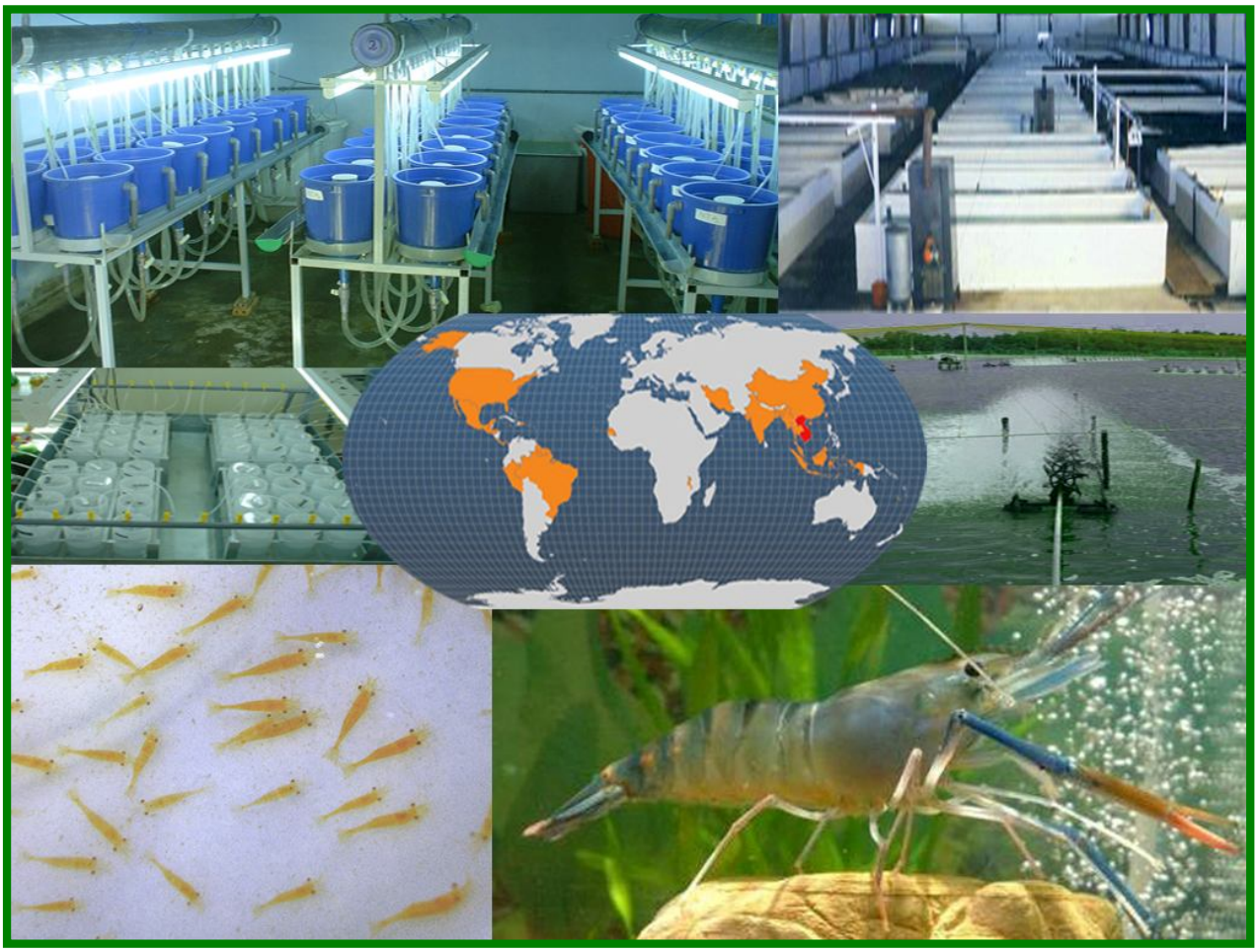

OPTIMIZATION OF HATCHERY PROTOCOLS FOR *MACROBRACHIUM ROSENBERGII* CULTURE IN VIETNAM

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LIST OF ABBREVIATIONS AND UNITS

AHL	Acyl Homoserine Lactone
AI	Autoinducer
AI–2	Autoinducer 2
ANOVA	Analysis of Variance
ARC	*Artemia* Reference Center
ATP	Adenosine Triphosphate
BTC	Belgian Technical Cooperation
CFU	Colony Forming Unit
cm	Centimetre
DGGE	Denaturing Gradient Gel Electrophoresis
DW	Dry Weight
EC	Enrichment Culture
EC5(D)	Enrichment Culture isolated from *Dicentrarchus labrax*
EC5(L)	Enrichment Culture isolated from *Lates calcarifer*
ELISA	Enzyme–linked Immunosorbent Assay
FAO	Food and Agriculture Organization
g	Gram
h	Hour
HHL	Hexanoyl Homoserine Lactone
ICB	Immunocolony Blot
ind.	Individual
kg	Kilogram
l	Litre
LB	Luria–Bertani
LC_{50}	Lethal Concentration for 50% of the population
LSI	Larval Stage Index

MA	Marine Agar
MA–R	Marine Agar supplemented with 100 mg/L of Rifampicin
MB	Marine Broth
min	Minute
mm	Millimetre
MOPS	3–(N–morpholino) propanesulfonic acid
n	Number of replicates
NSS	Nine Salt Solution
PHA	Polyhydroxyalkanoate
PHB	Poly–β–hydroxybutyrate
PL	Postlarvae
ppt	Part per thousand
QS	Quorum Sensing
RAC	Residual AHL Concentration
RIA2	Research Institute for Aquaculture No.2
RR	Rifampicin–Resistant
RS	Rifampicin–Sensitive
s	Second
SCFAs	Short Chain Fatty Acids
SE	Standard Error
SPSS	Statistical Package for the Social Sciences
TCBS	Thiosulphate–Citrate–Bile Salt–Sucrose
v/v	Volume per volume
w/v	Weight per volume
µg	Microgram
µl	Microlitre
µm	Micrometer

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CHAPTER 1

GENERAL INTRODUCTION

The giant freshwater prawn *Macrobrachium rosenbergii* (de Man) is a commercially important species in several Southeast Asian and South Pacific countries, Northern Oceania, and Western Pacific islands, both as a food item for local consumption as well as a valuable export product. Freshwater prawn farming can be conducted by unskilled rural people on small establishments and prawns are consumed domestically by all social classes. It is an activity that can play a role in poverty reduction and the empowerment of women. There has been a very rapid global expansion of freshwater prawn farming since 1995. The global farmed production of *M. rosenbergii* is expected to significantly exceed 400,000 tonnes by 2010. A majority of the seed used for grow out farming of *M. rosenbergii* comes from hatcheries. However, a major constraint in the large scale aquaculture of this species is the scarcity of seed at required quantities and in all locations. Existing hatcheries are not producing up to their installed capacity due various constraints. Availability of healthy and high-quality seeds has always been a major obstacle in the expansion of *M. rosenbergii* culture. The major factor that is hampering further development of the hatchery sector is the quality of hatchery–reared seed, culminating in low yield because of mass mortality. These problems may be caused by broodstock quality, zootechnical aspects of larval rearing and/or disease problems.

The overall objectives of the present thesis were to develop improved technical protocols for prawn seed production through investigating a number of factors believed to play an import role in larval rearing success. These included broodstock source, larval stocking densities, larval feeds and feeding regimes, *Artemia* replacement diets, and the use of novel strategies (acyl homoserine lactone–degrading enrichment cultures and poly–β–hydroxybutyrate) as a tool to control bacterial pathogens in prawn larval rearing.

The specific objectives and the thesis outline are as follows:

- To review the status of giant freshwater prawn farming and seed production and pinpoint the main bottlenecks for further expansion. Previous studies on broodstock rearing, zoo–technical and nutritional aspects of larval rearing, microbiological control approaches using homoserine lactone degrading bacteria and poly–β–hydroxybutyrate as a tool to control diseases in aquaculture seed production are reviewed (chapter 2).

- To compare the reproductive performance and offspring quality of *M. rosenbergii* broodstock from four different sources with the objective to determine which broodstock source is most suited for seed production under conditions prevailing in South Vietnam. This knowledge may add to the development of improved hatchery protocols and seed quality of *M. rosenbergii* culture and serve as a starting point to set up a selective breeding programme (chapter 3).

- To investigate the effects of larval stocking density and feeding regime on larval growth, survival, duration of the larval rearing cycle and larval quality of *M. rosenbergii*. The purpose of this study was to optimize larval stocking densities and feeding regimes to not only reduce production cost, but also maximize productivity of the hatchery. The resulting knowledge may add to the development of improved larval rearing techniques for more efficient hatchery production of *M. rosenbergii* (chapter 4.1)

- To optimize the use of supplemental diets and the replacement rate of live feed. Accordingly, a series of experiments were performed in the present study

to evaluate the use of formulated larval diets to supplement or replace *Artemia* nauplii partially in freshwater prawn rearing (chapter 4.2).

- To confirm that AHL has a negative effect on prawn survival and development and those AHL–degrading ECs can counteract this negative effect. In addition, we wanted to verify whether it was possible to grow the EC at the expense of glycerol released by hatching *Artemia* (chapter 5.1).

- To investigate the effect of two different mixtures of AHL–degrading enrichment cultures (ECs) from European sea bass (*Dicentrarchus labrax*) EC5(D) and from Asian sea bass (*Lates calcarifer*) EC5(L) on *Macrobrachium rosenbergii* larval performance and on the microflora in the rearing water as well as inside the prawn gut with and without *Vibrio harveyi* administration into the rearing water (chapter 5.2).

- To investigate the effect of PHB (administered via the live food) on the survival and growth of larvae of the giant freshwater prawn *Macrobrachium rosenbergii* and on the microbiota (total bacteria and vibrios) associated with the larvae. To demonstrate the application potential, loading of the *Artemia* nauplii with PHB was combined with lipid enrichment, a technique which is routinely applied in many fish and crustacean hatcheries (chapter 5.3).

- The results communicated in the various chapters are discussed and the main conclusions are mentioned. Additionally, the perspectives for further research are given in chapter 6.

CHAPTER 2

LITERATURE REVIEW

1. General status of freshwater prawn farming

1.1 General introduction

1.1.1 Nomenclature (New, 2002)

The giant freshwater prawn, *Macrobrachium rosenbergii* (De Man 1879), was one of the first species of the *Macrobrachium* genus to become scientifically known, the first recognisable illustration appearing in 1705. The nomenclature of freshwater prawns, both on a generic and a species level has had quite a muddled history. In the past, generic names have included *Cancer* (*Astacus*) and *Palaemon*. Previous names of *M. rosenbergii* have included *Palaemon carcinus, P. dacqueti*, and *P. rosenbergii* and it was not until 1959 that its present scientific name, *Macrobrachium rosenbergii* became universally accepted. The family tree of the giant freshwater prawn is:

Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea
Class	Malacostraca
Order	Decapoda
Suborder	Pleocyemata
Infraorder	Caridea
Family	Palaemonidae
Subfamily	Palaemoninae
Genus	*Macrobrachium*
Species	*M. rosenbergii* (De Man, 1879)
English name	Giant freshwater prawn

1.1.2 Distribution

The giant freshwater prawn *Macrobrachium rosenbergii* is native to Southeast Asia, South Pacific countries, northern Oceania, and western Pacific islands (New and Singholka, 1982; New, 2002). The adults of the species are usually found in freshwater bodies such as the lower reaches of rivers and lakes, ditches, canals and pools connected to the sea. *M. rosenbergii* was the species most used for commercial farming and consequently is the one which has been introduced to more countries. Following its import into Hawaii from Malaysia in 1965, where the pioneer work of Ling (1969) was translated into a method for the mass production of postlarvae (PL) by Fujimura and Okamoto (1972), it has been introduced into almost every continent for farming purposes (New, 2000a). *M. rosenbergii* is now farmed in many countries; the major producers are Bangladesh, Brazil, China, Ecuador, India, Malaysia, Taiwan, Vietnam and Thailand (FAO, 2002). More than thirty other countries reported production of this species in the year 2000. Vietnam is a major producer (New, 2000b). In addition, there are also valuable capture fisheries for *M. rosenbergii*, for example in Bangladesh, India, and several countries in Southeast Asia.

Fig. 1. Main producer countries (orange) of *Macrobrachium rosenbergii* (FAO, 2002)

1.1.3 Habitat, biology and life history

This species lives in tropical freshwater environments influenced by adjacent brackish water areas. It is often found in extremely turbid conditions. Gravid females migrate downstream into estuaries, where eggs hatch as free–swimming larvae in brackish water. The mating (copulation) of adults results in the deposition of a gelatinous mass of semen (referred to as a spermatophore) on the underside of the thoracic region of the female's body (between the walking legs). Successful mating can only take place between ripe females, which have just completed their pre–mating moult (usually at night) and are therefore soft–shelled, and hard–shelled males. All of the various types of males are capable of fertilising females but their behaviour is different. Detailed descriptions of the mating process are given in Ismael and New (2000) and Karplus *et al*. (2000). Under natural conditions, mating occurs throughout the year, although there are sometimes peaks of activity related to environmental conditions. In tropical areas these coincide with the onset of the rainy season, whereas in temperate areas they occur in the summer.

Although freshwater prawns require brackish water in the initial stages, most of their lifecycle is spent in turbid, riverine systems. There are four distinct phases in the life cycle: egg, larvae, postlarvae, and adult. Females become sexually mature before six months of age. Within a few hours after mating, eggs are laid and transferred to the underside of the tail where they are kept aerated and cleaned. The eggs remain attached to the abdomen until they hatch. The bright-yellow to orange colour of newly spawned eggs gradually changes to orange, then brown, and finally to grey-black. Eggs hatch 20 - 25 days after spawning based on the temperature. After hatching, larvae are released and swim upside down and tail first. Although larvae can survive for 48 hours in fresh water, they must be transferred to brackish water (9 to 19 parts

per thousand) for optimum survival. Larvae undergo 11 moults over a period of 15 to 40 days before transforming into postlarvae. The rate of this transformation depends upon food quantity and quality, temperature, and other water quality variables. Larvae feed primarily on zooplankton and larval stages of aquatic invertebrates. They change to principally bottom dwelling, crawling individuals. Postlarvae can tolerate a range of salinities. The term juvenile is used to describe the freshwater prawn between postlarvae and adult. (FAO, 2002)

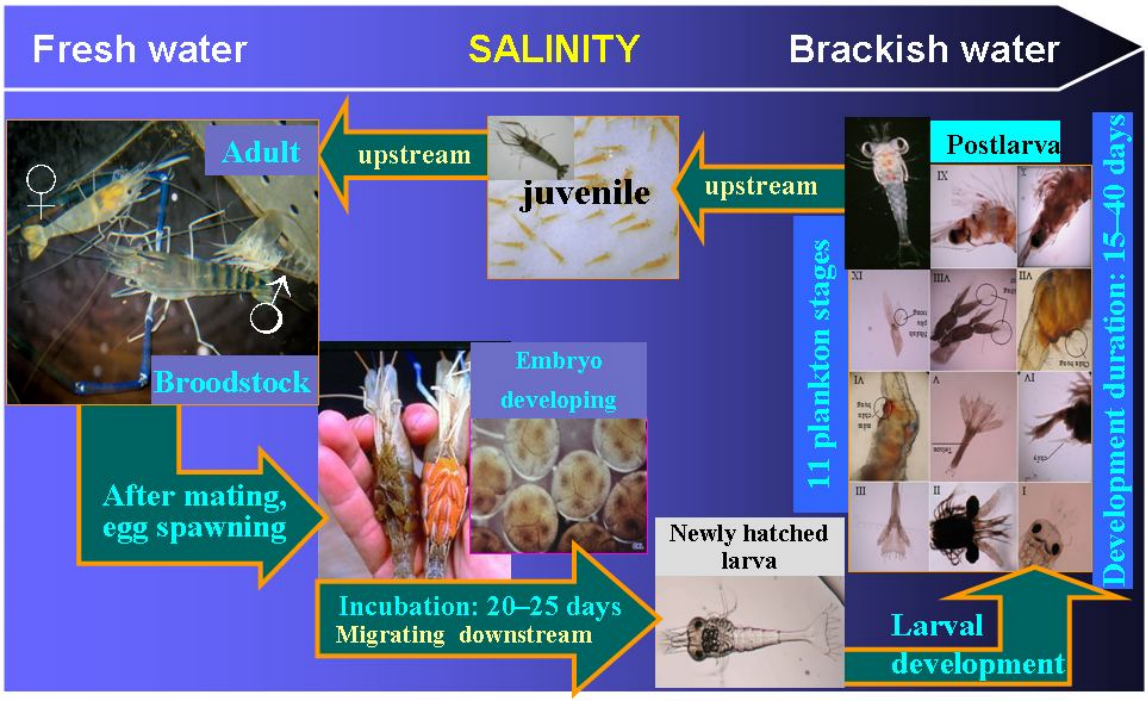
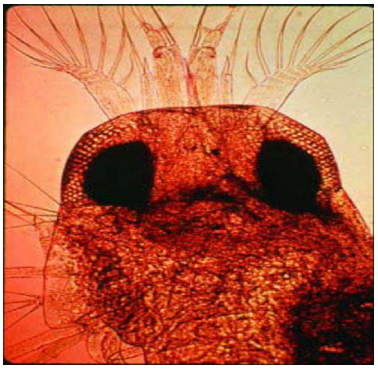
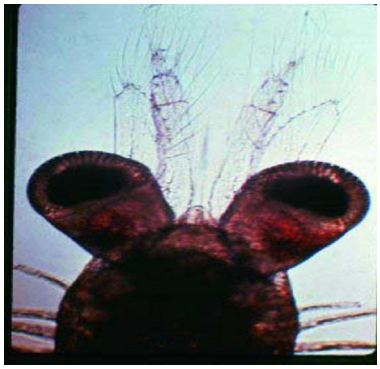


Fig. 2. Life cycle of *Macrobrachium rosenbergii*

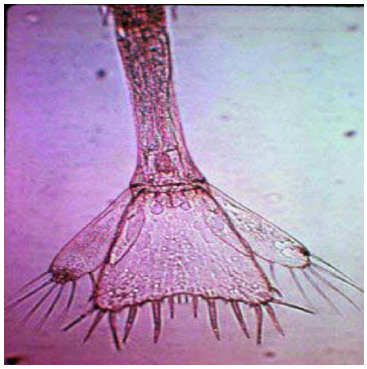
Before metamorphosis into postlarvae (PL), the larvae pass through eleven stages (Fig.3). After metamorphosis, PL assume a more benthic life style and begin to migrate upstream towards freshwater. Larvae swim actively tail first, ventral side uppermost. From PL onwards prawns swim forwards, dorsal side uppermost.



