rotifers are withdrawn earlier from a mixed diet. This nutrient inadequacy in our study was due to the inability of larvae to catch *Artemia* after moulting (Baylon et al., 2004).

As rotifers are withdrawn from a mixed diet with *Artemia*, a diet of *Artemia* alone could not provide the larvae with the nutrients required for post-moult that resulted in high mortality during metamorphosis from Z5 to megalopa (Baylon, 2009). Similarly, the larvae of *S. serrata* that were fed low-quality *Artemia* exhibited an increased percentage of moulting failure from Z5 to megalopa (Suprayudi et al., 2002a). Zeng et al. (2004) reported that feeding poor quality of diets such as starved *Artemia* to *S. paramamosain* at a later zoeal stage caused the appearance of additional Z6 larvae that resulted in low survival. This diet also resulted in morphologically abnormal first-stage crab with close-set eyes in swimming crab (*P. tritubercalartus*) (Dan et al., 2016b).

The present study confirmed that feeding a mixture of rotifers and *Artemia* until Z5 could reduce morphological variation and formation of immature (abnormal) megalopa of *S. paramamosain* (Quy et al., 2018a) and resulted in improved megalopa survival and metamorphosis rates (Quy et al., 2018b). Consistent with our findings, high megalopa production has been reported for zoeae of *S. serrata* and *P. pelagicus* that were fed a mixture rotifers and *Artemia* (Baylon & Failaman, 1999; Redzuari et al., 2012). A feeding regime of a mixture of rotifers and *Artemia* can provide a balanced nutrition for the post-moult larvae (Baylon, 2009), and it also can reduce the likelihood of *Artemia* starvation, as *Artemia* could feed on rotifer excrement (Dan et al., 2016b). Moreover, presence of both rotifers and *Artemia* in the rearing water resulted in appropriate accumulation of nutrition as *Artemia* represented the main nutritional source for tissue growth prior to moulting, and rotifers satisfied the nutritional requirements for acceptable survival after the moulting of crab larvae (Baylon et al., 2004). Based on our results presented in chapters 4 and 5, prolonged inclusion of rotifers with *Artemia* until the last zoeal stage is a suitable feeding regime for *S. paramamosain* that can improve megalopa production in mud crab hatcheries.

In summary, the benefit to crab larvae in the presence of both rotifers and *Artemia* for a designated time together could be attributed to adequate accumulation of nutrients as Dan et al. (2013) showed that the lack of nutritional accumulation during zoeal stages had a negative effect on the survival after the megalopa stage.

Effect of supplementing probiotics during the zoeal stages of mud crab

Our previously published research on megalopa (Quy et al., 2018a) and similar to as shown by (Jamali et al., 2015) in Pacific white shrimp larvae have proven that the feeding probiotic-enriched both rotifers and *Artemia* increased the survival and metamorphosis rates of the larvae. The probiotics can be administered at early larval stages either through direct addition to the rearing water or via live food, (Skjermo & Vadstein, 1999). High exposure to desirable microbes in the form of probiotics is beneficial to the indigenous microflora within the digestive tract of the larvae (Gatesoupe, 1999; Rengpipat et al., 1998), leading to increased activities of digestive enzymes (Ziaei-Nejad et al., 2006). This can also restrict harmful bacteria growth in the gut of the larvae, as well as in the rearing water (Talpur et al., 2013) such as *Vibrio* spp. (Talib et al., 2017) and necrosis bacteria (Dan & Hamasaki, 2015), which

could cause disease and mass mortality of mud crabs during the larval stages (Dan & Hamasaki, 2015; Liessmann, 2005).

Previous studies have demonstrated that probiotics supplementation led to increase growth of post-larvae of *F. indicus* (Ziaei-Nejad et al., 2006), *P. monodon* (NavinChandran et al., 2014; Uddin et al., 2013), and *L. vannamei* (Wang, 2007), due to the enhancement of digestive enzyme activities. On the contrary, other studies on probiotic-supplemented *F. indicus* larvae from Z1 to Z3 (Ziaei-Nejad et al., 2006), and *L. vannamei* larvae from Z3 to mysis (Zhou et al., 2009), did not report any enhancement of growth, which was attributed to the limited protease enzyme activity. The present results also confirmed that the administration of probiotics via live food caused no improvements in the growth of mud crab larvae from Z1 to megalopa (Quy et al., 2018a), probably due to the limited digestive enzyme activity (Holme et al., 2006b).