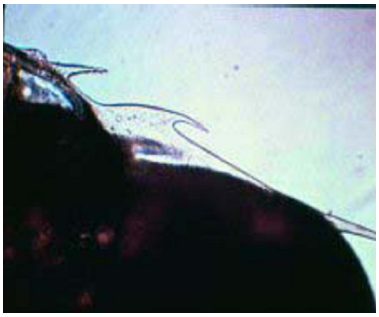
Stage 1: The eyes with sessile



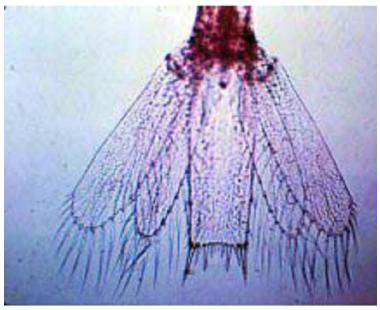
Stage 2: The eyes with stalked



Stage 3: One dorsal tooth in rostrum and first appearance of uropod

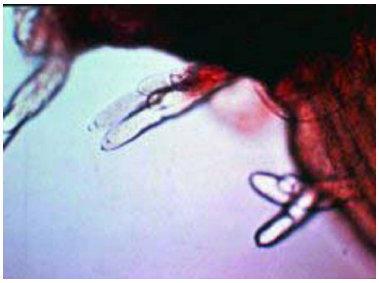


Stage 4: Two dorsal teeth and biramous with setae

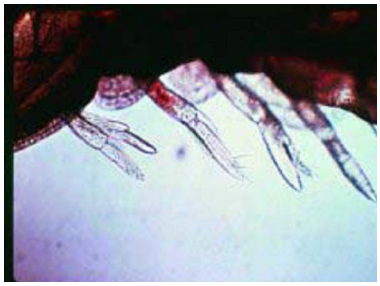


Stage 5: Antennal flagellum with 2 or 3 segments and more elongated and narrower of telson

Stage 6: Antennal flagellum with 4 segments; Telson more narrow and first pleopod appearance of buds



Stage 7: Antennal flagellum with 5 segments and biramous appearance with bare

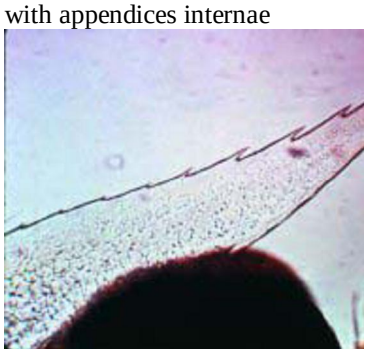


Stage 8: Antennal flagellum with 7 segments; biramous with setae

Stage 9: Antennal flagellum with 9 segments and endopods with appendices internae

Stage 10: 3 or 4 more dorsal teeth; Antennal flagellum with 12 segments and 1st & 2nd fully chelated

Stage 11: many dorsal teeth and Antennal flagellum with 15 segments



Postlarva stage: rostrum has dorsal and ventral teeth; behavior predominantly benthic, as in adults

Fig. 3. Micro-photographs and simplified key of the eleven larval stages and postlarva stage of *M. rosenbergii* (FAO, 2002)

From metamorphosis onwards prawns can also walk, not only on the substratum but also over damp areas including stones by river edges, up vertical surfaces (small waterfalls, weirs, etc.) and across land. Larvae mostly consume zooplankton (mainly minute crustaceans), very small worms, and larval stages of other crustaceans. Postlarvae and adults are omnivorous, eating algae, aquatic plants, molluscs, aquatic insects, worms, and other crustaceans. Males and females have different growth rates and males exhibit heterogenous individual growth (HIG); these are vitally important factors in grow–out management. Three distinct male morphotypes (and a number of intermediary types) exist: small males (SM), orange claw males (OC), and blue claw males (BC). The normal male developmental pathway is SM → OC → BC. BC males have extremely long second pereiopods; those of OC males are golden coloured; SM have small, slim, almost translucent claws. The type and behaviour of the males affects the growth rates of other prawns. The transition from rapidly growing OC to the slowly growing BC morphotype follows a "leapfrog" growth pattern. An OC metamorphoses into a BC only after it has become larger than the largest BC in its vicinity. The presence of this new BC male then delays the transition of the next OC to the BC morphotype, causing it to attain a larger size following its metamorphosis. BC males dominate OC males, regardless of their size, and suppress the growth of SM.

1.1.4 Rational

Freshwater prawn farming can be conducted by unskilled rural people on small establishments and prawns are consumed domestically by all social classes. This differs considerably from marine shrimp farming, which is controlled by a small number of individuals and is primarily for export (New *et al.*, 2000). It is an activity that can play a role in poverty reduction and the empowerment of women. It has aided

in increasing job opportunities for women and increasing their contribution to household income in developing countries (Ahmed, 2005). Women who participated had been allowed some economic independence and a more important role in household decision making ranging from the education of children to family planning (Ahmed, 2005).

1.2 Status of freshwater prawn farming

The giant freshwater prawn is commercially one of the most important crustaceans, being widely fished and reared in ponds and rice fields throughout its natural distribution and beyond (New, 2000a). By 1987 global production of farmed *M. rosenbergii* was estimated to be around 27,000 tons per annum (New, 1990). A large amount of that production has taken place in China and there has been rapid expansion in India and Bangladesh (New, 2005). Today, it is widely cultured all over the world. In the last decade, annual average of *M. rosenbergii* production rose by some 9–35.5% in value. In 1993, the overall production was 17,164 tons, worth US$ 116,799,000 and in 2005 it reached 205,033 tones with a net value of US$ 896,263,000 (FAO, 2007). Giant freshwater prawn farming is thus a major contributor to global aquaculture, both in terms of quantity and value. While the vast majority of *M. rosenbergii* is farmed in Asia, significant producers also include Africa, Israel, and Central and South America. Production also occurs in countries that at first seem unlikely candidates for a tropical species. Russia has also recently begun to culture the species using heated effluents from electrical power plants. There has been a very rapid global expansion of freshwater prawn farming since 1995 (Fig. 4). This is mainly because of the huge production of China but also, in the last few years, because of a rapid take–off of farming in India and Bangladesh. China is by far the

leading producer with over 128,300 tons. Vietnam was the second in the list with 28,000 tons.

Global expansion is difficult to predict, since it depends mainly on the evolution of consumer demand. However, even if a very modest expansion of 10 percent year^{-1} occurs, global farmed production of *M. rosenbergii* will have significantly exceeded 400,000 tonnes by 2010.

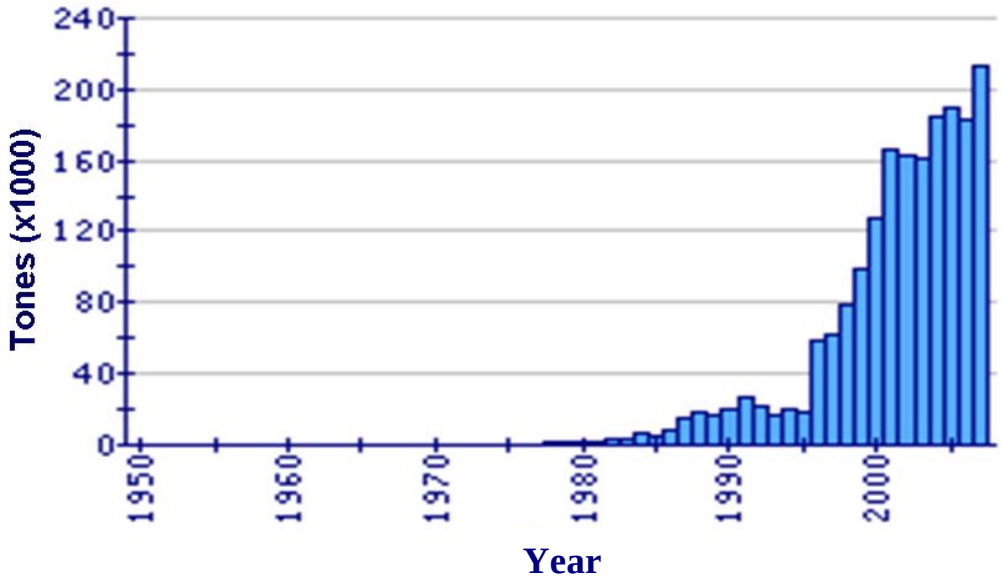


Fig. 4. Global aquaculture production of *M. rosenbergii* (FAO, 2008)

In Vietnam, the giant freshwater prawn *Macrobrachium rosenbergii* is indigenous to the Mekong Delta and is becoming an increasingly important target species. The culture of this species has been a traditional activity. *M. rosenbergii* is cultured in many ways, but combined rice–prawn farming and fence culture are the most important production models. Freshwater prawn farming began in the early 1980s in Vietnam, to supplement the capture fisheries production. The scale of farming activities is now substantial but it is difficult to assess the volume of its national production. According to New (2005), Vietnam was the second in the top 15 producers of freshwater prawn in 2001, with a production of 28,000 tons. However,

the actual production level is a little uncertain because it is included in the general FAO category "freshwater crustaceans not elsewhere included" while no specific figures for farmed *M. rosenbergii* production appear in the FAO statistics. Estimated output under this category increased from 10,000 tons in 1990 to 16,500 tons in 1995. Since 1996, actual data in this statistical category reported to FAO by Vietnam ranged between 2,500 tons and 6,250 tons in the period 1996–2005. It is not clear whether this entire category consists of freshwater prawns or even if all the production has been recorded and reported. The production of farmed freshwater prawns in the Mekong Delta region, the main farming area, had risen from 2500 tons in 1995 to 3000 tons in 1998 and was still expanding in 1999. One publication issued by the Ministry of Fisheries of Vietnam in 2003 stated that the national production of farmed freshwater prawns was 10,000 tons in 2002. Thus, the statistical situation is rather cloudy and will remain so until Vietnam separates its farmed freshwater prawn production from other aquaculture statistical categories when reporting to FAO. However, it seems likely that Vietnamese production already well exceeds 10,000 tons $year^{-1}$. The total area of rice–prawn culture was estimated to be around 750 ha in 2003 and fence culture consisted of 1516 units in 2002 (Phuong *et al*., 2004). Semi–intensive pond culture is also increasingly performed. The giant freshwater prawn is a high–value species, its culture by impoverished farmers is considered to have the potential to raise income and contribute to enhance rural development. Therefore, Vietnam's Ministry of Fisheries has put forth that the annual production of *M. rosenbergii* must reach 60,000 tons using 32,000 ha by 2010 (Government of Vietnam, 2000).

Typically, *Macrobrachium* have been reared in conjunction with rice production in Vietnam, based on wild fry and little or no feed, achieving low productivity (150–300

kg ha^{-1} $crop^{-1}$). Now that hatchery raised PL are more readily available, many farmers in the Mekong Delta are using both trash fish and artificial feeds for a 6 month culture period (leaving another 6 months for two crops of rice in one year) to achieve 1000–1500 kg ha^{-1} $crop^{-1}$ (Ridmontri, 2002). All major seafood processors in the Mekong Delta and some feed companies have become involved in hatchery development and feed supply for freshwater prawn farming, and see processed prawns as an important export item.

1.3 Responsible aquaculture practices

The culture of *Macrobrachium* spp. is less likely to have a detrimental impact because freshwater prawns cannot be reared at densities as high as those commonly used in marine shrimp farming. Productivity is generally lower, management is less labour intensive, and the potential for the abuse or waste of resources is minimal, and (unlike the inland culture of marine shrimp) the grow–out of *Macrobrachium* does not make agricultural land saline. Specific negative effects of *M. rosenbergii* culture on the environment have yet to be documented. Adherence to the FAO Code of Conduct for Responsible Fisheries would ensure that it remains sustainable and responsible. It is particularly well–suited to small long–term family businesses, can be practiced by relatively unskilled fishing and rural people, generates products which may be consumed by all social classes, and is amenable to integration with crop production.

2. The situation of freshwater prawn seed production and problem definition

2.1 Global status

A major constraint in the large scale aquaculture of *M. rosenbergii* is the adequate supply of seed at required quantities and in all locations. The majority of seed used in grow out farming of *M. rosenbergii* comes from hatcheries (Murthy *et al*. 2004).

Existing hatcheries are not producing up to their installed capacity due to various constraints. Availability of healthy and high–quality seedlings has always been a major obstacle in the expansion of *M. rosenbergii* culture. One of the major factors that is hampering the quality of hatchery–reared seed, culminating in low yield because of mass mortality, is disease. Outbreaks of diseases in prawns are often attributed to bacterial infections (Sung *et al*., 2000; Phatarpekar *et al*., 2002; Al–Harbi, 2003). Average survival in commercial hatcheries in India is about 30% (Murthy and Satheesha 1998; Murthy *et al*., 2004). Mass mortality of larvae in the hatcheries restricts the regular production of high quality post–larvae. Such mortality is often attributed to opportunistic pathogenic bacteria. (Skjermo and Vadstein, 1999). *Vibrio*'s have been reported as the causative agents for numerous disease outbreaks (Alavandi *et al*., 2004; Kennedy *et al*., 2006) and are often reported as a major problem in prawn hatcheries (Nayak and Mukherjee, 1997; Jayaprakash *et al*., 2006). Antibiotics and disinfectants, the conventional approaches to control bacteria populations in prawn hatcheries, are quite popular. However, they have begun to be withdrawn from the market in many countries owing to concerns about public health and environmental safety (Schneider *et al*., 2003).

The major disease problems affecting *Macrobrachium rosenbergii* generally occur because of poor treatment of the intake water, poor husbandry, overcrowding, poor sanitation, and non–existent or inadequate quarantine procedures. The measures to combat these problems are referred to as improved husbandry. The table below (FAO, 2002) lists the most important diseases.

Table 1. Common diseases and control measures in prawn farming and/or hatchery. In some cases antibiotics and other pharmaceuticals have been used in treatment but their inclusion in this table does not imply an FAO recommendation (FAO, 2002).

DISEASE	AGENT	TYPE	SYNDROME	MEASURES
MMV (*Macrobrachium* Muscle Virus)	Parvo–like virus	Virus	Infected tissue becomes opaque, with progressive necrosis; affects juveniles	Improved husbandry (IH)
WSBV (White spot Syndrome BaculoVirus)	Baculovirus	Virus	White spots; affects juveniles	IH
Unnamed viral disease	Nodavirus	Virus	Whitish tail; affects larvae	IH
Black spot; brown spot; shell disease	*Vibrio*; *Pseudomonas*; *Aeromonas*	Bacteria	Melanised lesions; affects all life stages, but more frequently observed in juveniles & adults	IH; oxolinic acid; nifurpurinol
Bacterial necrosis	*Pseudomonas*; *Leucothrix*	Bacteria	Similar to black spot but only affects larvae, especially stages IV & V	IH; nifurpurinol; erythromycin; penicillin–streptomycin; chloramphenicol
Luminescent larval syndrome	*Vibrio harveyi*	Bacterium	Moribund & dead larvae luminescent	IH; chloramphenicol; furazolidone
White postlarval disease	Rickettsia	Bacterium	White larvae, especially stages IV and V	IH; oxytetracycline; furazolidone; lime, prior to stocking
Unnamed fungal infection	*Lagenidium*	Fungus	Extensive mythelial network visible through exoskeleton of larvae	IH; trifluralin; merthiolate

Unnamed fungal infection (often associated with IMN – see below)	*Fusarium solani*	Fungus	Secondary infection; affects adults	IH
Unnamed yeast infections	*Debaryomyces hansenii*; *Metschnikowia bicuspidata*	Fungi	Yellowish, greyish or bluish muscle tissues in juveniles	IH
Protozoan infestations	*Zoothamnium*; *Epistylis*; *Vorticella*; *Opercularia*; *Vaginicola*; *Acineta*; *Podophyra*; etc.	Protozoans	External parasites that inhibit swimming, feeding and moulting; affect all life stages	IH; formalin; merthiolate; copper–based algicides
IMN (Idiopathic Muscle Necrosis)	[environmental disease]	Unknown	Whitish colour in striated tissue of tail and appendages; when advanced, necrotic areas may become reddish; affects all life stages	IH
MCD (Mid–Cycle Disease)	[undetermined aetiology]	Unknown	Lethargy; spiralling swimming; reduced feeding and growth; bluish–grey body colour; affects larvae, especially stages VI and VII	IH; hatchery disinfection
EED (Exuvia Entrapment Disease), sometimes known as MDS (Moult Death Syndrome)	[undetermined aetiology]	Unknown but probably multiple causes, including nutritional deficiency	Localised deformities; failure to complete moulting; affects late larval stages; also seen in postlarvae, juveniles & adults	IH; dietary enrichment

2.2 In Vietnam

The first hatchery was established in 1975 (Thang, 1995). However, initial research on the larval rearing of *M. rosenbergii* commenced in the early 1980s. Basic research using rearing systems such as the open clear water system, the closed clear water system and the green water system was conducted (Phuong *et al*., 2006). At that time, the open clear water system was attributed better results when compared to other systems, and it was applied in most commercial hatcheries. Several hatcheries were then established near Ho Chi Minh City and in the Mekong Delta. However, these hatcheries faced difficulties because they required larger amounts of sea water for the daily exchange of water. Since 1998 a new rearing system referred to as 'the modified stagnant green water system' has been applied and transferred widely through the Mekong Delta and to other parts of Vietnam. The advantage of this system is maintenance of water quality by natural microalgae (particularly *Chlorella* spp.) in the rearing water which reduce water demand. The survival of larvae until they reach the postlarvae–15 stage (15 days after metamorphosis) in the green water system averages about 40% (ranging 30–75%) compared to 35–50% for open systems, or 15.2–66.2% for recirculating systems (Phuong *et al*., 2006).

Currently however, lack of a stable seed supply was a significant obstacle to the further expansion and development of rice–prawn and pond farming systems. In order to address the needs of this burgeoning industry, basic and applied research has been conducted in order to develop appropriate seed production technology and verify the use of artificial seed in rice–prawn farming in the Mekong Delta (Phuong *et al*., 2006).

3. Broodstock

Broodstock of giant freshwater prawn usually refers only to the females that are kept in hatcheries until their eggs hatch. Freshwater prawn eggs are carried under the tail of the adult female prawn (known as 'berried' or ovigerous females) and are easily visible. In the tropics, berried females can be obtained year round from farm ponds containing adult animals but the quantity of berried females available may vary according to the time of year. They can be obtained by cast netting but are frequently selected at times of partial or total harvest. Berried females can also be obtained from rivers, canals and lakes in areas where they are indigenous (native). Some hatcheries prefer to use berried females from natural waters based on the belief that wild females produce better quality larvae than pond–reared ones. However, collecting ovigerous females from the wild often results in considerable egg loss during transport, therefore many hatcheries prefer to use adjacent rearing ponds for their broodstock supplies.

3.1. Genetic considerations

Freshwater prawns that originate from eggs that hatch early appear to have an advantage in grow–out because they are the first to establish themselves as dominant blue claw males (BC). However, there is no evidence that these 'early hatchers' have any genetic advantage over the 'late hatchers'. Therefore it would be pointless to select larvae from only one part of the spawning period to stock larval tanks. Moreover, selecting eggs from only one part of the spawning period could lead to a reduction in genetic variation and an increase in inbreeding. Proper genetic resource management combines selection and conservation of genetic diversity (Tave, 1996, 1999).

Most farmers select larger females, which usually carry more eggs, but this may not be good practice. Selecting fast–growing, berried females from ponds three months after they were stocked, rather than choosing large females six months after stocking, has a positive genetic effect on weight at harvest. Collecting the faster growing females and rearing them in dedicated broodstock ponds would enable to use selection to improve grow–out performance and also give the ability to hold the animals until their clutch size becomes larger (after later mating moults). Cutting off one of the eyestalks (ablation) of female broodstock increases the number of mature females in a captive broodstock and diminishes the time between each spawn. Young females (about 4 months old after stocking at PL size) spawn about 20 days after eyestalk ablation and spawn again after about 30 days (New, 2002).

Yen (1999) conducted a study on the effects of broodstock origin on the survival of larvae applying water exchange methods. this author found that the survival of larvae until the postlarva–15 stage was 51.9% and 16.5% for broodstock of Thai and Vietnamese origin, respectively. Amrit and Yen (2001) reported that PL survival (up to 36 days) was much higher (33%) when broodstock *M. rosenbergii* from Thailand were used instead of those from Vietnam (<2%). Furthermore, cross–bred Thai females and Vietnam males resulted in an even higher PL survival (39%). This work indicates the importance of comparative work on the various strains of freshwater prawns, and the potential improvement that could be gained not only from selection and cross breeding but also from examining whether different strains have different rearing requirements.

Broodstock development in ponds adjacent to a hatchery is a feasible and efficient option. It allows the hatchery access to suitable sized berried prawns throughout the year and also reduces transportation stress and cost of using wild stocks.

Thanh *et al*. (2009) studied the growth performance of three strains of giant freshwater prawn that originated from geographically separated locations in a complete (3×3) diallel cross (cross–red) as a starting point for a stock improvement program for the industry in Vietnam. Evaluating reproductive characteristics and offspring quality of different prawn strains could also be considered as a first step in the development of selective breeding programs. These authors rightly noted that in this respect, very little efforts have been made on crustacean species. In their study they found significant differences in growth performance between crosses.

During grow–out there is tendency for the performance (growth rate, survival, FCR) of farmed *Macrobrachium rosenbergii* to decline after several production cycles when the berried females used in the hatcheries have been drawn from grow–out ponds. This phenomenon, caused by inbreeding and sometimes called as genetic degradation, has been noticed in a number of countries including Martinique, Taiwan Province of China, and Thailand. In countries where *M. rosenbergii* is indigenous the problem has occurred because of the 'recycling' of animals (broodstock for hatcheries being obtained from grow–out ponds and the process being repeated for many generations). In countries where this species is not indigenous the problem may be worse because the farmed stock normally originated from a very small number of females (or PL), that were introduced to the country many years ago. When the problem of declining yields (and therefore incomes) occurs, it naturally results in the initial enthusiasm of farmers fading. The solution to the problem must be two–fold: using more wild broodstock, and genetic improvement (FAO, 2002).