In addition, the present study also found that enrichment of only one type of live feed was not be beneficial to the survival or metamorphosis of mud crab larvae. This could be due to the fact that the concentration of probiotics in the diet is low when only one of the components is enriched, resulting in diminished probiotic effects (Dan & Hamasaki, 2015; Tseng et al., 2009). Besides, our earlier study (Quy et al., 2018b) showed that feeding a mixture of un-enriched rotifers and *Artemia* until Z4 and Z5 was not significant different in survival, metamorphosis rates and growth of megalopa. However, when combining probiotics enrichment of live food with extended inclusion of rotifers in the diet, the feeding a mixture of rotifers and *Artemia*, irrespective of enrichment, until Z5, rather than Z4, significantly improved megalopa survival, metamorphosis rates and growth (Quy et al., 2018a). It indicated that there were possibly beneficial effects as combining probiotic enrichment of live food and extension of rotifers inclusion.

Selection of suitable practical diets for megalopae

Survival and moulting of megalopa are considered important criteria to evaluate larval mud crab diets (Genodepa et al., 2004b). The present study showed that the locally available diets such as LA, MSM, LFF, and CF can be used as feed in megalopa nurseries, either individually or as combinations, such as LA with MSM, LFF, or CF. Their suitability is reflected by the successful moults and high survival

that was observed for first crablets. Previous studies have reported that MBD containing different protein sources such as squid meal, dried rotifers (Genodepa et al., 2004b), *Artemia* meal, or fish meal (Holme et al., 2006b) can be used as an exclusive feed for megalopa stage with no adverse effects on crablet survival. These findings highlight that *Scylla* spp. can accept a wide variety of food types from the megalopa stage onwards.

The development of diets that are globally accepted or locally available ingredients could improve the hatchery viability for the production of crablets (Holme et al., 2006b). Our results demonstrated that locally available diets including LA, MSM, or LFF can be used in a similar manner to MBD to feed megalopa without adverse effects on crablet survival. Reduced costs may be achieved compared with MBD, as the latter contains expensive and sometimes unavailable ingredients such as rotifers, *Artemia*, squid, and fish meal.

Selection of efficient rearing systems for mud crab larvae

Water quality parameters are considered the most important factors for the success of crustacean hatcheries (Bray and Lawrence, 1992). Clear water systems with regular water exchange are commonly used for larval rearing, due to their simple management and the removal of wastes through water exchanges (Deb, 1998). This type of system is appropriate for coastal areas where abundant clear seawater supply is available (Hirayama, 1974). A clear water rearing system utilising quality seawater could result in a good larval survival (Nghia, 2004). However, such areas are limited and are affected by run-off containing agricultural and industrial chemicals, disease vectors, or antibiotic-resistant bacteria from nearby aquaculture areas. These contaminants can cause the failure of mud crab hatcheries. Apart from these impediments, a dramatic drop in salinity occurs during the rainy season in South of Vietnam, which could delay the production of mud crab seed, or increase the cost due to having to purchase and transport seawater from elsewhere. To overcome this, the introduction of closed systems with limited water exchange is necessary in order to enable the hatchery operators to produce crablets year-round, and avoid a collapse in production caused by the risks associated with an incoming water source.

Closed water rearing systems such as green, recirculating, and biofloc water systems have been successfully used for *M. rosenbergii* (Lober & Zeng, 2009; Piamsak &

Somkiate, 1980), *L. vannamei* (De Lorenzo et al., 2016a; De Lorenzo et al., 2016b; Manan et al., 2016), and *P. monodon* hatcheries (Beard & Wickins, 1980; Millamena et al., 1991). Our results demonstrated that a closed system could be used to rear mud crab larvae from Z1 to crablets; however, the suitable type of each rearing system would depend on the specific larval developmental stages (Fig. 8.1).