3.2 Broodstock nutrition

The nutrition of broodstock *M. rosenbergii* has not received the same level of attention as that of marine shrimp. Information about specific nutritional requirements

is limited and probably results from the lack of a need for routine maintenance of captive broodstock in many commercial hatcheries. Eggs can be easily hatched from females obtained from either grow–out pond or capture fisheries. Broodstock nutrition would probably become a focus of research if interest in domestication through genetic improvement develops. In addition the knowledge that the nutritional history of broodstock influences the health and performance of larvae underlie the importance of understanding broodstock nutrition, particularly as the need for consistency in commercial production arises (D'Abramo and New, 2009).

A successful semi–purified broodstock diet (44% protein, 9% lipid) for *M. rosenbergii* was used in reproductive performance trials by Cavalli *et al*. (2001). The formulation included, inter alia, casein, lobster meal, soy protein isolate, squid meal, shrimp meal, crab meal, synthetic amino acids, soybean oil, fish oil, a protein extract attractant, astaxanthin, soy lecithin, vitamins and minerals, together with a carrageenan binder and anti–oxidants. The authors used this semi-purified diet to study requirements for a number of nutrients. Cavalli *et al*. (1999) studied the effect of linoleic acid (18:2*n*–6) and *n*–3 highly unsaturated fatty acids (HUFA) on the reproduction performance and offspring quality of the freshwater prawn during 180 days. They found that feeding *M. rosenbergii* broodstock with high levels of 18:2*n*–6 and *n*–3 HUFA (13 and 15 mg g^{-1} DW of food respectively) improved fecundity, egg hatchability, and the overall quality of the larvae. Larvae hatched from females fed diets containing HUFA exhibited a higher tolerance to stressful conditions created by high levels of ammonia. However, manipulation of the level of dietary phospholipid in broodstock diets (Cavalli *et al*., 2000) had no significant effect on larval quality.

In addition, these authors showed that under proper rearing conditions, *M. rosenbergii* females are able to spawn up to five times within a 180–day period, compared with

three to five times as reported for wild populations. They also indicated that the number of viable larvae produced might be increased by *in vitro* incubation. However, disinfecting the eggs may be necessary to replace the loss of the anti–microbial secretions of the incubating female.

Das *et al*. (1996) evaluated 12 diets for broodstock rearing. These diets were composed of various ingredients and contained different levels of gross energy, protein and lipid. Based on results for fecundity and per cent hatching rate of the eggs, the recommended broodstock diet contained 40% crude protein and a gross energy level of 400 kcal $(100\ g)^{-1}$ diet. The other diets that produced high fecundity and hatching rate contained 40% protein and a gross energy/protein ratio of 92 to 99. Increases in fecundity were realised when the level of dietary fishmeal were increased to 16-18%. Dietary protein with an essential amino acid index similar to that of the eggs may contribute to the overall quality of broodstock diets.

4 Larval rearing: zootechnical and nutritional aspects

4.1 Rearing systems and larval density

Some seed (postlarvae; juveniles) is still obtained from the capture fishery where *M. rosenbergii* is indigenous, but most is now hatchery–reared. The first larval stage is just less than 2 mm long and grows through 11 larval stages, to almost 8 mm at metamorphosis into postlarvae. Individual metamorphosis can be achieved in as little as 16 days but usually takes much longer, depending on environmental conditions. In commercial hatcheries, most larvae metamorphose by day 32–35 at the optimum temperature (28–31°C). Larval rearing typically occurs in 12 g l^{-1} brackish water, and hatcheries are either flow–through (where a proportion of the rearing water is regularly replaced) or recirculating (where a variety of systems involving physical and

biological filters are used to minimise water use). Either type of hatchery may be inland or coastal. Inland hatcheries produce brackish water by mixing freshwater with seawater transported from the coast, brine trucked from salt pans, or artificial seawater. Some flow–through hatcheries use a "green–water" system, which involves fertilization to encourage the growth of phytoplankton (mainly *Chlorella* spp.), which is believed to improve water quality and increase larval survival; others operate a "clear–water" regime. Feeding systems vary widely but typically include brine shrimp (*Artemia*) fed several times per day at first, diminishing to a single daily feeding by larval stage X. Prepared feed (usually an egg custard containing mussel or fish flesh, squid, or other ingredients) is introduced at stage III–V and its feeding frequency is increased towards metamorphosis.

There are several studies on larval rearing of giant freshwater prawn aiming at the improvement of larval survival using different rearing methods, feeds, stocking density, and origin of prawn. Since a few reports have reported rearing of *M. rosenbergii* larvae at higher densities, there might also be room to intensify larval rearing of prawn. However, increasing larval density, feed ration and feeding frequency consequently need to be appropriately adjusted. Thang (1993) found that the survival of larvae until the postlarvae–15 stage could attain 35–50% for open systems, or 15–66% for recirculating systems. Hung and Phuc (1999) reported that rearing larvae using clear–water methods showed a variation of survival of larvae until postlarvae–15 from 24 to 53%. Tinh (1999) studied the effects of stocking density on the survival of larvae until the postlarvae–15 stage using clear–water methods, finding that at a stocking density of 80–100 larvae l^{-1}, survival could reach 40%.

Other studies investigated the interaction of larval stocking density and larval rearing system. Phuong *et al*. (2003) reported larval survival of 53, 29, 32 and 27% when stocking larvae at 30, 60, 90 and 120 l^{-1} respectively in a recirculation system. Meanwhile, in a modified stagnant green water system, larval survival was significantly increased to 92, 46, 46 and 32% when larval stocking at 30, 60, 90 and 120 l^{-1} respectively. Based on the survival, although lower stocking densities yield higher survival, it can be calculated that medium densities provide greater numbers of postlarvae upon completion of the rearing cycle. Very high stocking levels lead to decreased survival and thus decreased efficiency (Phuong *et al*., 2006). Larval survival relates to the cannibalism of *M. rosenbergii* larvae which depends on the feeding regime and/or feed ration (Coyle *et al*. 2004).. Ohs *et al*. (1998) showed different proportions of cannibalism, namely 35.0 and 9.3% in freshwater prawn larvae fed artificial diets and live *Artemia* nauplii, respectively. Diaz *et al*. 1990 compared survival of *M. rosenbergii* juveniles of which the tip of the propodus of both chelae was removed with untreated animals. Survival of the treated group was 92%, while that of the untreated group was only 25%.

4.2 The role of live food and feeding regime

The food for culture of *Macrobrachium rosenbergii* larvae consists basically of newly hatched *Artemia* nauplii (Lavens *et al*., 2000) supplemented with inert food (Valenti and Daniels, 2000). Most freshwater prawn larvae do not feed on the first day (hatching day). However, it is recommended to provide some *Artemia* nauplii in the late afternoon of the first day because some larvae begin to eat early. From day 2 until day 4, *Artemia* nauplii are provided five times per day, with the last and main feeding in the evening. After that, the number of *Artemia* nauplii feeding per day can gradually be reduced until, by day 10, only *Artemia* nauplii are given at the evening

feeding time. The evening food should be given as late as possible (18.00–19.00). The amount of *Artemia* nauplii given at each feeding time depends on the visual examination of the rearing tanks. Freshwater prawn larvae do not actively search for food, which is why *Artemia* nauplii (that swim actively in the same part of the water column as the larvae) are such a suitable feed. The ideal is therefore to have *Artemia* nauplii always present in the tanks in sufficient numbers for the larvae to encounter. The amount of *Artemia* nauplii required at any one time depends primarily on the tank volume, not on the number of larvae present, although the latter of course controls the rate at which *Artemia* nauplii are consumed. There should be about 3–6 *Artemia* nauplii ml^{-1} directly after feeding, depending on the age of the prawn larvae, and 1 nauplii ml^{-1} left in the water just before the next feeding time. If there is more than 1 nauplii ml^{-1} at the latter time then they have been overfed or the larvae are not feeding well. If there is less than 1 nauplii ml^{-1}, then the amount of *Artemia* nauplii should be increased.

Nowadays, a variety of larval feeds are used during the hatchery phase of production of the giant freshwater prawn. Apart from nauplii of the brine shrimp (*Artemia* spp.), freshwater cladocerans (*Moina* spp.), fish eggs, squid flesh, frozen adult *Artemia*, flaked adult *Artemia*, fish flesh, egg custard, worms and commercial feeds are also used. Many alternative feeding systems exist and are applied with locally available feeds.

Generally, live food supplies all the necessary nutrients for development and can contribute with exogenous digestive enzymes that aid in digestion (Kurmaly *et al*., 1990; Jones *et al*., 1993; Kamarudin *et al*., 1994). Feed consumption by larvae is dependent upon numerous factors related to site-specific culture conditions, and scientific knowledge is not enough to derive an adequate feeding schedule.

Hatcheries, in general, either maintain a constant *Artemia* supply (about 5 nauplii ml^{-1} day^{-1}) or increase the density over the culture period (Aquacop, 1983; Daniels *et al*., 1992; Valenti *et al*., 1998).

Seed production of giant freshwater prawn, depends heavily on live *Artemia* nauplii, which are both costly and nutritionally inconsistent (Sorgeloos and Leger, 1992). Knowledge of nutritional requirements of larval *M. rosenbergii* is an essential prerequisite for the formulation of a nutritionally adequate and balanced diet. However, dietary information is scarce although larval rearing techniques have been successful and improved over the last 30 years (Ling, 1969). Since *Artemia* nauplii that have molted into the second instar stage are non–selective particle feeders, simple methods have been developed to incorporate different kinds of products into the *Artemia* prior to feeding to predator larvae. This method of "bioencapsulation", also called *Artemia* enrichment or boosting, is widely applied in marine fish and crustacean hatcheries for enhancing the nutritional value of *Artemia* with essential fatty acids. As the nutritional quality of commercially available *Artemia* strains is relatively poor in some essential highly unsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), it is essential and common practice to enrich these live prey organisms prior to feeding to the larvae. Many researchers have developed various enrichment products and procedures using selected microalgae, micro–encapsulated products, yeasts, self–emulsifying concentrates, and micro–particulate products, either singly or in various combinations (Léger *et al*., 1986). In the 1980s, most attention was dedicated to the presence of EPA in *Artemia* as a guarantee for successful production of marine fish larvae (Watanabe *et al*., 1983; Léger *et al*., 1985). However, in the late 1980s and early 1990s, more attention was paid to the level of DHA, since good survival appears to be

correlated with EPA, but DHA tends to improve larval quality and growth. Several studies revealed the importance of DHA, more particularly the requirement for high DHA/EPA ratio in promoting growth, stress resistance, and pigmentation (Reitan *et al*., 1994; Lavens *et al*., 1995).

The application of enriched live food and supplementation with marine oil that is rich in n–3 fatty acids in artificial diets has proved to improve the larval growth of *M. rosenbergii* (Sorgeloos and Leger, 1992; Alam *et al*., 1995). However, high–quality marine oils are costly and often unavailable. To reduce the cost of dietary components, successful approaches include the blending of marine oils with vegetable oils or animal fat (Borlongan and Parazo, 1991).

4.3 The approaches of live food replacement

The need for live food is considered a limiting factor in commercial hatchery operations. Although *Artemia* has proven to be successful for raising the larvae of many species, including *Macrobrachium*, inherent problems persist. Imported *Artemia* cysts are predominantly used, which are expensive and uncertain in availability. In addition, seasonal environmental fluctuations in the Great Salt Lake (USA), a major cyst producing site, are a constant concern to the aquaculture industry (Lavens and Sorgeloos, 2000). Complete dependency on *Artemia* as feed not only makes hatchery operations expensive, but also unsustainable. Hence, there is a need to look for alternative acceptable diets to replace *Artemia* and reduce the cost of larval prawn rearing. Accordingly, there were several studies to evaluate formulated inert larval diets to replace *Artemia* partially in freshwater prawn hatchery.

Nutritionally complete, formulated diets are seen as attractive and valuable alternatives to live food. Therefore, numerous attempts have been made to develop

formulated diets that effectively replace live food, but most have not been successful. Often the diets are used as supplements because growth and survival are compromised when used exclusively. Formulated diets developed for larvae fall into three categories: micro–encapsulated, micro–bound, and micro–coated (Tucker, 1998). To achieve long–term survival and growth of larvae, a diet must be attractive, palatable, digestible, and at least equivalent to the nutrient composition of live food. Equally important are physical characteristics such as water stability and neutral buoyancy (Bengston, 1993).

The development of a nutritionally sound and locally available artificial diet is highly desirable to reduce hatchery operational costs and thus pave the way for the further development of freshwater prawn aquaculture. Hien *et al*. (2005) developed a simple custard diet containing egg, powdered milk, and different sources of lipids. A basic recipe containing powdered milk (53.8% w/w), chicken egg yolk (41.7% w/w), 3% squid oil and 1.5% lecithin yielded satisfactory survival and body length of postlarvae. Increasing dietary lecithin from 1.5% to 3.0% did not increase larval growth rates; for this reason, supplementation at 1.5% was considered most effective in terms of cost. D'Abramo *et al*. (2002) also reported the successful development of a formulated micro–bound feed to replace the use of *Artemia* for larval stages V–XI and for PL *M. rosenbergii*. The formulated diet was moist (60% moisture) and could not be reduced to particles below 0.25mm unless previously dried. The diet was energy dense (46% protein and 37% lipid).

Kovalenko *et al*. (2002) also developed a micro–bound diet that replaces live food for the larval culture of the freshwater prawn. A high moisture (63–71%), semi–purified micro–bound diet containing alginate was compared with newly hatched live *Artemia*

nauplii as an exclusive diet for the culture of larval *M. rosenbergii* from 5th stage to postlarvae. The growth of larvae fed the micro–bound diet was 90% of that achieved for larvae fed newly hatched nauplii of *Artemia*. Survival of larvae fed the micro–bound diet was 77.3%, and was not significantly different from that of *Artemia* fed larvae. Composed of readily available ingredients, the diet contained 46.2% crude protein and 37.4% crude lipid, was easy to prepare and had good water stability. The diet has the potential to serve as an economically practical alternative to the fluctuating cost, nutrient uncertainty and labour associated with the use of *Artemia* nauplii hatched from cysts. The characteristics of the diet suggest high potential for successful application to the larviculture of other fish and crustacean species, in either the existing or a modified state (Tidwell *et al*., 2005).

Lipids are known to play an essential role in the metabolism of crustaceans to reproduction and early larval development (Teshima, 1972, 1997; Middleditch *et al*., 1980; Teshima and Kanazawa, 1983; Harrison, 1990). Apart from being a major source of metabolic energy and the main form of energy storage, lipids also supply essential fatty acids needed for the maintenance and integrity of cellular membranes, and serve as precursors of steroid and moulting hormones (Teshima, 1972; Harrison, 1990). For the freshwater prawn *Macrobrachium rosenbergii*, however, most studies that have investigated lipid metabolism and requirements have been restricted to larval and juvenile stages (Devresse *et al*., 1990; Sheen and D'Abramo, 1991; Teshima *et al*., 1992, 1997; D'Abramo and Sheen, 1993; Querijero *et al*., 1997; Roustaian *et al*., 1999).

5. The use of N–acyl homoserine lactone (AHL)–degrading bacteria as a tool to control diseases in aquaculture seed production.

5.1 Quorum sensing – a means of bacterial communication

Bacteria communicate with one another using chemical signalling molecules. They release, detect, and respond to the accumulation of these molecules, which are called autoinducers. Detection of autoinducers allows bacteria to distinguish between low and high cell population density and to control gene expression in response to changes in cell numbers. This process, termed "quorum sensing", allows a population of bacteria to co–ordinately control gene expression of the entire community. Many bacterial behavioural responses are regulated by quorum sensing, including symbiosis, virulence, antibiotic production, and biofilm formation (Schauder and Bassler, 2001).

5.2 Disruption of quorum sensing (QS)

There are two major strategies for the control of bacterial infection, either to kill the organism or to attenuate its virulence such that it fails to adapt to the host environment and is readily cleared by the innate host defences. The discovery that bacteria employ low–molecular–weight pheromones, namely quorum sensing (QS) molecules, to regulate the production of secondary metabolites and virulence determinants offers a target for the second approach (Finch *et al*., 1998).

Disruption of bacterial quorum sensing has been proposed as an alternative anti–infective strategy and several techniques that could be used to disrupt quorum sensing have been investigated. These techniques comprise:

(1) the inhibition of signal molecule biosynthesis;

(2) the application of quorum sensing antagonists;

(3) the chemical inactivation of quorum sensing signals;

(4) signal molecule biodegradation by bacterial enzymes;

(5) the application of quorum sensing agonists.

The few reports that deal with disruption of quorum sensing of aquatic pathogens, together with the results obtained with human and plant pathogens indicate that this approach has potential in fighting infections in aquaculture (Defoirdt *et al*., 2004).

5.2.1 Inhibition of signal molecule biosynthesis

A first quorum sensing disrupting technique aims at inhibiting signal molecule biosynthesis. In many cases, homologues of the *Vibrio fischeri* LuxI protein catalyse the biosynthesis of Gram–negative N–acyl homoserine lactone (AHL) signal molecules, using acyl–acyl carrier proteins (for the acyl chain) and S–adenosylmethionine (for the homoserine lactone moiety) as substrates (Whitehead *et al*., 2001). In an attempt to block AHL biosynthesis, Parsek *et al*. (1999) found that analogues of S–adenosylmethionine (such as S–adenosylcysteine) could inhibit activity of the *Pseudomonas aeruginosa* LuxI homologue RhlI by up to 97%. Database research revealed that no AHL synthase sequence motifs were present in other enzymes with S–adenosylmethionine binding sites. Therefore, it might be possible to use the S–adenosylmethionine analogues as specific quorum sensing inhibitors, without affecting other vital processes in prokaryotic and eukaryotic organisms.

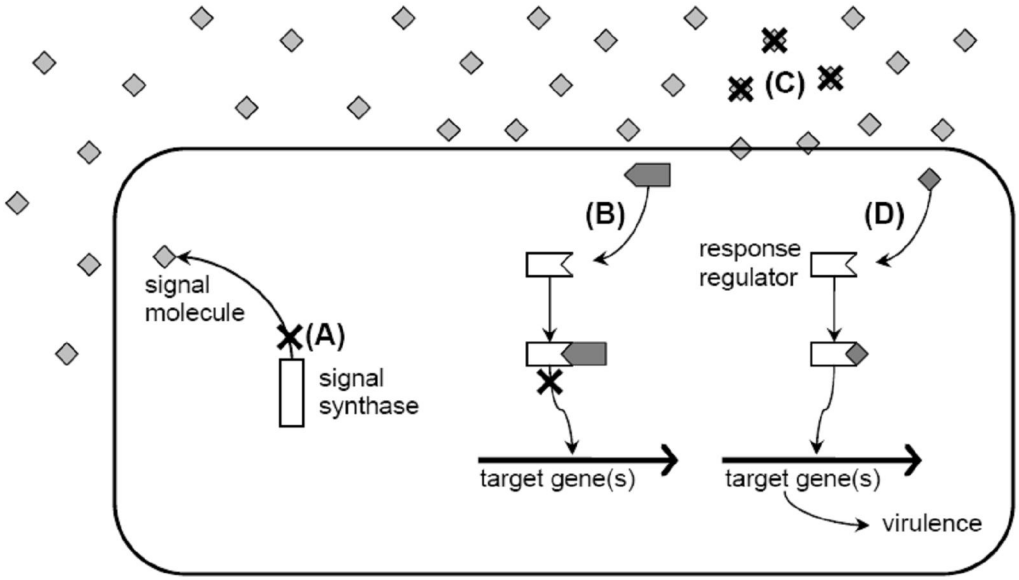


Fig. 5. Schematic overview of different strategies that have been developed to disrupt bacterial quorum sensing. (A) Inhibition of signal molecule biosynthesis by the application of substrate analogues. **(B)** Blocking signal transduction by the application of quorum sensing antagonists. **(C)** Chemical inactivation and biodegradation of signal molecules. **(D)** Application of quorum sensing agonists to evoke virulence factor expression under unfavourable conditions (Defoirdt *et al*., 2004).

Signal molecule biosynthesis inhibitors seem to be promising quorum sensing disrupting compounds. However, further research is necessary to elucidate whether these inhibitors would be useful to combat infections in aquaculture since there are currently no data about their impact on virulence expression of pathogenic bacteria.

5.2.2 Application of quorum sensing antagonists

It was the applied research for compounds capable of preventing or disrupting bacterial biofilm formation that led to the discovery of the first quorum sensing "signal–mimic" compounds. The marine red alga *Delisea pulchra* was found to produce substances that were highly effective in preventing biofouling (De Nys *et al*., 1995). The active compounds comprise a range of halogenated furanones. Givskov *et*

al. (1996) recognized that these halogenated furanones were similar in structure to N–acyl homoserine lactones. They subsequently demonstrated that these halogenated furanones inhibited AHL–regulated behaviours in a variety of Gram–negative bacteria. Thus, the furanones appear to mimic the AHL signals of these bacteria, and most likely do so by binding to the AHL receptor, LuxR–like protein (Manefield *et al*., 1999).

Although all of the *D. pulchra* quorum sensing mimic compounds have inhibitory effects, higher plants secrete a variety of signal mimics that stimulate quorum sensing–regulated behaviours as well as mimic substances that inhibit quorum sensing–regulated behaviour (Teplitski *et al*., 2000). The compounds responsible for these signal–mimic activities in higher plants have not been identified yet, but their effects seem to be specific to quorum sensing–regulated gene expression (Bauer and Robinson, 2002).

A large number of other AHL–based molecules were reported to have quorum sensing inhibiting activity. The AHLs with longer acyl chains identified as autoinducers in one quorum sensing system antagonize receptors normally agonized by AHLs with shorter acyl chains, as reported in *Aeromonas hydrophila*, *Chromobacterium violaceum* and *Vibrio fischeri*. An analogous array of racemic sulfonamides, some of which were antagonists of LuxR in *V. fischeri*, was also reported recently (review by Persson *et al*., 2005).

Altogether, the results mentioned above indicate that the application of quorum sensing antagonists in aquaculture might constitute an alternative approach for controlling infections caused by pathogens that regulate virulence factor expression by quorum sensing. Apart from that, the results obtained in the researches with the macro–alga *Delisea pulchra* and with the unicellular algae *Chlamydomonas* and

Chlorella indicate that algae might be useful to control infections in aquaculture as they disrupt the quorum sensing systems of pathogenic bacteria.

5.2.3 Chemical inactivation of quorum sensing molecules

The only chemical inactivation that has been studied so far is the reaction with oxidized halogen antimicrobials. These antimicrobials, at a concentration of approximately 0.14 mM, were found to decrease the concentration of 3–oxo–substituted AHLs to about 25% after 1 min incubation, but had no effect on unsubstituted ones (Borchardt *et al.*, 2001).

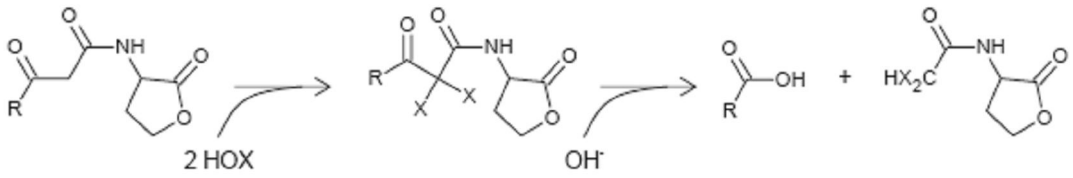


Fig. 6. Reaction between a 3–oxo AHL and halogen antimicrobials (HOX; hypobromous or hypochlorous acid) at pH 6. First, two α–halogenation reactions occur, yielding 2,2–dihalo–3–oxo AHL. Subsequently, the acyl chain is hydrolised, yielding a fatty acid and 2,2–dihalo–N–ethanoyl–L–homoserine lactone. The R is an alkyl group consisting of between 3 and 13 carbons which can have an oxo or hydroxyl substitution at the second carbon. Redrawn after Michels *et al*. (2000).

5.2.4 Enzymatic inactivation and biodegradation of quorum sensing molecules

The ability to degrade AHLs is widely distributed in the bacterial kingdom. *Variovorax paradoxus* isolated from soil by enrichment culture, is able to utilize AHL compounds as sole carbon, nitrogen and energy source. There may be various bacterial species in natural environments that can metabolize AHLs and disrupt QS regulation in nearby bacteria (Leadbetter and Greenberg, 2000). Some mechanistic insight how one bacterial species might interfere with QS regulation in another

species are provided in recent studies (Dong *et al.*, 2002; Dong and Zhang, 2005). These authors found that about 5% of the several hundred soil bacteria tested were able to inactivate AHLs. The AHL inactivation activity in a *Bacillus cereus* isolate was due to its synthesis and secretion of a lactonase capable of opening the homoserine lactone ring of AHLs, thereby reducing the effectiveness of the signal molecules by about 1000–fold (Bauer and Robinson, 2002).

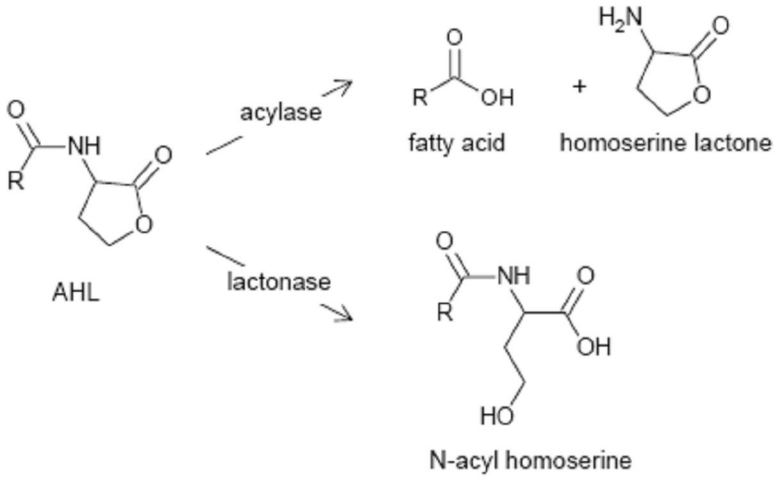


Fig. 7. Enzymatic inactivation of AHLs. Cleavage of the amide bond by an AHL acylase enzyme yields a fatty acid and homoserine lactone. Cleavage of the lactone ring by an AHL lactonase enzyme yields the corresponding acylated homoserine. The R is an alkyl group consisting of between 3 and 13 carbons which can have an oxo or hydroxyl substitution at the second carbon (Defoirdt *et al.*, 2004).

The data mentioned above indicate that bacteria that are able to degrade quorum sensing signal molecules might be useful as biocontrol agents in aquaculture. Hence, it is of interest to investigate whether signal molecule degraders would be good probionts. Apart from that, the reports dealing with AHL–degrading *Bacillus* spp. (Dong *et al.*, 2000; Molina *et al.*, 2003; Dong *et al.*, 2004) suggest that the positive effect of *Bacillus* spp., used as probionts in aquaculture (Moriarty, 1998; Rengpipat *et*

al., 2003), might partly be due to inactivation of quorum sensing molecules –apart from the production of growth–inhibiting substances.

5.2.5 Application of quorum sensing agonistic analogues

Mäe *et al*. (2001) have tested an opposite strategy. They activated quorum sensing–regulated virulence factor expression by using quorum sensing agonists. The idea behind this strategy is that by adding the signal molecule of a pathogen, virulence factor expression would be activated at low population density. Subsequently, the virulence factors could trigger the activation of the host defence system allowing resistance to develop (Defoirdt *et al*., 2004).

5.3 Combination of techniques in a general quorum sensing–disrupting strategy

Many pathogenic bacteria have been reported to regulate virulence by AHL– or AI–2–mediated quorum sensing. The list includes plant, animal and human pathogens, such as *Agrobacterium tumefaciens* (Hwang *et al*., 1994), *Clostridium perfringens* (Ohtani *et al*., 2002), *Neisseria meningitidis* (Winzer *et al*., 2002), *Pseudomonas aeruginosa* (Duan *et al*., 2003; Smith and Iglewski, 2003), *Streptococcus pneumoniae* (Joyce *et al*., 2004; Stroeher *et al*., 2003), *Streptococcus pyogenes* (Lyon *et al*., 2001) and different species belonging to the genera *Aeromonas* (Swift *et al*., 1999), *Erwinia* (Loh *et al*., 2002) and *Vibrio* (Milton *et al*., 2006). One appealing option could therefore be to develop a strategy that disrupts quorum sensing in all those species. This could be achieved by designing compounds that affect all types of quorum sensing–regulated response regulators (such as halogenated furanone analogues) or by combining techniques that disrupt AHL–mediated quorum sensing (such as AHL–degrading bacteria) with others that disrupt AI–2–mediated quorum sensing (such as LuxS inhibitors). However, nonspecific quorum sensing disruption might cause too

drastic disturbances in the microbial community that is treated and might also block favourable quorum sensing–regulated processes (such as for instance nodule formation by rhizobia; Pierson *et al*., 1997). From this point of view, it might be more sustainable to build a toolbox containing different quorum sensing–disrupting techniques and to apply one or a combination of the techniques only affecting the target species (or as few other species as possible). More knowledge about the effect of quorum sensing disruption on the (functioning of the) microbial communities to be treated will be necessary in order to make a sound decision on which techniques can and which cannot be applied in a certain situation.

5.4 The impact of quorum sensing disruption on the virulence of Vibrio harveyi

Unlike most other Gram–negative bacteria, *Vibrio harveyi* was found to use a multi–channel quorum sensing system (Fig. 8). The first channel of this system is mediated by the Harveyi Autoinducer 1 (HAI–1), an acylated homoserine lactone (AHL) (Cao and Meighen, 1989). The second channel is mediated by the so–called Autoinducer 2 (AI–2), which is a furanosyl borate diester (Chen *et al*., 2002). The chemical structure of the third autoinducer, called Cholerae Autoinducer 1 (CAI–1) (Henke and Bassler, 2004b). All three auto-inducers are detected at the cell surface and activate or inactivate target gene expression by a phosphorylation–/dephosphorylation signal transduction cascade. *In vitro* experiments with quorum sensing mutants indicated *Vibrio harveyi* quorum sensing system is a three–way coincidence detector, with bioluminescence of the cells dependent on the levels of the three signal molecules (Henke and Bassler, 2004b)

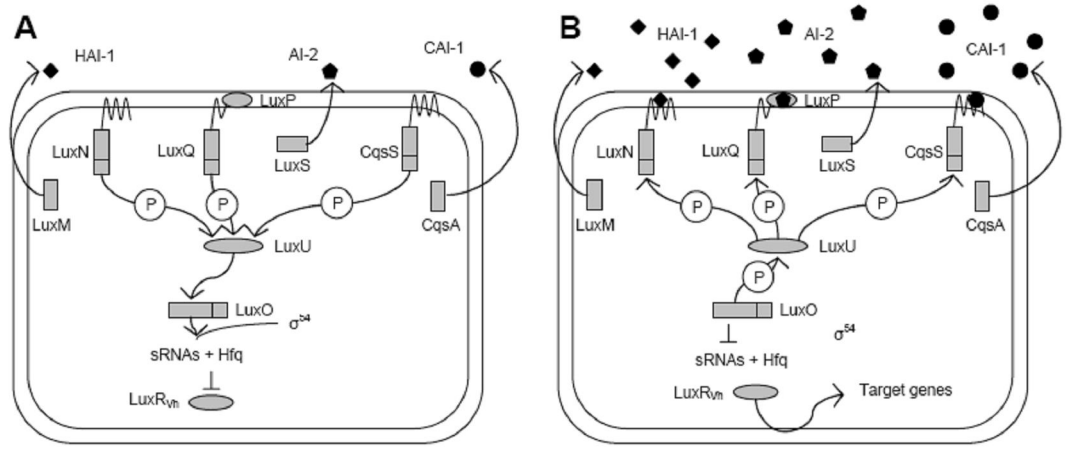


Fig. 8. Quorum sensing in *Vibrio harveyi*. The LuxM, LuxS and CqsA enzymes synthesise the autoinducers HAI–1, AI–2 and CAI–1, respectively. These autoinducers are detected at the cell surface by the LuxN, LuxP–LuxQ and CqsS receptor proteins, respectively. **(A)** At low signal molecule concentration, the receptors autophosphorylate and transfer phosphate to LuxO via LuxU. Phosphorylation activates LuxO, which together with σ54 activates the production of small regulatory RNAs (sRNAs). These sRNAs, together with the chaperone Hfq, destabilise the mRNA encoding the response regulator LuxRVh. Therefore, in the absence of autoinducers, the LuxRVh protein is not produced. (B) In the presence of high concentrations of the autoinducers, the receptor proteins switch from kinases to phosphatases, which results in dephosphorylation of LuxO. Dephosphorylated LuxO is inactive and therefore, the sRNAs are not formed and the response regulator $LuxR_{Vh}$ is produced (adapted from Henke and Bassler (2004b)).

5.5 Using acyl homoserine lactone (AHL)–degrading bacteria

Diverse bacterial pathogens (including aquatic pathogens) employ signal molecules to regulate the production of virulence factors (Baruah *et al*., 2009). However, screening for bacteria capable of producing enzymes, which inactivate the signal compound, blocking the QS systems of their competitors, has unverified potential for disease control in aquaculture (Defoirdt *et al*., 2004). Disruption of these signal molecules can

significantly decrease virulence factor production in bacteria without interfering with their growth (Finch *et al*., 1998; Hentzer *et al*., 2003; Rasch *et al*., 2004), and it may be a particularly useful method in aquaculture (Defoirdt *et al*., 2004, 2005). One of the approaches proposed for QS disruption is the isolation of bacteria that degrade signal molecules involved in QS. Bacteria capable of utilizing AHL molecules as sole sources of carbon and nitrogen can be used as potential quenchers of QS–regulated functions in pathogenic bacteria. Bacteria capable of degrading AHL–type signal molecules have been reported extensively in the literature (Leadbetter and Greenberg, 2000; Dong *et al*., 2002; Uroz *et al*., 2003). Hence, it is of interest to investigate whether these types of bacteria could be used as a new type of probiotic, a live microbial adjunct that is beneficial to the host (Defoirdt *et al*., 2007a).

Tinh *et al*. (2008b) investigated the use of two enrichment cultures of AHL–degrading bacteria in controlling the overall microbial activity in turbot larvae. The enrichment culture was effective in improving turbot larvae survival under the experimental conditions, *i.e.* when the survival of turbot larvae was compromised through the daily addition of AHL molecules (1 mg l^{-1}). The addition of AHL was presumably stimulating the virulence of opportunistic pathogenic bacteria. The low concentration of AHL could have a negative effect on turbot larval survival, apparently through the prevailing microbial community, while enrichment culture can counteract this. Assuming that in more intensive larviculture systems, the often observed high mortality is related to the presence of QS molecules and QS–induced virulence factors, the addition of a QS degrader could be beneficial. The authors suggested that QS interference might become part of novel non–antibiotic based strategies to overcome high mortalities in the industrial larval production.

6. The use of poly–β–hydroxybutyrate (PHB) as a tool to control diseases in aquaculture seed production.

6.1 Introduction

Polyhydroxyalkanoates (PHA's) are structurally simple macromolecules synthesised by numerous micro organisms as a carbon and energy reserve material under conditions of nutrient stress, usually when an essential nutrient such as nitrogen is limited in the presence of an excess carbon source. It thus acts as a carbon and energy reserve during periods of carbon shortage (Madison and Huisman, 1999). Patnaik (2005) illustrated that optimum carbon to nitrogen ratio should be about 20:1. PHA's are accumulated as discrete granules to levels as high as 90% of the cell dry weight (Anderson and Dawes, 1990). More than 90 different monomer units have been described as constituents of PHA's, with the alkyl side chain not necessarily being saturated; aromatic, unsaturated, halogenated, epoxidised and branched monomers have been reported as well (Madison and Huisman, 1999). The best known member of PHA is poly–β–hydroxybutyrate (PHB), containing repeat units of (R)–3 hydroxybutyrate (Lee, 1996). PHA is the polymer of β–hydroxy short chain fatty acid (SCFA) with PHB being the polymer of β–hydroxy butyric acid. PHA is polyester of various hydroxyalkanoates that are synthesized by many gram–positive and gram–negative bacteria from at least 75 different genera. Of all PHA's, poly–β–hydroxybutyrate (PHB, Fig. 9) is the most extensively characterised polymer (Lee, 1996).

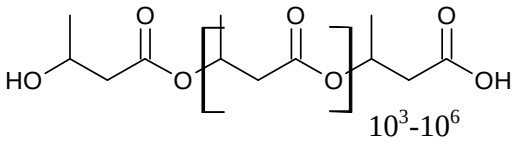


Fig. 9. General structural formula of poly–β–hydroxybutyrate.

6.2 Biodegradation of polyhydroxyalkanoate (poly–β–hydroxybutyrate)

PHA exists in amorphous and crystalline state in and outside the cell. PHA shows different mechanism for intracellular and extracellular degradation. The rate of biodegradation is influenced by various factors such as the microbial population present in a given environment, temperature, moisture level, pH, nutrient supply as well as composition, crystallinity and surface area of the PHA itself.

Extracellular degradation is carried out by various micro-organisms excreting extracellular PHA depolymerase (Choi *et al*., 2004) that hydrolyse PHA into water–soluble oligomers and monomers (hydroxyacid) and subsequently utilise these resulting products as carbon source for growth. The rate of PHA degradation by various microbial depolymerases decreases with increasing crystallinity and the rate of enzymatic degradation is largely independent of molecular weight (Doi *et al*., 1990; Quinteros *et al*., 1999; Yoshie *et al*., 1999). Intracellular PHA depolymerases are unable to hydrolyse extracellular crystalline PHA and thus different enzyme systems like extracellular PHB depolymerase are needed (Khanna and Srivastava, 2004).

Degradation of PHA inside the gastrointestinal tract can be carried out via enzymatic and chemical hydrolysis (Yu *et al*., 2005). Enzymatic hydrolysis is generally carried out by PHA depolymerases produced by various microorganisms such as *Aspergillus fumigatus, Pseudomonas fluorescens, Comamonas sp.* and others (Khanna and Srivastava 2004). However, abiotic decomposition of PHA polymer in the gut is a relatively slow process and may take several weeks (Choi *et al*., 2004; Yu *et al*., 2005).

A wide variety of micro organisms are known to produce polymers of the fatty acid ß–hydroxybutyrate as an intracellular energy and carbon storage compound (Anderson and Dawes, 1990; Madison and Huisman, 1999). Poly– ß–hydroxybutyrate (PHB) is deposited intracellularly in the form of inclusion bodies in a fluid, amorphous state (Amor *et al*., 1991). After death and cell lysis, the polymer is released in a partially crystalline state (Doi, 1995). The ability to degrade extracellular PHB depends on the secretion of extracellular PHB depolymerase enzymes and is widely distributed among bacteria and fungi (Jendrossek, 1998; Jendrossek and Handrick, 2002). The extracellular PHB depolymerase of *Comamonas testosteroni* is well characterised; the enzyme was found to hydrolyse PHB into ß–hydroxybutyrate monomers (Mukai *et al*., 1993; Kasuya *et al*., 1994; Shinomiya *et al*., 1997). Apart from microbial degradation, PHB has also been shown to be degraded in animal tissues (Saito *et al*., 1991; Gogolewski *et al*., 1993; Freier *et al*., 2002) and to be hydrolysed under acidic and alkaline conditions (Yu *et al*., 2005).