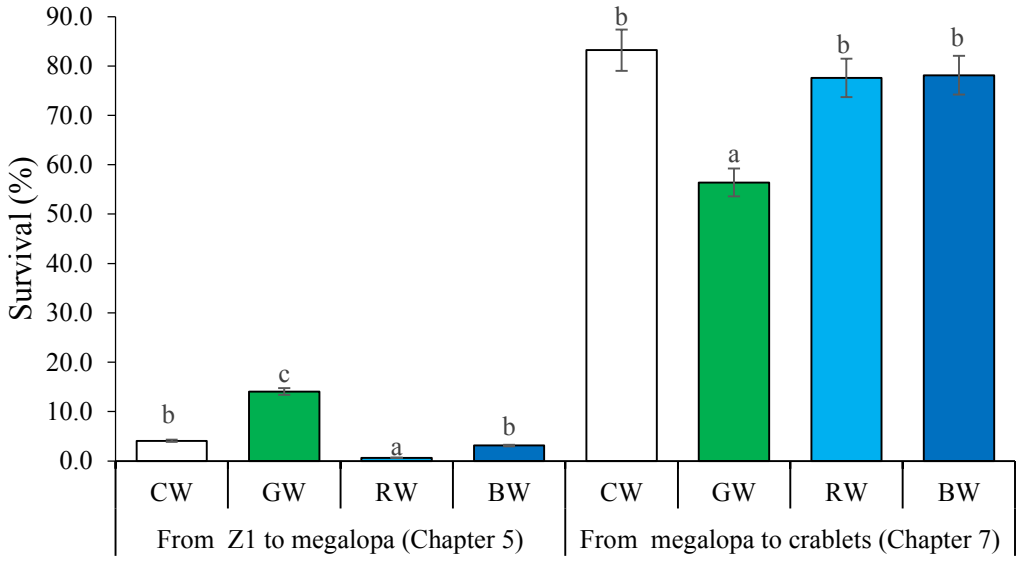


Figure 8.1: Survival of mud crab larvae using different water systems from zoea to megalopa stage and from megalopa to crablet stage.

Key: CW: Clear water; GW: Green water; RW: Recirculating water and BW: Biofloc water systems. Significant differences were found among all treatments with different superscript letters ($P < 0.05$) in the bar chart within the chapter.

The present study showed that green water supplemented with *Chlorella* spp. increased in megalopa survival and resistance to air exposure during the transport stress test. This could be due to the algae fulfilling the essential requirements for live food enrichment and also control of bacterial growth (Nghia et al., 2007a). This finding is in agreement with previous studies on *S. serrata* (Brick, 1974).

On the other hand, the present results revealed that the use of a recirculating water system adversely affected the early larval survival. Reduced quality and quantity of live food in rearing water due to filtered out by the physical filter, be vulnerable to physical damage of early larvae due to trapped on the screening net and decreased trace element or deposited waste products in this system could be one of the causes leading to low survival of zoea.

In the successive phases from megalopa to the crablets, recirculating and biofloc water systems can be used in place of the clear water system without any impact on the crablet survival. The recirculating water system can produce high-quality crablets, indicated by their high tolerance to ammonia. Good and consistent quality of the recirculating water has been seen to improve production of shrimp postlarvae (Esparza-Leal et al., 2015).

The current research showed that the green water culture system was suitable for growing zoea to megalopa stage rather than growing crablets from megalopae. As dead algae can interfere more with bottom-dwelling megalopa rather than free-swimming zoea, the effect of dead algae can be more pronounced on megalopa and onward stages. The visual observation also indicated that during the crablet production filamentous algae was more prevalent in the rearing systems, thus interfering with movement of megalopa. Further, the collapse and degradation of filamentous algae in the system could have contributed in the drop of water quality (Baylon & Failaman, 1999) resulting in higher mortalities.

In the current study, the biofloc water system similar to clear water system was less suitable than green water system for rearing megalopae from Z1, but more suitable to raise crablets from megalopae than green water system. Biofloc water system could replace the clear water system for rearing larvae from Z1 to megalopa, or from megalopa to crablets. However, it is worth noting that floc particles in this system could result in decrease of larval survival due to poor nutrient quality (Nguyen, 2014).

Our results suggest that a green water system should be utilised for rearing mud crabs from the early larval Z1 to megalopa stage, and a clear water system can be applied to raise megalopa to crablet stage. However, when seawater supply is limited for hatcheries either due to pollution or unsuitable salinity, the recirculating and biofloc water systems are recommended for rearing megalopae into crablets.

Conclusions

1. Feeding *S. paramamosain* larvae a diet of rotifers mixed with *Artemia* until the Z3 or Z4 stages leads to improved metamorphosis of megalopa, and inclusion of rotifers in the diet until Z5 increases the survival of megalopa.
2. Enriching both *Artemia* and rotifers with *Bacillus* as probiotics leads to improve survival and metamorphosis.
3. No interactive effects exist between probiotic enrichment of live food and extended inclusion of rotifers in the diet; however, an additive effect of the two these two factors could exist as reflected by improving survival, growth, and metamorphosis of megalopa.
4. Locally and globally available diets (LA, MSM, LLF, and CF) resulted in successful moults and high survival from megalopa to first crablet stage hence, suitable for use in megalopa nurseries. However, LA is the most suitable diet for this phase.
5. A green water system results in increased survival and improved quality of megalopa, and is suitable for rearing Z1 to the megalopa stage, while a recirculating water system has adverse effects on megalopa production.
6. During crablet production, the green water rearing system adversely affects the megalopa development.
7. Recirculating and biofloc water systems can be used in place of a clear water system to enable year-round seed production with no adverse effects on the larval development.

Recommendations

Based on the outcomes of this research, following recommendation are made:

1. The feeding regime should include the prolonged inclusion of rotifers until the last zoeal stage.
2. Probiotic enrichment of both rotifers and *Artemia* should be applied for all the larval stages development.

3. Locally and globally available diets such as live *Acetes*, minced shrimp meat, locally formulated, and commercial feed should be used for the nursery phases of megalopa.
4. A green water system is recommended for rearing larvae from Z1 to megalopa; however, this system is discouraged for the late larval stages from megalopa onwards.

The following recommendations for future research are also made:

1. Further research is required to evaluate whether extending the inclusion of rotifers in the diet until the last zoeal stage will exert a positive carry-over effect for the megalopa and crablet stages.
2. Further work should focus on the nutritional and economic values of locally and globally available diets that can replace expensive diets such as *Artemia,* MBD for megalopa production nurseries.
3. Further investigation needs to be conducted into the optimal schedule for feeding of live food in a biofloc water system.
4. Further studies should be carried out at a pilot and/or commercial scale to ensure that the findings of this research can be applied and replicated under commercial hatchery conditions.

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Appendix 1: List of publication and conference abstracts

Journal articles:

1. Quy, O. M., Fotedar, R., & Thy, H. T. T. (2018b). Extension of rotifer (*Brachionus plicatilis*) inclusions in the larval diets of mud crab, *Scylla paramamosain* (Estampodor, 1949): Effects on survival, growth, metamorphosis and development time. *Modern Applied Science,* **12**, 65-74. https://doi.org/10.5539/mas.v12n1p65

2. Quy, O. M., Fotedar, R., & Thy, H. T. T. (2018a). Effects of extended-rotifers inclusion and live food-enrichment with probiotics on the survival, metamorphosis, development time and growth of mud crab, *Scylla paramamosain* (estampador) larvae. *American Journal of Applied Sciences,* **15**), 375-386. https://doi.org/10.3844/ajassp.2018.375.386

3. Quy, O. M., Fotedar, R., & Thy, H. T. T. Impact of different rearing systems on survival, growth and quality of mud crab (*Scylla paramamosain*) megalopae reared from early zoeae . *Aquaculture International,* **27**, 1673-1687.

4. Quy, O. M., Fotedar, R., & Thy, H. T. T. will be submitted soon. Selection of locally available diets for rearing crablets of mud crab (*Scylla paramamosain*) from megalopa.

Conferences abstracts:

1. Quy, O. M. & Thy, H. T. T. (2018). Four rearing systems for mud crab larvae (*Scylla paramamosain*): a comparison based on survival, growth and quality of megalopa. *5th International Conference on Fisheries and Aquaculture (ICFA 2018)*. 23rd – 24th August 2018 in Colombo, Sri Lanka.

2. Quy, O. M., Fotedar, R., & Thy, H. T. T (2018). Extension of rotifers (*Brachionus plicatilis*) inclusions and enrichment of live food with probiotic in the larval feeding regimes of mud crab (*Scylla paramamosain*): effects on survival, growth, metamorphosis and development time. *World Aquaculture Society Conference*, August 25-29, Montpellier, France.