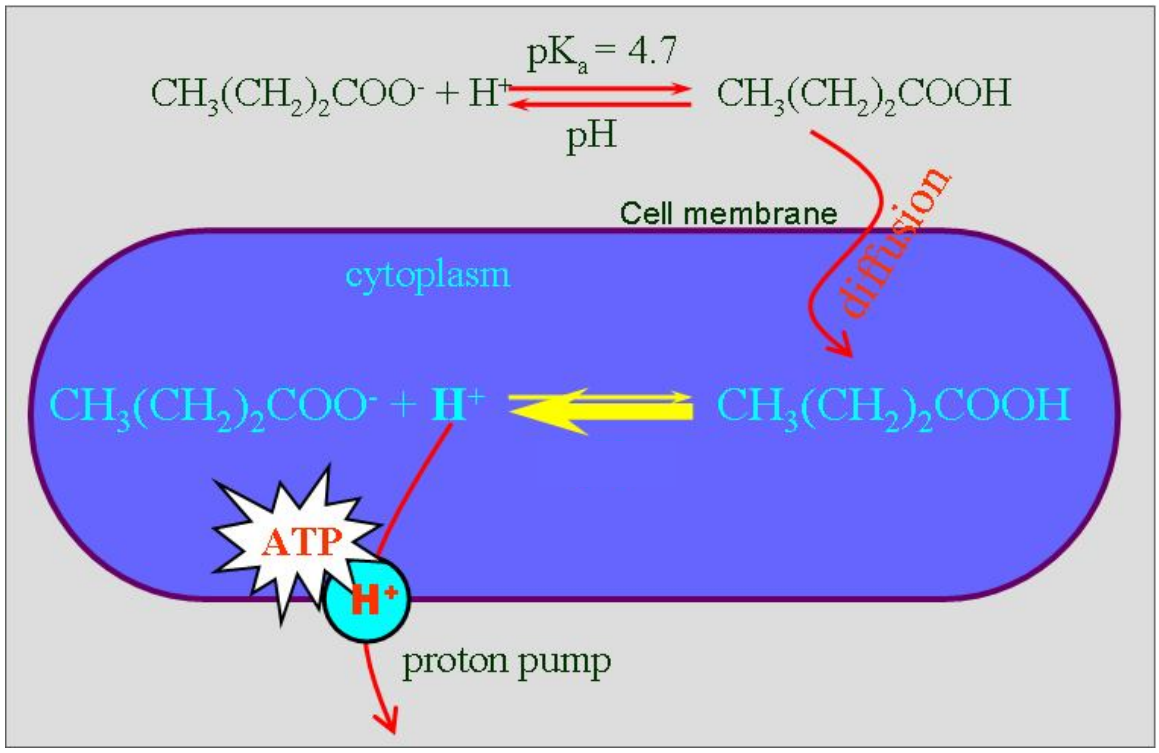
6.3 Mechanism of antibacterial activity of poly–β–hydroxybutyrate

If the polymers of β–hydroxy SCFA could be degraded in the gut, they could have similar beneficial effects as has been described for SCFA: i.e. the working mechanism of SCFA with respect to their antibacterial activity is related to the reduction of the intracellular pH. The antibacterial activity increases with decreasing pH value. SCFA are lipid soluble in undissociated form, enabling them to enter the microbial cell. However, carrier mediated transport mechanism seems to be involved as well in the membrane transport of these organic acids. Once in the cell, the acid releases the proton in the more alkaline environment thereby decreasing intracellular pH. This influences the metabolism of microbes, inhibiting the action of important enzymes and forces the bacterial cell to use energy to release protons. The acid anion seems to

be very important regarding the antibacterial effect of SCFA and their salts. Another possibility is that SCFA may interfere with membrane structure and membrane protein of bacteria in such a way that electron transport is uncoupled and ATP production is diminished (Fig**.** 10).

Fig. 10. Mechanism of bacteriostatic activity of short–chain fatty acids (taking butyric acid as example). The fatty acids pass the cell membrane in their undissociated form and dissociate in the cytoplasm. As a consequence, the cells have to expend energy to export the excess of protons (Defoirdt *et al*., 2006b).

In a recent study, PHB particles inhibited the growth of *Vibrio campbellii* and



provided energy to *Artemia* (Defoirdt *et al.*, 2007b). It indicated that the PHB particles could be (partially) degraded into β–hydroxybutyrate monomers and oligomers in the *Artemia* gut. Degradation of PHA's can occur via one or several mechanisms, including enzymatic hydrolysis and chemical decomposition or hydrolysis (Yu *et al.*, 2005). Abiotic decomposition of PHB is a relatively slow process under mild conditions and the degradation rate is affected by factors such as

pH, temperature and polymer crystallinity. Freier *et al*. (2002) found that PHB slowly decomposes in phosphate buffer, following first order kinetics. However, the addition of digestive enzymes (pancreatin) increased the decomposition from 10% to 30% after 12 weeks.

An *in vitro* study with *Vibrio* by Defoirdt *et al*. (2006b) showed the influence of pH on the growth inhibition by formic acid, acetic acid, propionic acid, butyric acid, and valeric acid. It was verified that for these fatty acids, at a concentration of 20mM, the growth inhibitory effect against *V. campbellii* increased with decreasing pH. At pH 5, the growth of the mentioned pathogen was completely inhibited, while at pH 6 growth was delayed and at pH 7, no inhibition was seen.

6.4 The use of poly–β–hydroxybutyrate (PHB)–accumulating bacteria

PHB–accumulating bacteria in a study conducted by Halet *et al*. (2007) offered a preventive and curative protection to *Artemia* against luminescent vibriosis, suggesting that the application of bacteria loaded with PHB may be an effective method for the protection of *Artemia* upon infection with luminescent vibriosis. When the *Artemia* culture water was enriched with the PHB–accumulating bacterial strain, it significantly enhanced the survival of brine shrimp challenged with pathogenic *V. campbellii* strain LMG21363. This is likely to be mediated by the partial degradation of the PHB polymer into β–hydroxybutyrate in the *Artemia* gut. If occurring, this PHB degradation was probably due to physio–chemical activity or mediated by enzymes present in gut of *Artemia*. Since the *Artemia* nauplii were cultured axenically, it is not likely that the degradation of PHB in the gut was due to microbial activity.

Defoirdt *et al*. (2007) investigated whether PHB could be used as a biocontrol agent to fight luminescent vibriosis. The addition of commercial PHB particles (average diameter 30 μm) or PHB–containing bacteria offered a significant protection against pathogenic *Vibrio campbellii* (Defoirdt *et al*., 2007; Halet *et al*., 2007). Moreover, a complete protection was noticed at 1000 mg l^{-1} commercial PHB particles or 10 mg l^{-1} PHB contained in bacterial cells. The fact that lower PHB concentrations were needed when PHB–containing bacteria were used could be explained by differences in crystallinity and particle size.

Because the costs to license new bacterial control agents for use in animal feeds are quite high (Hong *et al*., 2005), it might be worthwhile to screen bacteria that have a "generally recognised as safe" (GRAS) status for PHA–production instead of licensing new strains. PHB production has been reported for lactic acid bacteria and bacilli belonging to probiotic species (Aslim *et al*., 1998; Yilmaz *et al*., 2005). Hence, PHA enrichment might constitute an added value to these already approved probiotics.

CHAPTER 3

BROODSTOCK

CHAPTER 3

Comparison of reproductive performance and offspring quality of giant freshwater prawn (*Macrobrachium rosenbergii*) broodstock from different regions

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Abstract

An experiment was conducted to compare reproductive performance and offspring quality of *Macrobrachium rosenbergii* broodstock from four different sources: (1) Vietnam wild; (2) Vietnam pond–cultured; (3) Hawaii pond–cultured and (4) China pond–cultured *M. rosenbergii* females were individually followed for 180 days in three 1,200–l fresh water recirculation systems and fed a commercial diet. Ovarian development, moulting and spawning events were checked daily. In addition a number of egg and larval quality parameters were determined. The breeding frequency, fecundity, egg laying success rate, egg dimensions and egg hatchability were not significantly different between animals from the four different sources. However, there were significant differences in terms of offspring quality between the different broodstock sources. Individual dry weight, larval development rate, time to reach the postlarval stage, postlarval survival and tolerance to ammonia toxicity were all better in offspring originating from China pond–reared and Vietnam pond–reared broodstock sources compared to those originating from Vietnam wild and Hawaii pond–reared sources. Moreover, offspring quality from Chinese and Vietnamese pond–reared broodstock proved more stable in terms of ammonia tolerance over three consecutive reproduction cycles. In general, the pond–reared broodstock from China and from Vietnam resulted in better offspring quality than the Hawaii pond–reared and Vietnam wild broodstock. These results indicate that broodstock sourcing deserves proper attention in hatchery operations of *M. rosenbergii*. It furthermore proves that domesticated (pond–reared) animals are not necessarily inferior as breeders as compared to wild–sourced animals. The results may also point out the potential to selectively breed stocks with improved characteristics adapted to the local culture environment.

Keywords: *Macrobrachium rosenbergii*, reproduction, larval quality, broodstock origin

1. Introduction

The giant freshwater prawn *Macrobrachium rosenbergii* (de Man) is the largest species in the genus and is the most favored species for farming. Broodstock of *M. rosenbergii* were introduced from Malaysia to the Anuenue Fisheries Research Centre in Hawaii–USA in 1965 (Hedgecock *et al*., 1979). Later, broodstock from Hawaii and SE Asia were introduced into many regions where *M. rosenbergii* was not indigenous, including North and South America, Africa, Europe and parts of Asia to initiate culture industries (New, 2000a). Freshwater prawns are suitable candidates for inclusion in polyculture systems, and in integrated aquaculture–agriculture. The relatively long larval rearing period (18–35 days) and low larval survival are however disadvantages to commercial culture of this species (Maclean and Brown, 1991).

In Vietnam, the giant freshwater prawn is becoming an increasingly important aquaculture species, as its culture, especially in rice fields, is considered to have the potential to raise income among impoverished farmers. The aquaculture production of *M. rosenbergii* reached over 10,000 tons per year in 2002, having increased from about 3000 tons in the 1990s (Phuong *et al*., 2006). The lack of a stable seed supply has been an important obstacle to the further expansion and development of *M. rosenbergii* culture (Phuong *et al*., 2006). Farmers have traditionally depended on wild sources to obtain seed for aquaculture but are now faced with dwindling resources and a shortage of natural spawners (Wilder *et al*., 1999). Poor performance (in terms of survival and metamorphosis rate) of larvae from wild captured parent stock remains a bottleneck (Thang, 1995; Amrit and Yen, 2003). At present about 52% of the hatcheries use wild broodstock, the remaining rely on farmed broodstock (Phuong *et al*., 2006).

Despite four decades of domestication (Ling and Merican, 1961; New, 2000a, b), little information is published concerning the effects of many generations of domestication on cultured stock (e.g. inbreeding level). Amrit and Yen (2003) compared performance of larvae originating from Thai and Vietnamese *Macrobrachium* breeders. Larvae from Thai breeders were found to have higher survival and develop more uniformly as compared to larvae from Vietnamese breeders. As Thai prawns were pond–reared and Vietnamese prawns were wild sourced, this study could however not separate the effect of geographical origin from the effect of wild versus captive source. There is still controversy whether it is better to use wild or pond–reared breeders and local or imported prawn breeders. Wild breeders are generally considered better, but quality may vary depending on capture techniques and transport conditions. Moreover, breeders from different geographical origin might have different characteristics in terms of reproduction and offspring quality.

Evaluating reproductive characteristics and offspring quality of different prawn strains could also be considered as a first step in the development of selective breeding programs. Thanh *et al*. (2009) rightly noted that in this respect, very little efforts have been made on crustacean species.

To date, there are five national hatcheries in Vietnam for the production of *M. rosenbergii*. Hatchery output nevertheless is still insufficient to meet demands both in terms of both quantity and quality. Therefore, large numbers of *M. rosenbergii* postlarvae (PL) are imported from China to supply the farmers. Ongrown specimens from this Chinese source are used as pond–reared breeders in hatcheries. Early 2005 the Fishery Department of An Giang province imported *M. rosenbergii* PL from Hawaii which are believed to have better quality in terms of reproductive and growout performance.

In the current study, an experiment was conducted to compare the reproductive performance and offspring quality of *M. rosenbergii* broodstock from four different sources: Vietnam wild; Vietnam pond–reared; Hawaii pond–reared and China pond–reared with the objective to determine which broodstock source is most suited for seed production under conditions prevailing in South Vietnam. This knowledge may add to the development of improved hatchery protocols and seed quality of *M. rosenbergii* culture and serve as a starting point to set up a selective breeding programme.

2. Materials and methods

2.1 Broodstock sources

Adults of the giant freshwater prawn *M. rosenbergii* were selected from four different sources: (1) Vietnam wild breeders (VW) were captured in the Cua Dai river, Ben Tre province, belonging to the lower section of the Mekong river system; (2) Vietnam pond–reared breeders (VP) were collected from growout ponds which had been stocked with postlarvae originating from wild breeders from Cu Chi, Ho Chi Minh peri–urban; (3) Hawaii pond–reared breeders (HP), introduced from Malaysia into Hawaii in 1965, but kept for many generation in captivity since, were introduced to Vietnam in early 2005 as postlarvae for on–farm trials in the Mekong Delta and collected as broodstock at the end of a culture trial from a local farm in An Giang province; and (4) China pond–reared breeders (CP) were imported from China in April 2005 as postlarvae by Nguyen Thi Suong (prawn hatchery owner in Thanh Phu district, Ben Tre province) and were grown out in culture ponds in Binh Dai district, Ben Tre province, South Vietnam and collected as broodstock at the end of grow–out culture period. The individual weight and length of the animals were not significantly different between the sources at the beginning of the experiment. Numbers of initial

breeders for each source (n) were: VW (n = 67); VP (n = 86); HP (n = 54) and CP (n = 75).

2.2 Experimental conditions and set–up

2.2.1 Broodstock system

Three separate recirculation units were set up, each one containing one 1000–l holding tank (1.4 x 2.4 x 0.3 m) and one 200–l overhead biological filter tank. The biofilter was filled with coral stone. An airlift system provided aeration and aided the water to pass through the filter media. Water was continuously pumped from the central chamber of the broodstock holding tank into the biofilter tank and then returned to the holding tank by gravity.

The holding tank was divided into twenty–four square rearing compartments (0.35 x 0.35 m) and one central pump compartment, with each compartment containing one female. There were 3 replicate units with in total 18 females per source of broodstock.

2.2.2 Broodstock back up system

A recirculation system, similar to the one described above was arranged to maintain extra animals from the four sources for replacing any mortalities that occurred during the initial phase of the experimental period. In this way, from each source an extra 20 females and 10 males were maintained separately.

2.2.3 Broodstock rearing conditions

Broodstock rearing methodology roughly followed the techniques described by Cavalli *et al*. (1999). The prawns were randomly selected and stocked into the three experimental units. Freshwater was continuously pumped from the central

compartment of the holding tank into the biological filter and flowed back to the holding tank by gravity. Water was exchanged at a rate of approximately 20% per day after removing waste and uneaten feed by siphoning. NH_4–N; NO_2–N, and NO_3–N levels were maintained below 0.2, 0.1, and 10.0 mg l^{-1} respectively. The photoperiod was set at 12h light at an intensity of 600 lux with fluorescent lamps at the water surface. Temperature was maintained at 28±1 ^oC. Prawns were fed *ad libitum* with a commercial formulated shrimp diet twice a day (at 8.00h and 17.00h). The formulated diet contained 400 g kg^{-1} crude protein, 77 g kg^{-1} total lipids, and 108 g kg^{-1} ash. (Shrimp Maturation, Uni–President, Viet Nam).

2.2.4 Larval rearing system

A larval rearing system was installed following the design of Cavalli *et al.* (2001). The set–up consisted of three separate recirculation systems. Each had 16 12–l cylindro–conical jars connected to a single recirculation system containing a settlement tank (100 l) and connected to a submerged biological filter tank (200 l) which was filled with plastic media. Airlifts provided the necessary oxygen in the biofilter. Water from the biological filter tank flowed into a reservoir tank from which it was pumped back to the larval rearing tanks. The water entered the larval tanks from the bottom at a flow rate of approximately 0.2–0.3 l min^{-1}. The total volume of the recirculation system was approximately 600 l. Water was exchanged at a rate of approximately 10% per day after removing wastes and uneaten feed by siphoning. NH_4–N; NO_2–N, and NO_3–N levels were maintained below 0.2, 0.1, and 10.0 mg l^{-1} respectively. Water salinity was adjusted by diluting seawater to 12 g l^{-1} with deionized water. Gentle aeration was applied in all rearing jars. A fluorescent lamp system was installed, providing around 900–1000 lux at the water surface for 12h

day^{-1}. From each spawner, triplicate groups of 600 newly–hatched larvae were stocked. Larval stocking density was 50 larvae l^{-1}. Average water temperature was 30±1 ^{o}C. Newly–hatched *Artemia franciscana* nauplii (Great Salt Lake strain) were offered at a density of 10–15 ml^{-1} from day 2 to day 7. The *Artemia* ration was split over two feedings at 7.00h and 17.00h. From day 8 until metamorphosis to PL, the larvae were also fed a supplemental commercial diet (Brine Shrimp Flakes, O.S.I., USA) containing 530 g kg^{-1} crude protein, 90 g kg^{-1} total lipid, 110 g kg^{-1} ash, 90 g kg^{-1} moisture, and 20 g kg^{-1} fibre. The commercial diet was fed to satiation five times per day (7.00, 9.00, 11.00, 13.00 and 15.00h) while *Artemia* nauplii were fed once a day at 17.00h.

2.3 Evaluation parameters

2.3.1 Reproductive parameters

Initial mean weight and total length of females and males were recorded. Ovarian development was classified according to colour, size and outline of the ovary following the description by Chang and Shih (1995). Moulting and the duration of the intermoult period of the females were recorded. If the moulted female had developed ovaries she was allowed to mate with a hard–shelled male for 3 hours. Spawning events were recorded for each female. Within each broodstock group, for approximately 50% of the spawns (randomly selected), the egg clutches were removed 7 days after spawning in order to estimate egg fecundity. The eggs were then incubated *in vitro* in order to estimate egg hatchability. The remaining 50% of the berried prawns were allowed to incubate their eggs until hatching. From these prawns the larvae were collected for further rearing purposes in order to assess larval quality.

To determine the total weight of the egg clutch, the females were first blotted dry with paper tissue. Then the eggs were removed and the egg mass and total (somatic) wet weight of the female was determined. With these values the egg clutch somatic index (ESI: egg clutch–weight somatic–weight^{-1}) was calculated. The ESI was determined at day 7 after spawning. Fecundity was determined as follows: Three egg sub–samples were weighed and the number of eggs in each sub–sample was then counted to determine the individual egg weight and the total number of eggs per clutch. Fecundity was both expressed as the number of eggs spawn^{-1} and the number of eggs g^{-1} female. A number of females from each broodstock source that were classified at phase V of ovary development were sacrificed to determine the gonadal somatic index (GSI: gonad–weight somatic–weight^{-1}). Spawning frequency and survival (%) of the females over the 6 months rearing period were also calculated. Egg laying success rate (%) was calculated as the ratio of successful spawning events (eggs still retained 7 days after spawning) and total number of spawns.

2.3.2 Egg quality

- *Egg weight*

Egg wet weight (µg) was determined when calculating the number of eggs per egg clutch (see above). Egg dry weight (µg) was obtained by placing three egg samples in the drying stove at 60 °C for 48h. Egg moisture content was then calculated based on the egg wet and dry weight estimates.

- *Egg hatchability*

Hatching were estimated *in vitro*. From each egg clutch obtained in the first broodstock subgroup (eggs removed), three samples of eggs containing around 100–200 eggs were incubated in 200–ml glass cones containing diluted seawater at a salinity of 6 g l^{-1}. Moderate aeration was provided in each cone. Hatching was

calculated from the number of live larvae and dead eggs 24 hours after hatching. *In vivo* hatching (subgroup 2) was also determined based on the average larval fecundity (larvae g^{-1} female) in subgroup 2 and the average egg fecundity (egg g^{-1} female) in subgroup 1. Where:

Egg fecundity (eggs g^{-1} female) = Total eggs / female weight

Larval fecundity (larvae g^{-1} female) = Total larvae / female weight

Hence, for this parameter no standard deviation could be calculated.

2.3.3 Larval quality

Larval quality was assessed based on weight, development rate, survival, and tolerance to ammonia stress of the larvae. From each spawn, triplicate groups of several hundred larvae were reared to the postlarval stage. The larval quality was determined at day 8 after hatch and at postlarvae stage: Larval wet and dry weight of 3 times 50 larvae were determined. For dry weight, larvae were dried at 60 °C for 48h. Survival of the larvae at day 8 and upon metamorphosis to postlarvae were also determined.

At day 5, 10 and 15, a larval stage index (LSI) was estimated according to Maddox and Manzi (1976). For this, at least 30 larvae from each treatment were sampled to determine the average larval stage. Determination of the larval stage was based on the description by Uno and Kwnon (1969).

$$LSI = \sum S_i / N$$

Where: S_i is the stage of the larvae (i = 1 to 11)

N is number of larvae examined

The time of the first postlarvae appearance and the duration of metamorphosis (T_s) were determined.

$$T_s = T_{90} – T_{10}$$

Where: T_{90}: the time needed for 90% of the larvae to reach postlarva stage

T_{10}: the time needed for 10% of the larvae to reach postlarva stage

Larvae were also subjected to an ammonia toxicity test. For this test, 5 groups of 30 larvae were exposed to four increasing concentrations of total ammonia and a control (no ammonia added) for 24 hours. A series of 15 (5 groups x 3 replicates for each treatment) 1–l glass cones were filled with water at 28±1 ^{o}C and 12 g l^{-1} salinity. pH value in each glass cone was determined before and after testing. The concentration of non–ionized ammonia (NH_3) was estimated according to the general formula for the mean values of pH, salinity and temperature as presented by Armstrong *et al*. (1978):

$$[NH_3] = [TAN] / (1 + 10^{[pK–pH]})$$

Where: pK = 9.31 at temperature of 28 ^{o}C and salinity of 12 g l^{-1}

pH = mean of values measured at the beginning and the end of test.

Based on the mortality after 24 hours, the mean lethal concentrations for 50% of the population (24h–LC_{50}) were estimated. The ammonia toxicity test was performed on newly-hatched larvae, 8-day old larvae and postlarvae.

2.4 Statistical analyses

Duration of intermoult periods, reproductive performance parameters, and offspring quality parameters were analyzed by analysis of variance (one–way ANOVA) and, if significant differences were found ($P<0.05$), the least significant differences (Weller–Duncan) test was applied. All percentage data were normalized by square root–arcsine, but only non–transformed means are presented. Correlations were determined using linear regression analysis.

3. Results

3.1 Broodstock reproductive performance

Average values of water temperature, pH, dissolved oxygen and ammonia were 28±1 ^{o}C, 7.1 to 7.8, 5.4±0.4 mg l^{-1} and <0.1 mg l^{-1}, respectively throughout the experimental period. The reproductive performance of *M. rosenbergii* females of the different sources is presented in Table 1. The experimental design enabled us to individually follow the reproductive cycle of *M. rosenbergii* females over at least 7 moults and 5 consecutive spawns over the 180–days survey period. During that period some females of the Chinese source moulted 9 times; while this was 8 times for the Vietnam pond–reared and Hawaii pond–reared sources; and 7 times for the Vietnam wild source. The breeding frequency of the females reached up to 7 times for the Vietnam pond–reared and China pond–reared sources; 6 times for the Hawaii source and only 5 times for the Vietnam wild source. Over the 180 days of the experiment, the Hawaii pond–reared broodstock had the lowest survival (70%), which was significantly different ($p<0.05$) from the Vietnam pond–reared source (93%). The other two broodstock sources demonstrated a similar value of 78%. There was a significantly ($p<0.05$) longer average intermoult period of 36 days for Vietnam wild females compared to the three other broodstock sources which ranged around 30–31 days. On the other hand, there were no significant differences ($p>0.05$) in interspawn period between the broodstock sources. Egg laying success rate also did not vary significantly between the treatments, ranging from 80 to 94%. Within each broodstock source, there was also no significant difference in intermoult and interspawn period nor egg laying success rate between the subgroup in which the eggs were removed and the subgroup which was allowed to incubate their eggs until hatching.

The fecundity expressed as number of eggs per gram female weight was approximately 1,100 eggs g^{-1} female for all four broodstock sources. However, this parameter was highly variable between individual females (SD up to 364). The gonado–somatic index (GSI) of broodstock that were sampled at stage V of ovary development showed no differences between the broodstock sources and ranged from 6.9 to 8.1%. While, the egg clutch somatic index (ESI) ranged from 9.3 to 10.2% (Table 1).

Table 1. Reproductive performance (mean±SD) of *M. rosenbergii* females from different sources

Parameter	Broodstock source			
	VW	**VP**	**HP**	**CP**
Weight (g)	37.4±7.9[a] (18)	31.8±4.7[a] (18)	34.9±5.1[a] (18)	37.8±7.3[a] (18)
Survival (%)	78±9[ab] (3)	93±6[a] (3)	70±6[b] (3)	78±11[ab] (3)
Intermoult period (days)	36±7[b] (26)	30±5[a] (20)	31±8[a] (23)	30±5[a] (25)
Interspawn period (days)	52±13[a] (20)	50±35[a] (16)	44±11[a] (18)	53±20[a] (18)
Egg laying success rate (%)	94±12[a] (3)	83±17[a] (3)	81±19[a] (3)	80±22[a] (3)
Eggs per spawning event (eggs $female^{-1}$)	49,040[a] ±18,903 (26)	41,078[a] ±8,630 (31)	38,137[a] ±9,942 (30)	41,662[a] ±12,641 (23)
Fecundity (eggs g^{-1} female)	1,178±364[a] (26)	1,111±184[a] (31)	1,055±260[a] (30)	1,113±284[a] (23)
Gonadal somatic index (%)	8.1±2.6[a] (8)	6.9±1.5[a] (8)	7.3±1.5[a] (8)	7.9±2.3[a] (8)
Egg clutch somatic index (%)	10.2±3.0[a] (26)	10.0±1.4[a] (31)	9.3±2.0[a] (30)	9.6±2.4[a] (23)

Different letters within rows denote significant differences ($p<0.05$), (n) = number of sample.

The individual wet weight and dry weight of the eggs were similar between the treatments, ranging from 86.8 to 90.1 µg egg^{-1} and 41.7 to 44.8 µg egg^{-1} respectively.

Also the moisture content of the eggs was the same between the treatments, ranging from 57.9 to 59.7%. The (*in vitro*) egg incubation periods were also similarly between the broodstock sources. It took approximately 21 days at a water temperature of 30±1 °C for the eggs to hatch. The egg *in vitro* hatching of the treatments ranged from 65 to 72%, which was higher than the *in vivo* hatching, which ranged from 49 to 54%. (Table 2)

Table 2. Egg quality parameters (mean±SD) of *M. rosenbergii* females from different sources.

Parameter	Broodstock source			
	VW (n=20)	**VP** (n=16)	**HP** (n=18)	**CP** (n=18)
Egg wet weight (µg)	87.2±7.4[a]	90.1±7.2[a]	89.1±7.5[a]	86.8±6.2[a]
Egg dry weight (µg)	41.7±5.2[a]	44.8±4.4[a]	43.8±4.3[a]	42.5±5.5[a]
Egg *in vitro* hatching (%)	68±15[a]	65±19[a]	66±16[a]	72±16[a]
Egg *In vivo* hatching (%)	54	49	52	53

n= number of replicates

Overall, there were no major differences in most of the reproductive parameters in terms of reproductive capacity and fecundity between the different broodstock sources. Also the characteristics of the eggs originating from the different broodstock sources were similar in terms of weight and hatching properties.

3.2 Larval quality

Larval performance was assessed on a second group of breeders that were allowed to maintain and incubate their egg clutch until hatching. The females produced on average between 17,000 and 24,000 larvae per hatching event (Table 3). The number of newly–hatched larvae per female body weight unit was around 600 larvae g^{-1}, and

was not different between the different broodstock sources. The dry weight of the newly–hatched larvae of the Vietnam wild and China pond–reared sources was significantly higher than for the Hawaii pond–reared source ($p<0.05$). When the larvae were ongrown, there were also significant differences in the dry weight of eight–day–old larvae. Larvae from the China pond–reared source had the highest dry weight (148 µg), followed by these originating from Vietnam pond–reared breeders (118 µg) and Vietnam wild breeders (99 µg); while the lowest weight was observed for larvae from the Hawaii source (75 µg). A similar trend was observed for the survival on day 8 (Table 3).

The larval stage index (LSI) on day 5 and 10 showed that larval development of larvae from the China pond–reared and Vietnam pond–reared sources was significantly faster than for larvae of the Vietnam wild and Hawaii pond–reared sources (Table 3). On day 15, LSI was very different ($p<0.05$) between the treatments, descending in the order Vietnam pond–reared, China pond–reared, Hawaii pond–reared and Vietnam wild. Based on the duration of the rearing period from newly–hatched larvae to the first postlarvae appearance, two distinct groups could be identified. The group of Vietnam pond–reared and China pond–reared breeders resulted in significantly shorter rearing periods (17 and 19 days, respectively) than the group of Vietnam wild and Hawaii pond–reared breeders (24 and 26 days, respectively) ($p<0.05$). The duration of larval metamorphosis from 10% up to 90% of the Hawaii pond–reared source was 8 days, which was significantly longer than for larvae from the three others sources. The survival up to postlarva stage of the Vietnam pond–reared (66%) and China pond–reared (61%) sources was significantly higher than for the Vietnam wild (29%) and Hawaii pond–reared (18%) sources ($p<0.05$).

Table 3. Offspring quality parameters (mean±SD) of *M. rosenbergii* females from different sources.

Parameter	Broodstock source			
	VW	**VP**	**HP**	**CP**
Larvae per hatching event (n=16)	23,744[a] ±11,683	17,399[a] ±577	19,000[a] ±6,404	22,046[a] ±7,395
Larvae fecundity (larvae g^{-1} female, n=16)	635[a] ±277	549[a] ±164	546[a] ±172	586[a] ±187
Newly–hatched larvae dry weight (µg, n=16)	25±2[a]	23±4[ab]	20±4[b]	24±6[a]
Larvae dry weight on day 8 (µg) (n=16)	99±18[b]	118±35[b]	76±10[c]	148±45[a]
Larval survival on day 8 (%, n=16)	87±8[b]	83±11[b]	74±8[c]	92±6[a]
Larval stage index at day 5 (n=8)	3.9±0.4[c]	4.3±0.5[a]	4.0±0.4[b]	4.2±0.4[a]
Larval stage index at day 10 (n=8)	6.4±1.0[b]	7.5±1.1[a]	6.5±0.7[b]	8.1±0.9[a]
Larval stage index at day 15 (n=8)	7.2±1.1[d]	9.4±0.9[a]	8.2±0.8[c]	9.1±0.8[b]
First PL appearance (days, n=8)	24±3[b]	17±2[a]	26±1[b]	19±3[a]
Duration of metamorphosis (days, n=8)	6±1[a]	6±2[a]	8±1[b]	4± 1[a]
Survival up to PL (%, n=8)	29±14 [b]	66±4 [a]	18±8 [b]	61±8[a]

Different letters within rows denote significant differences ($p<0.05$), (n= number of replicates).

Overall, the larval development results revealed that the larvae originating from *M. rosenbergii* breeders from Vietnam and China cultured in ponds presented considerably better results than those from wild Vietnamese and pond–reared Hawaiian breeders.

3.3 Ammonia toxicity

Ammonia tolerance, expressed as 24h–LC_{50}, was used as a criterion for the evaluation of larval quality. There were significant differences in the LC_{50} for non–ionized ammonia between the treatments ($p<0.05$) both for newly–hatched larvae and for 8 day–old larvae (Table 4). The results showed that larvae from the Chinese broodstock source had a considerably higher tolerance to ammonia compared to larvae from pond–reared and wild Vietnamese, and Hawaiian breeders. In contrast, there was no difference in ammonia tolerance of newly–metamorphosed postlarvae between the different broodstock sources.

Table 4.. 24h–LC_{50} (mg l^{-1} NH_3; mean±SD) of larvae originating from different broodstock sources.

Parameter	Broodstock source			
	VW	VP	HP	CP
Newly–hatched	0.64^{a}	0.68^{a}	0.42^{b}	0.71^{a}
larvae (n=16)	±0.27	± 0.26	± 0.15	± 0.26
8–day old larvae	0.49^{bc}	0.61^{ab}	0.44^{c}	0.76^{a}
(n=14)	± 0.14	± 0.24	± 0.14	± 0.28
Postlarvae	0.50^{a}	0.58^{a}	0.54^{a}	0.68^{a}
(n=6)	± 0.24	± 0.03	± 0.10	± 0.07

Different letters within rows denote significant differences ($p<0.05$), (n= number of replicates).

In Fig. 1 the ammonia tolerance of newly–hatched larvae is presented in function of the spawn order in order to evaluate the stability of the larval quality over successive spawns. The results show that newly–hatched larvae from Vietnam wild and Hawaii pond–reared sources from the first spawn were significantly more tolerant compared with those from the next two reproduction cycles. On the other hand, although

tolerance generally also decreased slightly, no significant difference in response of newly–hatched larvae originating from the Vietnam pond–reared and China pond–reared sources was observed in function of spawn order.

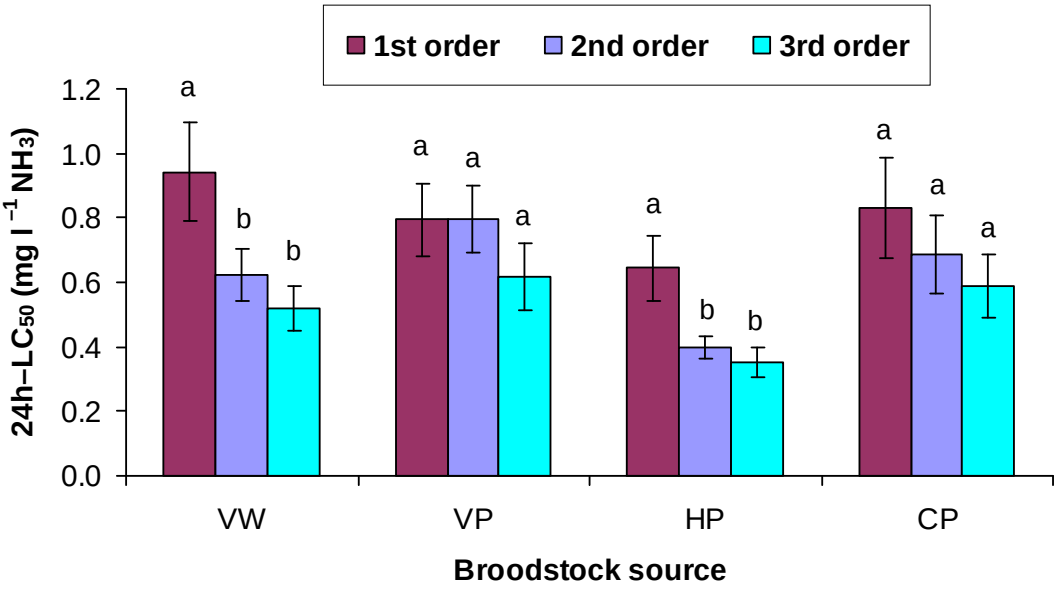

Fig. 1. 24h–LC_{50} (mg l^{-1} NH_3, mean±SD) of newly–hatched larvae originating from different broodstock sources in function of spawn order. Different letters within each broodstock group denote significant differences ($p<0.05$).

4. Discussion

The results of this study showed that the comparison of reproductive performance between of *M. rosenbergii* breeders from four very different sources was largely the same in terms of reproductive capacity and fecundity. To our knowledge, there are no previous studies that compared reproductive parameters of different strains of freshwater prawns or prawns from different (geographical) origin. Several studies however compared reproductive performance of wild and pond–reared penaeid shrimp broodstock. A lower fecundity has been commonly observed for pond–reared shrimp broodstock, but it has been indicated that this could be an effect of differences

in shrimp size rather than source (Menasveta *et al*., 1993, 1994; Cavalli *et al*., 1997; Palacios *et al*., 2000). However, when comparing shrimp of similar size, Browdy *et al*. (1986) reported lower fecundity for pond–reared *Penaeus semisulcatus.* Others obtained similar values for wild and pond–reared broodstock sources, e.g. for *Penaeus monodon* (Menesveta *et al*., 1994); and for Kuruma shrimp, *Penaeus japonicus* (Bate) (Preston *et al*., 1999). In the present study fecundity varied around 1100 eggs g^{-1} female, and was not significantly different among treatments. In a series of nutritional experiments, Cavalli *et al*. (1999, 2001) reported fecundity values around 1,450 eggs g^{-1} female for females with an average weight of 26.2±5.1 g. This author mentioned that the efficiency of egg production tended to decrease with increasing female size. The larger size of the females used in the current study (average 31–38 g) could thus account for the lower fecundity observed. Similar data were reported by Costa and Wanninayake (1986) and Rao (1991) who indicated that in wild *M. rosenbergii* populations in Sri Lanka and India, smaller females produced a higher number of eggs per unit body weight. These authors explained that egg size increased with increasing body size of the spawner, resulting in fewer eggs being produced. In addition, differences in feeding practices between different studies probably also affect reproductive performance. For example in the studies of Cavalli *et al*. (1999), supplementation of the broodstock diet with fatty acid resulted in improved reproductive performance. In our study, a common commercial feed without any supplement was fed to the breeders.

In the natural environment, *M. rosenbergii* may spawn up to 4 times or more per year (Ling, 1969; Rao, 1991). In captive conditions, Wickins and Beard (1974) showed that one female spawned 4 times in 170 days. Cavalli *et al*. (2001) reported that one female demonstrated a capacity to breed up to 5 times over 180 days. In the current

study, the breeding capacity reached up to 7 times in 180 days for the Chinese breeders; 6 times for the Vietnamese and Hawaiian pond–reared sources and 5 times for the Vietnamese wild source. This result seems to indicate that pond-reared prawns may be better than wild animals in terms of breeding frequency. Wild animals grew up under natural conditions which may differ from the captive conditions and therefore they may need some time to acclimate and adapt to the new conditions. Moreover, optimal and stable environmental conditions and a more balanced and constant nutrition in culture conditions may also play a significant role in this (Cavalli *et al*., 1999).

In contrast to reproductive parameters, in the current study, many indicators showed differences in terms of offspring quality between the different broodstock sources. In general, the pond–reared Chinese and Vietnamese breeders resulted in better offspring quality than the broodstock source from Hawaii and the wild Vietnamese breeders. Possible reasons for the better results with the former two broodstock sources may lay in the fact that these animals are pond–reared and the fact that they originate from the same geographical area as where the current experiment was performed. Amrit and Yen (2003) argued that the observed differences in offspring quality between domesticated Thai and wild Vietnamese breeders should be brought back to genetic differences due to their geographical origin and the domestication process. Wild breeders need to adapt to the captive conditions and moreover are not acquainted to formulated feeds. This could have led to stress and may have impaired their nutritional condition. This may very likely be responsible for the lower breeding frequency and offspring performance observed for these wild breeders. It has been demonstrated that domesticated strains often perform better over time as they become adapted to the artificial culture environment. This has been demonstrated for example

for channel catfish (Burnside *et al*., 1975) and African catfish *Heterobranchus longifilis* (Nguenga *et al*., 2000). The better performance of pond–reared animals may also be the consequence of a rapid selection for animals that are adapted to grow in captivity. This suggests that, despite earlier claims, wild *M. rosenbergii* are not necessarily a better source of breeders than pond–reared animals.

The pond–reared Hawaiian broodstock source on the other hand resulted in a lower survival and generally lowers offspring quality compared to the other domesticated sources. This may point out that they are less adapted to the conditions used in this experiment. The Hawaiian strain (originally from Malaysia), although already in captivity for many generations, was only recently introduced to southern Vietnam and, therefore, differences in environmental optima may have affected offspring fitness under the present experimental conditions. These results seem to partly contradict the results of Thanh *et al*. (2009) who found superior growth performance when comparing exactly the same Hawaiian *M. rosenbergii* strain with two different pond–reared strains from Vietnamese origin. Differences in experimental conditions may of course account for this. Also, Thanh *et al*. (2009) focussed on growout performance, while the current study focussed on reproductive and larval rearing performance. This demonstrates that comparing suitability of strains is not straightforward and furthermore stresses the importance of investigating the complete rearing cycle when comparing breeder sources.

Within the same broodstock source, the egg clutch somatic index values were higher than the gonado–somatic index values. This is logical, as the embryos are expected to be heavier than the oocytes when still inside the body of the animal. The eggs increase in volume caused by uptake of water and by the development of the eggs after

fertilization. The *in vitro* egg hatching in this study was not different between broodstock sources and ranged from 65 to 72%, which proved higher than the *in vivo* hatching, which ranged from 49 to 54%. Differences in hatching between *in vitro* and *in vivo* incubation were reported in several studies. Cavalli *et al.* (2001) reports that egg loss is considered to be partially due to consumption by the females, to the continual sloughing off of dying eggs due to epizootic infestations and to the loose nature of the larger grey eggs, which would render them more prone to physical losses. Wickins and Beard (1974) reported that egg loss during *in vivo* incubation could amount to 31% of the eggs initially deposited in the brood chamber. From our results we estimated that egg loss during *in vivo* incubation was about 23%. Within each broodstock source treatment, there were no significant differences in moulting and spawning frequency between the group in which the eggs were removed and the groups which were allowed to incubate their egg clutch until hatching. Hence, removing the egg clutch from the females did not affect the reproductive performance. In contrast, Damrongphol *et al.* (1991) and Cavalli *et al.* (2001) reported that removing the eggs from females increased their reproduction output through an increased breeding frequency.