Appendix 2: Abstract of published papers

1. Quy, O. M., Fotedar, R., & Thy, H. T. T. (2018b). Extension of rotifer (*Brachionus plicatilis*) inclusions in the larval diets of mud crab, *Scylla paramamosain* (Estampodor, 1949): Effects on survival, growth, metamorphosis and development time. *Modern Applied Science,* **12**, 65-74.

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Extension of Rotifer (*Brachionus Plicatilis*) Inclusions in the Larval Diets of Mud Crab, *Scylla Paramamosain* (Estampodor, 1949): Effects on Survival, Growth, Metamorphosis and Development Time

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Abstract

The study evaluated the effects of extended dietary inclusions of rotifers (*Brachionus plicatilis*) on the survival, metamorphosis rate, growth and development time in the larvae of mud crabs (*Scylla paramamosain*). The five most commonly published feeding regimes of mud crab (*S. paramamosain*) larvae were selected and tested by including rotifers onto them. Mud crab larvae in the first feeding regime were fed exclusively with *Artemia* nauplii (control or regime A), while those in feeding regimes 2, 3, 4 and 5 were fed rotifers starting from zoea 1 (Z1) to various development stages of mud crab larvae whereas feeding with *Artemia* nauplii was commenced from the Z2 stage until the end of the trial (megalopa stage). The results of the larval feeding trial for 24 days of culture showed the progressive decrease in survival of the larvae in all feeding regimes. Extended inclusion of rotifer feeding until Z5 stage resulted in significantly higher survival than in the control from 18 day after hatching onwards. Larval survival was negatively correlated (R^2 from 0.78 to 0.90) with the rearing time; however, different feeding regimes had no significant effect on this correlation. Extending inclusion of rotifer feeding until the Z3, Z4 and Z5 stages resulted in higher percentages of megalopa metamorphosis than in regime control, but did not significantly impact development time, carapace width, body length and wet weight of megalopa.

Keywords: Mud crab, *Scylla paramamosain*, *Artemia*, rotifer-extension, hatchery, larval feeding regime

1. Introduction

2. Quy, O. M., Fotedar, R., & Thy, H. T. T. (2018a). Effects of extended-rotifers inclusion and live food-enrichment with probiotics on the survival, metamorphosis, development time and growth of mud crab, *Scylla paramamosain* (estampador) larvae. *American Journal of Applied Sciences,* **15**, 375-386.

American Journal of Applied Sciences

Original Research Paper

Effects of Extended-Rotifers Inclusion and Live Food-Enrichment with Probiotics on the Survival, Metamorphosis, Development Time and Growth of Mud Crab, *Scylla paramamosain* (Estampador) Larvae

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Abstract: The present larval nutrition study was conducted to evaluate the effects of feeding probiotic-enriched rotifers inclusion mixed with *Artemia* until zoeal stages 4 (Z4) and Z5 on the survival, metamorphosis, development time and growth of mud crabs, *Scylla paramamosain* larvae. The efficiency of the feeding regime was tested by two-factor analysis. A 4×2 factorial experimental design with eight combinations of enriched or un-enriched or a mixture of enriched and un-enriched live food (factor 1) in each of two feeding regimes (factor 2) in triplicate was setup. After 24 days of larval cultivation, there were no significant ($p>0.05$) interactive effects between the two selected factors on survival and metamorphosis rate of larvae. Larval survival in all treatments, under both factors, decreased gradually ($p<0.05$) during the cultivation period. From 15 Days After Hatching (DAH), the survival and metamorphosis rates of larvae fed both enriched rotifers and *Artemia* were higher ($p<0.05$) than those of the larvae without enriched live food. Similarly, from 18 DAH onwards, the extension of rotifers inclusion mixed with *Artemia* until Z5, irrespective of enrichment, showed higher ($p<0.05$) survival, metamorphosis and growth of megalopa than when the rotifers were included only until Z4 stage. Overall, an amalgamation of extending rotifer feeding and enriching them at the same time with *Artemia* can result in considerable improvements in survival and metamorphosis which can in turn, have beneficial impact on the technical feasibility of a commercial crab hatchery.

Keywords: Mud Crab, *Scylla paramamosain*, Hatchery, *Artemia*, Rotifers-Extension, Probiotics

3. Quy, O. M., Fotedar, R., & Thy, H. T. T. Impact of different rearing systems on survival, growth and quality of mud crab (*Scylla paramamosain*) megalopae reared from early zoeae . *Aquaculture International,* **27**, 1673-1687.

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Impact of different rearing systems on survival, growth and quality of mud crab (*Scylla paramamosain*) megalopae reared from early zoeae

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Abstract
This study aimed to compare the effects of four different rearing systems—namely clear water, green water, recirculating water and biofloc water systems—on survival, growth and quality of mud crab (*Scylla paramamosain*) megalopae reared from early zoeae. Twelve 60-L plastic buckets filled with 50 L of disinfected seawater were stocked with 20 larvae of zoea 1 (Z1) L^{-1}. The larvae were fed both probiotic-enriched L-strain rotifers (*Brachionus plicatilis*) and probiotic-enriched *Artemia* (*Artemia franciscana*) in all systems. After 20 days of culture, the green water system resulted in the highest survival to megalopae than all the other systems. The survival of megalopae reared under the biofloc water system was similar to that of the clear water system, but both systems exhibited higher survival than the recirculating water system. However, larval growth performance was not affected by the various rearing systems. The quality of megalopae produced under these systems was determined by ammonia and simulated transport stress tests. The ammonia stress test did not show a significant difference in the quality of megalopae, but the simulated transport stress test demonstrated a significant effect of rearing system on the quality of mud crab megalopae. The resistance to the air exposure until the end of the 48 h of transport was observed in the green water system. Overall, the results of the present study revealed that the green water system is the most suitable for rearing *Scylla paramamosain* larvae from Z1 to megalopa stage.

Keywords Clear water system · Green water system · Recirculating water system · Biofloc water system · Hatchery

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Appendix 3: Author's contributions

1. Quy, O. M., Fotedar, R., & Thy, H. T. T. (2018b). Extension of rotifer (*Brachionus plicatilis*) inclusions in the larval diets of mud crab, *Scylla paramamosain* (Estampodor, 1949): Effects on survival, growth, metamorphosis and development time. *Modern Applied Science,* **12**, 65-74. https://doi.org/10.5539/mas.v12n1p65

Chapter	Name of the author	Contribution (%)	Contribution	Signature
3	Moc Quy Ong	85	Conceptualised the experiment, designed and set up of the experiment, day to day feeding and data collection, data analysis and writing of the manuscript.	
	Ravi Fotedar	10	Participation in conceptualisation, supervised the research, revised the MS.	
	Thi Truong Thy Ho	5	Assisted in daily feeding of animals, critically discussed the operation of the experiment, proof-read the MS	

2. Quy, O. M., Fotedar, R., & Thy, H. T. T. (2018a). Effects of extended-rotifers inclusion and live food-enrichment with probiotics on the survival, metamorphosis, development time and growth of mud crab, *Scylla paramamosain* (estampador) larvae. *American Journal of Applied Sciences,* **15**), 375-386. https://doi.org/10.3844/ajassp.2018.375.386

Chapter	Name of the author	Contribution (%)	Contribution	Signature
4	Moc Quy Ong	85	Conceptualised the experiment, designed and set up the experiment, day to day feeding and data collection, data analysis and writing of the manuscript.	
	Ravi Fotedar	10	Participation in conceptualisation, supervised the research, discussed at every stage of data collection and revised the MS.	
	Thi Truong Thy Ho	5	Assisted in daily feeding of animals, helped in bringing the resources at one place, critically discussed the operation of the experiment, proof-read the MS	

3. Quy, O. M., Fotedar, R., & Thy, H. T. T. Impact of different rearing systems on survival, growth and quality of mud crab (*Scylla paramamosain*) megalopae reared from early zoeae . *Aquaculture International,* **27**, 1673-1687. https://doi.org/10.1007/s10499-019-00421-2

Chapter	Name of the author	Contribution (%)	Contribution	Signature
	Moc Quy Ong	85	Conceptualised the experiment, designed and set up the experiment, day to day feeding and data collection, data analysis and writing of the manuscript.	
6	Ravi Fotedar	10	Participation in conceptualisation, supervised the research, visited the facility in Viet Nam a few times, revised the MS.	
	Thi Truong Thy Ho	5	Assisted in daily feeding of animals, critically discussed the operation of the experiment, proof-read the MS	