The term 'larval quality' generally refers to the physiological condition of the larvae and is related to survival and growth rates during several larval developmental stages. Several variables at the broodstock management level are known or suspected to affect larval quality (Racotta *et al.*, 2003). In the current study, the weight of the newly–hatched larvae was different although the egg wet and dry weight was not different between the broodstock sources. When the larvae were raised to postlarvae, the differences in larval rearing performance between larvae from the Chinese and Vietnamese pond–reared sources and the Vietnamese wild and Hawaiian pond–reared

sources became more and more pronounced. This could clearly be demonstrated from the results of larval dry weight, larval stage index, time of appearance of postlarvae and survival to postlarval stage. This confirms earlier findings that larval quality is difficult to assess and might only become apparent further down the rearing cycle (Dhert *et al*., 1991).

Comparison of the ammonia tolerance (LC_{50}) of larvae at hatch and on day 8 showed that larvae originating from the Chinese breeders consistently presented the highest tolerance. In contrast, larvae from the Hawaiian source demonstrated the lowest tolerance. Cavalli *et al*. (2000a) reported that larval ammonia tolerance (LC_{50}) may vary due to many reasons such as differences in broodstock nutritional status or differences in larval quality between different batches associated with differences in amino acid content in the newly–hatched larvae. Tolerance to ammonia is especially relevant since animals are in culture conditions often subjected to relatively high concentrations of this toxic excretion product. Racotta *et al*. (2003) showed that penaeid shrimp larvae from pond–reared spawners had a higher resistance to ammonia stress compared to larvae from wild spawners. It could be hypothesized that this constitutes some kind of selection through the domestication process as captive stocks are regularly exposed to high ammonia concentrations. The current study also demonstrated that *M. rosenbergii* larval quality in term of ammonia tolerance tended to decrease with spawn order. This was very clear for offspring of Vietnamese wild and Hawaiian breeders, while for the Chinese and Vietnamese pond–reared sources no statistical differences were found over three consecutive spawns. This might point out an advantage of the Chinese and Vietnamese pond–reared broodstock sources in that they may be used as broodstock for a longer time with limited reduction in larval quality.

5. Conclusions

The reproductive performance of four different sources of *M. rosenbergii* broodstock (Vietnam wild, Vietnam pond–reared, Hawaii pond–reared and China pond–reared) did not significantly differ in terms of breeding frequency, fecundity and egg dimensions. However, larval quality of Chinese and Vietnamese pond–reared breeders was markedly better than that of Vietnamese wild and Hawaiian pond–reared animals in terms of larval development, survival and ammonia tolerance. Pond–reared broodstock may be an equally good or even better wild source. When introducing broodstock from different geographical locations its performance and fitness under the locally prevailing conditions should be carefully checked.

Acknowledgments

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CHAPTER 4

LARVICULTURE ZOOTECHNIQUES

CHAPTER 4

SECTION I

Effects of larval stocking density and feeding regime on larval rearing of giant freshwater prawn (*Macrobrachium rosenbergii*)

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Abstract

A series of larval rearing experiments were conducted to investigate the effect of larval stocking density and feeding regime on larval development and survival, and larval quality of *Macrobrachium rosenbergii* (de Man). For larval density, fixed initial stocking densities, as well as treatments where larval density changed during the rearing period (in function of the rearing volume), were set up. The factor feeding regime (based on *Artemia* nauplii) included both feed ration and feeding frequency. The results of the experiments showed that larval stocking density and feeding regime strongly affected larval development, survival, duration of the rearing cycle as well as larval quality. The best initial larval stocking density when using a constant water volume was 50–150 l^{-1}, while this could be increased up to 400 l^{-1} in case increasing water volume is used. A feeding frequency of 6 times per day by first–stage *Artemia* nauplii proved better than feeding only twice daily. Production efficiency in terms of the number of postlarvae produced per unit rearing volume and the number of *Artemia* nauplii used per postlarva produced in the different treatments is discussed. These optimized larval stocking densities and feeding regimes are expected to render freshwater prawn larval rearing more profitable and improve the resulting larval quality.

Key words: Larval stocking density, feeding frequency, growth, survival, larval quality, production efficiency.

1. Introduction

The giant freshwater prawn *Macrobrachium rosenbergii* (de Man) is a commercially important species in several Southeast Asian countries, both as a food item for local consumption as well as a valuable export product. Over the past 20 years farming techniques for *M. rosenbergii* have been studied and developed throughout the world (New and Valenti, 2000). In Vietnam, the giant freshwater prawn, which is indigenous to the Mekong Delta, is becoming an increasingly important target species (Phuong *et al*., 2006). To date, different rearing techniques are used for seed production of giant freshwater prawn in Vietnam. Some hatcheries use a clear water technique, while others rely on green water; some operate open flow through systems, while some apply recirculation systems.

M. rosenbergii larvae can be raised on a diet of only *Artemia* nauplii (Devresse *et al*., 1990; Lavens *et al*., 2000). This live food supplies all the necessary nutrients for development and can contribute with exogenous digestive enzymes that aid in digestion (Jones *et al*., 1993; Kamarudin *et al*., 1994). However, at the high water temperatures in the larval rearing tanks, freshly–hatched *Artemia* nauplii develop into Instar II metanauplii within 6–8 h. Sorgeloos at al. (2001) reported that feeding first–instar nauplii is better than starved second–instar metanauplii. Over a 24–h larval developmental period, individual dry weight and energy content of *Artemia* nauplii decrease with 16–34% and 22–34% respectively. Hence the nutritional value of uneaten *Artemia* nauplii remaining in the rearing tank gradually decreases as the live food develop into instar II stage (Vanhaecke *et al*., 1983). Optimal feed rations and feeding frequencies are therefore of utmost importance. Residence time of the *Artemia* nauplii in the rearing tank will also be affected by the larval density. To date

hatcheries in Vietnam mostly apply larval rearing densities ranging between 40 and 125 larvae l^{-1} but typically are 40–60 l^{-1} in green–water systems and 80–100 l^{-1} in open and recirculating systems (Phuong *et al.*, 2006). Initial stocking density for prawn larval production is however largely arbitrarily chosen based on the culturist's experience (Lim and Hirayama, 1993). Since a few studies have reported rearing of *M. rosenbergii* larvae at higher densities, there might also be room to intensify larval rearing of prawn. When increasing larval density, feed ration and feeding frequency consequently need to be appropriately adjusted. This is especially true for a cannibalistic species like *M. rosenbergii*. Overfeeding of *Artemia* nauplii in larval rearing systems will generate waste and elevate the concentration of toxic nitrogen compounds. In contrast, underfeeding will reduce growth rate and induce cannibalism (Barros *et al.*, 2003). Consequently, the feeding strategy also largely impacts production cost. Most hatcheries use a larval rearing density which remains stable throughout the rearing cycle. An alternative may be to start at higher densities, but gradually reduce this by increasing the water volume in the tank as the animals develop. It is anticipated that this could make feeding and hence production more economical.

The current experiments were designed to investigate the effects of larval stocking density and feeding regime on larval growth, survival, duration of the larval rearing cycle and larval quality of *M. rosenbergii*. The purpose of this study was to optimize larval stocking densities and feeding regimes to not only reduce production cost, but also maximize productivity of the hatchery. The resulting knowledge may add to the development of improved larval rearing techniques for more efficient hatchery production of *M. rosenbergii*.

2. Materials and methods

2.1 Experimental animals

Three experiments were conducted at the National Breeding Centre for Southern Marine Aquaculture, Research Institute for Aquaculture No.2 in Vung Tau city, Vietnam. *M. rosenbergii* breeders bearing yellow eggs were obtained from culture ponds in An Giang province, Southern Vietnam and acclimated to the hatchery conditions for egg incubation. 24 h after hatching, larvae were collected and stocked into the experimental tanks.

2.2 Experimental design

Experiment 1 consisted of eight treatments, which consisted of various combinations of different larval stocking densities and different feeding regimes. Larval rearing densities ranged from 50 to 200 larvae l^{-1}. The different feeding regimes were designed based on the standard feed ration (called "X"), which was used for rearing the larvae at 50 l^{-1}. For the different treatments, the number of *Artemia* nauplii fed in the standard feed ration (a) was multiplied with a factor, assuming that always 2 *Artemia* nauplii ml^{-1} remain in the water and are not consumed (Table 1). The experiment was conducted in a small–scale rearing set–up consisting of 1–l bottles. Each treatment was run in five replicates.

Experiment 2 consisted of eight treatments resulting from the combination of four different larval stocking densities and two different feeding frequencies. Feed ration was adjusted in function of larval rearing density. Treatments are outlined in Table 2. Experiment 2 was performed in pilot–scale 12–l cylindro–conical rearing tanks in a completely randomized block design with three replicates per treatment.

Experiment 3 consisted of four treatments based on different initial larval stocking densities (100 to 400 larvae l^{-1}), with the water volume gradually increased during the rearing cycle (Table 3). Experiment 3 was performed in 4–l rearing containers, but the actual rearing volume was, depending on the treatment, stepwise increased from 1 to maximum 4 l. Each treatment had five replicates.

2.3 Larval rearing procedures

Water salinity was adjusted by diluting seawater with deionized water to a salinity of 12 g l^{-1}. Gentle aeration was applied in all rearing tanks. A fluorescent lamp system was installed to provide around 900–1000 lux at the water surface for 12h day^{-1}. Average water temperature was controlled at 29±1 ^{o}C by using a thermostatic heater. pH of the water varied between 7.8–8.2; and dissolved oxygen in the rearing water was always over 5 mg l^{-1}. In all experiments an open clear water system was used with a daily water exchange of 30–50%.

Table 1. Experimental design in experiment 1: larval rearing density and feed ration

Treatment code	Larval density (larvae l^{-1})	Feed ration	*Artemia* nauplii ml^{-1} day^{-1}		
			Day 1–7	Day 8–15	Day 16–PL
50/X	50	a*	4	5	6
100/X	100	a	4	5	6
100/1.5X		1.5(a–2)+2	5	6.5	8
100/2X		2(a–2)+2	6	8	10
200/X	200	a	4	5	6
200/2X		2(a–2)+2	6	8	10
200/3X		3(a–2)+2	8	11	14
200/4X		4(a–2)+2	10	14	18

*: Standard feeding ration

Table 2. Experimental design in experiment 2: larval rearing density and feeding frequency

Treatment code	Larval density (larvae l^{-1})	Feeding frequency	*Artemia* nauplii ml^{-1} day^{-1})		
			Day 1–7	Day 8–15	Day 16–PL
50/2	50	2	4	5	6
50/6		6			
100/2	100	2	6	8	10
100/6		6			
150/2	150	2	8	11	14
150/6		6			
200/2	200	2	10	14	18
200/6		6			

Table 3. Experimental design in experiment 3: initial larval rearing density and rearing volume during the rearing cycle.

Treatment code	Initial larval density (larvae l^{-1})	Rearing volume (l) during rearing cycle			
		Day 1–5	Day 6–10	Day 11–15	Day 16–PL
D100	100	1	1	1	1
D200	200	1	1	2	2
D300	300	1	1.5	2.5	3
D400	400	1	2	3	4

2.4 Artemia preparation and feeding

During the complete rearing cycle *M. rosenbergii* larvae were exclusively fed *Artemia franciscana* nauplii (Great Salt Lake strain). *Artemia* nauplii were collected in instar–I

stage and kept in a refrigerator at 4–6 ºC with gentle aeration in order to maintain throughout the day instar–I stage nauplii for feeding. Feed rations are expressed as *Artemia* nauplii density in the rearing water (nauplii ml^{-1} day^{-1}). When exchanging water, the remaining *Artemia* from the previous day were removed by siphoning. After water exchange, fresh food was added.

In experiment 1, all treatments were offered feed twice a day at 7h00 and 17h00. In experiment 2, the feeding frequency was either 6 times (7h00, 10h00, 13h00, 16h00, 19h00 and 22h00) or 2 times (7h00 and 17h00) per day. The proportioning of the total feed amount in the treatments having 6 feedings was 25% at the first feeding and 15% for every next feeding, while this was 50% per feeding for treatments having only 2 feeding intervals. In experiment 3, the feed ration was the same for all treatments, namely 6, 8 and 10 *Artemia* nauplii ml^{-1} day^{-1} for day 1–7, 8–15 and 16–PL, respectively. Feeding was offered twice a day at 7h00 and 17h00.

2.5 Evaluation parameters

At day 10 and 15, larval development in each treatment was estimated as larval stage index (LSI) determined according to Maddox and Manzi (1976). For this the average larval stage of at least 30 larvae randomly sampled from each treatment was determined. The larval stages were recorded based on the description by Uno and Kwon (1969). The duration of the rearing cycle, which is the time (in days) needed from stocking to reach 90% postlarva stage, was also determined for each rearing tank. From day 5 up to day 25, larval survival was checked every other day in experiment 1 and every three days in experiment 3. At the termination of the experiment (90% of the larvae metamorphosed to postlarvae), the final survival to PL stage was determined for each treatment. From the survival data, the postlarva

production efficiency (number of postlarvae l^{-1}) was determined as a measure of productivity. The number of *Artemia* nauplii used per postlarva produced was also estimated. At the completion of rearing period for experiment 2, larvae were subjected to an ammonia toxicity test following the procedure described by Cavalli *et al*. (2000). As to assess the larval quality, the test was performed on postlarvae in a series of 1–l glass cones at 28±1 ^{o}C: replicate groups of 30 animals from each treatment were exposed for 24h to 4 increasing concentrations of total ammonia and a control (no ammonia added); the concentration of non–ionized ammonia (NH_3) was estimated according to the general formula for the mean values of pH, salinity and temperature as presented by Armstrong *et al*. (1978); based on the mortality, the mean lethal concentration for 50% of the population (24h–LC_{50}) was estimated.

2.6 Statistical analyses

Survival, larval stage index, duration of the rearing cycle and ammonia tolerance (24–h LC_{50}) were analyzed by analysis of variance (one–way ANOVA) and, if significant differences were found ($P<0.05$), the least significant differences (Weller–Duncan) test was applied. All percentage data were normalized by arcsine transformation, but only non–transformed means are presented. Two–way ANOVA was performed to determine the interaction between the experimental factors in experiment 2.

3. Results

3.1 Experiment 1

The larval stage index (LSI) was used as a specific indicator to assess larval growth and development. There were significant differences in LSI between treatments at day 10 and 15 of the experiment ($P<0.05$). At day 10, the treatments 100/X and 200/X

showed the lowest development rate. Subsequently, at day 15 treatments 100/X, 200/X and 200/2X presented significantly lower LSI values than the treatment 50/X. There were also significant differences in the duration of the rearing cycle between the treatments ($P<0.05$). Generally, the time needed to reach 90% postlarvae in the treatments ranged from 27 to 33 days. The treatments 100/X and 200/X and 200/2X which already demonstrated a slower development rate also needed a longer time to reach the postlarval stage (Table 4).

Table 4. Larval stage index and duration of the rearing cycle of *M. rosenbergii* larvae reared at different densities and fed different rations (experiment 1).

Treatment code	Larval stage index		Duration rearing cycle (days)
	Day 10	Day 15	
50/X	6.9 ± 0.1^{ab}	8.1 ± 0.2^{a}	27.0 ± 0.9^{a}
100/X	6.2 ± 0.1^{c}	7.4 ± 0.2^{b}	30.0 ± 0.7^{b}
100/1.5X	6.7 ± 0.1^{bc}	7.5 ± 0.2^{ab}	28.6 ± 1.1^{ab}
100/2X	6.9 ± 0.2^{ab}	7.6 ± 0.2^{ab}	27.8 ± 0.8^{ab}
200/X	6.4 ± 0.2^{bc}	7.0 ± 0.2^{b}	33.2 ± 1.2^{c}
200/2X	6.9 ± 0.2^{ab}	7.2 ± 0.2^{b}	30.0 ± 1.0^{b}
200/3X	7.0 ± 0.2^{a}	7.5 ± 0.3^{ab}	28.8 ± 1.0^{ab}
200/4X	7.0 ± 0.2^{a}	7.7 ± 0.2^{ab}	30.4 ± 0.7^{bc}

Values are average ± SE, n = 5. Different superscript letters within columns denote significant differences ($P<0.05$).

Larval survival in experiment 1 was determined every two days from day 5 to day 25 of the rearing cycle (Fig. 1). There were significant differences ($P<0.05$) in larval survival between the different treatments at several sampling points (data not show). The treatments 100/2X and 200/3X generally showed a higher survival throughout the

experiment. In contrast, survival in treatment 200/X was the lowest. The final postlarval survival in the treatments was counted at the end of the rearing cycle when 90% of the larvae reached the postlarval stage. There were marked differences in final survival between the treatments ($P<0.05$). The treatment 100/2X gave the highest survival (44.2%), while the treatment 200/X yielded the lowest survival (12.7%). Also production efficiency (number of postlarvae produced per liter) showed a big difference between treatments. Efficiency reached the highest value of 48 PL l^{-1} in the treatment 200/3X, while the lowest value was obtained in treatment 50/X (21 PL l^{-1}) (Fig. 2). However, when evaluating production efficiency based on the number of *Artemia* nauplii used per postlarva produced, the treatments 100/2X and 100/1.5X had the best efficiency (around 5,300–5,400 nauplii to produce one postlarva). In contrast, the treatment 200/4X needed around 11,500 *Artemia* nauplii per postlarva (Fig. 3).

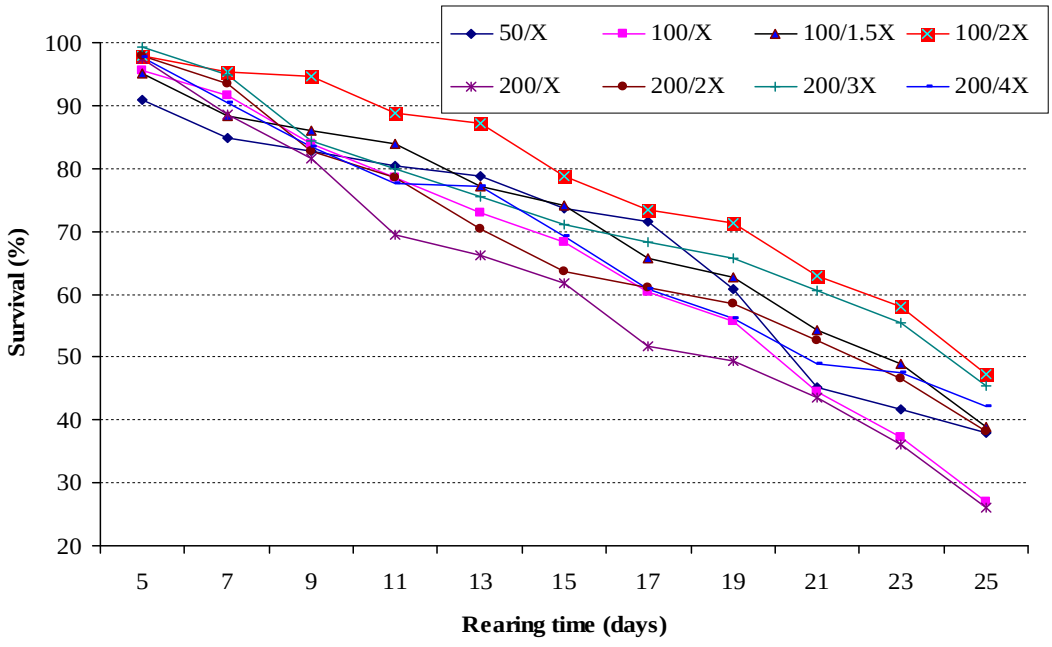

Fig. 1. Evolution of larval survival from day 5 until day 25 of *M. rosenbergii* larvae reared at different densities and fed different rations (experiment 1).

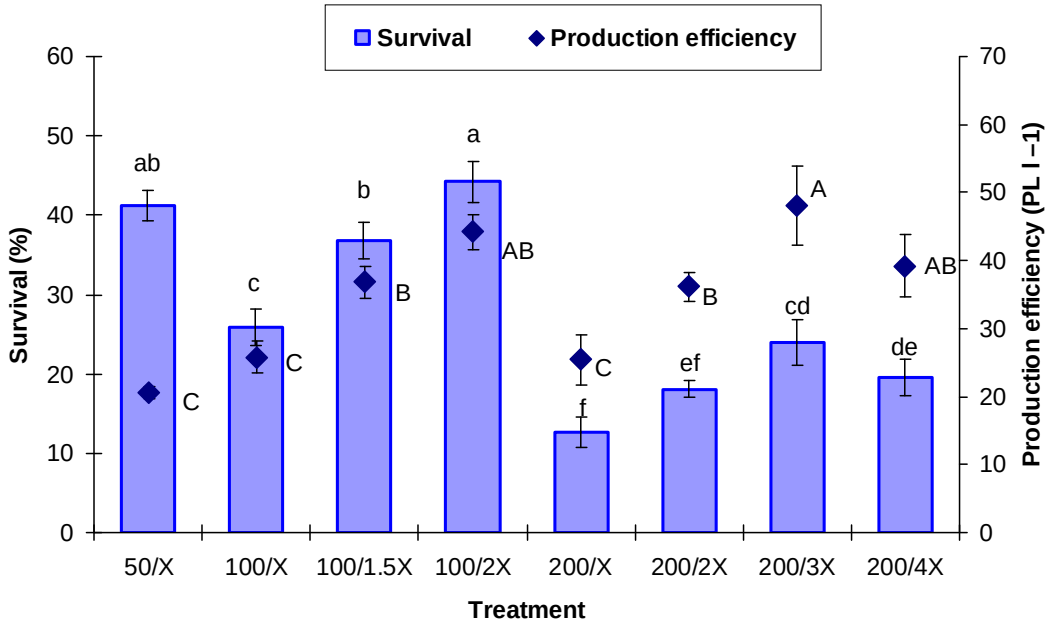

Fig. 2. Final postlarval survival and postlarva production efficiency of *M. rosenbergii* larvae reared at different densities and fed different rations (experiment 1). Values are average ± SE, n = 5. Different letters denote significant differences (P<0.05). Small and capital letters denote different comparisons.

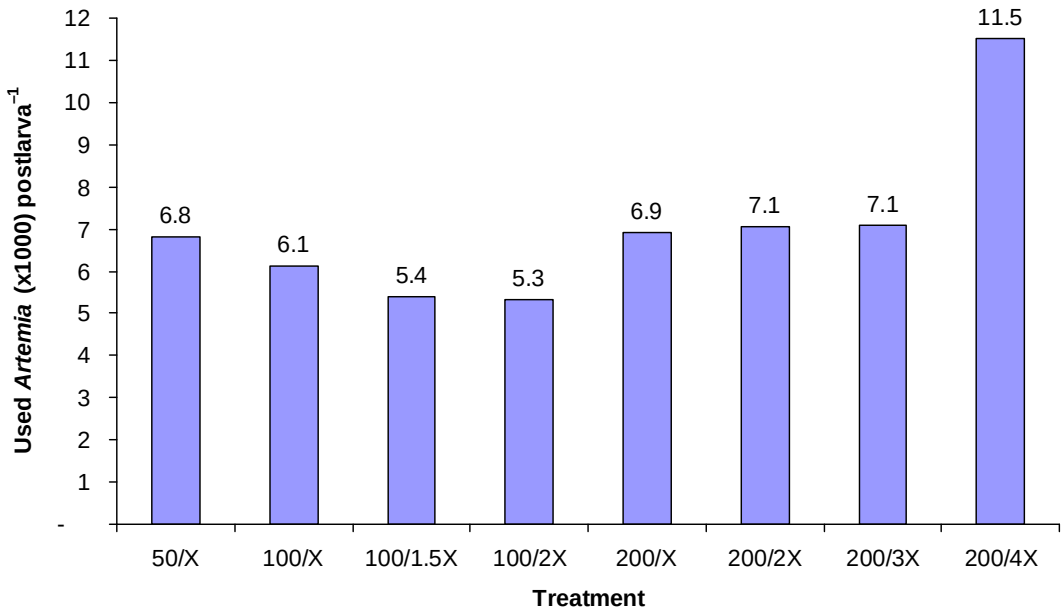

Fig. 3. Number of *Artemia* nauplii used per postlarva of *M. rosenbergii* larvae reared at different densities and fed different rations (experiment 1).

3.2 Experiment 2

In experiment 2, at day 10, the LSI of the larvae showed significant differences between treatments. Development was faster in treatments 50/6, 100/6, 150/6, 200/6 and 50/2 compared to treatments 150/2 and 200/2 ($P<0.05$). Both larval density and feeding frequency significantly affected development ($P<0.05$), however, no interaction was noted ($P>0.05$) between these two factors on the LSI at day 10. Differences in larval stage index between treatments became more pronounced at day 15 with the highest LSI value (9.6) in treatment 50/6 and the lowest value (8.2) in treatment 200/2. Interestingly, larval development in treatments 150/6 and 200/6 was similar to treatments 50/2 and 100/2. Two–factorial analysis on the factors larval density and feeding frequency did not reveal interaction between the two ($P>0.05$) (Table 5).

Metamorphosis from larva to postlarva and duration of the rearing cycle was largely influenced by larval development rate. The results showed that both larval density and feeding frequency affected the metamorphosis process and rearing cycle duration ($P<0.05$), however larval density seemed more decisive in this respect. Hence treatments 50/2, 50/6 and 100/6 demonstrated a significantly shorter larval rearing duration than the others treatments (Table 5). For this parameter, a significant interaction between the two factors was found ($P<0.05$).

Table 5. Larval stage index at day 10 and 15 and duration of the rearing cycle of *M. rosenbergii* larvae reared at different densities and with different feeding frequencies (experiment 2).

Treatment code	Larval stage index		Duration rearing cycle (days)
	Day 10	Day 15	
50/2	7.5±0.1^{ab}	9.0±0.1^{cd}	25.3±0.6^{a}
50/6	7.7±0.1^{a}	9.6±0.1^{a}	24.3±0.6^{a}
100/2	7.3±0.1^{bc}	8.6±0.1^{d}	27.7±0.6^{bc}
100/6	7.8±0.1^{a}	9.3±0.2^{ab}	25.3±0.5^{a}
150/2	7.1±0.1^{c}	8.2±0.1^{e}	28.0±1.0^{bc}
150/6	7.6±0.1^{ab}	8.9±0.1^{cd}	27.0±1.0^{b}
200/2	7.0±0.1^{c}	8.2±0.1^{e}	28.3±0.6^{c}
200/6	7.5±0.1^{ab}	8.8±0.1^{cd}	28.7±0.6^{c}
Density factor	P = 0.001	P = 0.000	P = 0.000
Frequency factor	P = 0.000	P = 0.000	P = 0.003
Interaction	P = 0.443	P = 0.832	P = 0.038

Values are average ± SE, n = 3. Different superscript letters within columns denote significant differences (P<0.05).

Larval stocking density had a strong effect on larval survival (P<0.05). Larval survival was as high as 66 and 56% in treatments 50/6 and 50/2, respectively (Fig. 4). However, survival decreased to low values of 16 and 11% in treatments 200/6 and 200/2 respectively. The results showed that feeding frequency had a significant effect on larval survival, with improved larval survival with increasing feeding frequency (P<0.05). However, there was no interaction between the two factors tested (P>0.05). Both factors also significantly affected postlarva production efficiency (P<0.05). However, there was no interaction between these two factors (P>0.05). The highest production efficiency value (38 PL l^{-1}) was found in the treatments 100/6 and 150/6,

and the lowest (22 PL l^{-1}) in treatment 200/2 (Fig. 4). There was a marked difference in production efficiency of respectively 27, 36 and 73% between treatments 100/6 and 150/6 (both 38 PL l^{-1}) when compared with treatments 150/2 (30 PL l^{-1}), 50/2 (28 PL l^{-1}) and 200/2 (22 PL l^{-1}). Evaluating the production efficiency based on the number of *Artemia* nauplii used to produce one postlarva showed a lot of variation between treatments. The lowest value of 3,800 *Artemia* nauplii per postlarva was found in treatment 50/6 while the highest value of 18,900 *Artemia* nauplii was found in treatment 200/2 (Fig. 5).

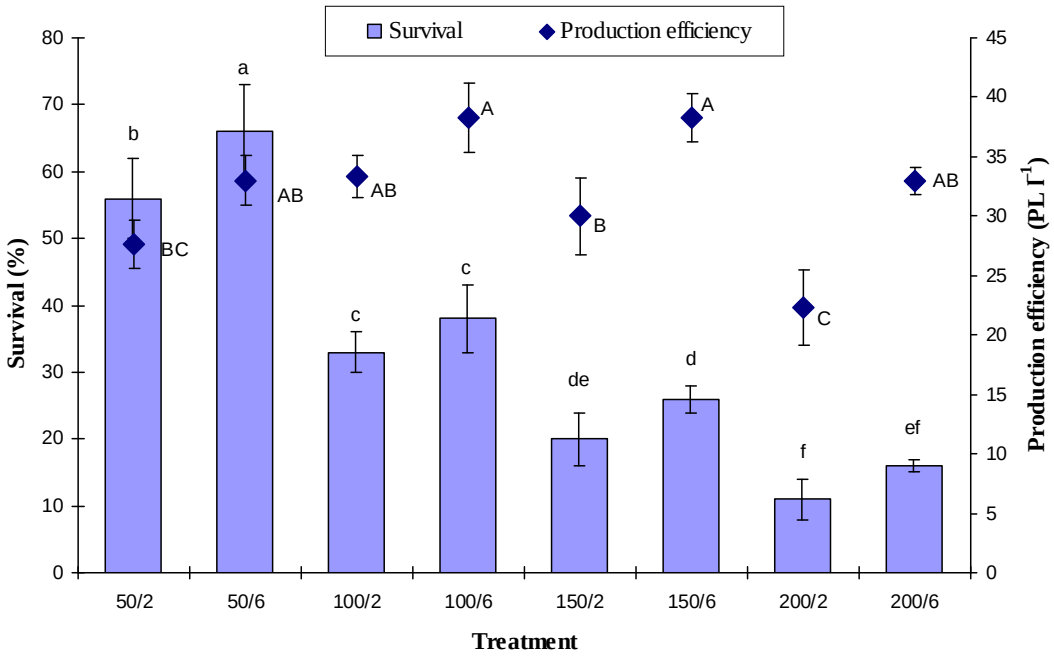

Fig. 4. Final survival and postlarva production efficiency of *M. rosenbergii* larvae reared at different densities and with different feeding frequencies (experiment 2). Values are average ± SE, n = 3. Different letters denote significant differences (P<0.05). Small and capital letters denote different comparisons.

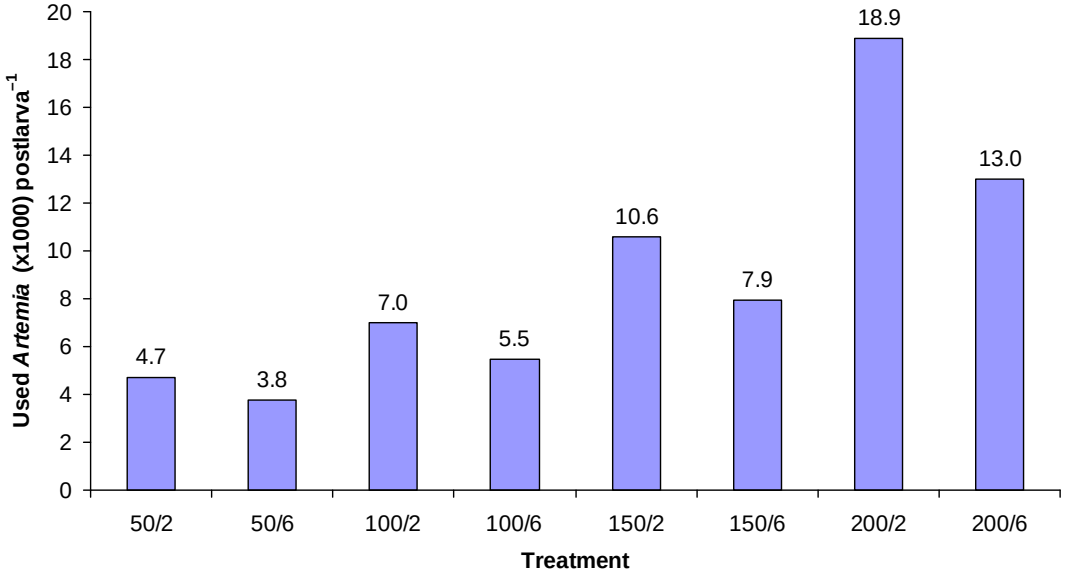

Fig. 5. Number of *Artemia* nauplii used per postlarva of *M. rosenbergii* larvae reared at different larval densities and with different feeding frequencies (experiment 2).

Results of the ammonia test showed significant differences in terms of tolerance of the larvae to ammonia between treatments. Larval density significantly affected larval quality ($P<0.05$), as ammonia tolerance decreased with increasing larval rearing density. However, there was no significant effect detected of feeding frequency on larval quality, nor was there any interaction between both factors ($P > 0.05$) (Fig. 6).

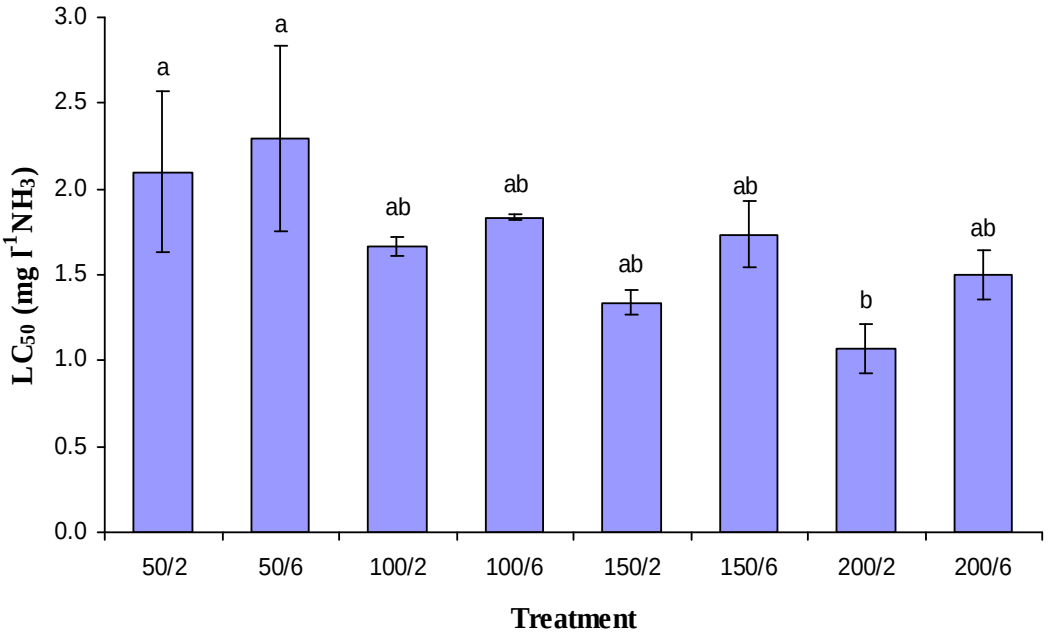

Fig. 6. Ammonia tolerance (expressed as 24hour LC_{50}–NH_3) of *M. rosenbergii* larvae reared at different densities and with different feeding frequencies (experiment 2). Values are average ± SE, n = 3. Different letters denote significant differences (P<0.05).

3.3 Experiment 3

Larval stage index at day 10 in treatment D100 was significantly higher than in the three other treatments. However, at day 15, larval stage index, larval survival and duration of the rearing cycle were not significantly different among treatments (Table 6 and Fig. 7). In most treatments substantial mortality occurred between day 13 and 16. Survival decreased from over 80% to less than 60% during these 3 days (Fig. 7). Production efficiency in terms of number of *Artemia* nauplii used to produce a postlarva was around 4,800, 4,600 and 4,200 in treatments D200, D300 and D400 respectively. In contrast, treatment D100 referred around 6,600 *Artemia* nauplii for the production of one postlarva (Fig. 8).

Table 6. Larval stage index at day 10 and 15, postlarval survival and duration of the rearing cycle of *M. rosenbergii* larvae reared at different densities (experiment 3).

Treatment code	Larval stage index		Duration rearing cycle (days)
	Day 10	Day 15	
D100	8.3±0.1[a]	9.1±0.2[a]	25.8±0.4[a]
D200	7.4±0.1[b]	8.9±0.1[a]	25.4±0.2[a]
D300	7.3±0.2[b]	9.2±0,1[a]	25.2±0.2[a]
D400	7.5±0.2[b]	9.1±0.2[a]	25.6±0.2[a]

Values are average ± SE, n = 5. Different superscript letters within columns denote significant differences (P<0.05).

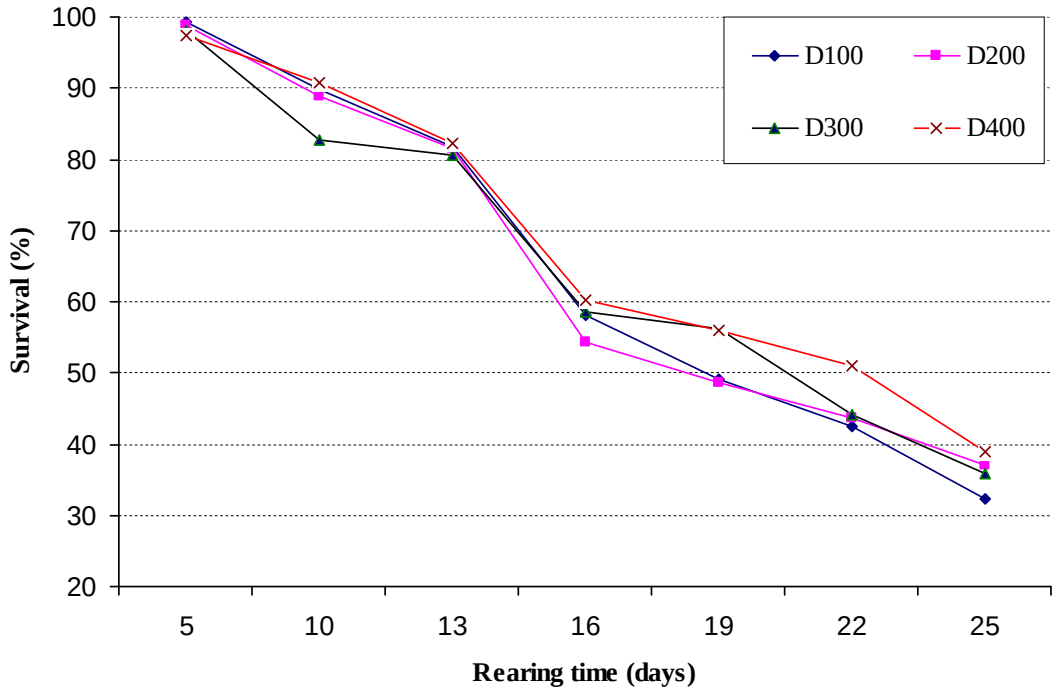

Fig. 7. Evolution of larval survival during the rearing cycle of *M. rosenbergii* larvae reared at different densities (experiment 3).

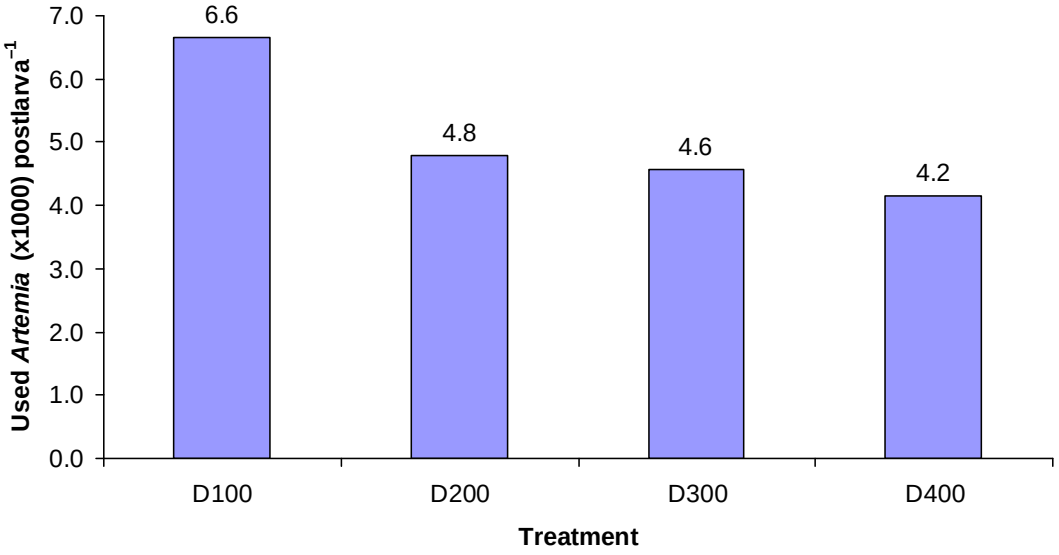

Fig. 8. Number of *Artemia* nauplii used per postlarva of *M. rosenbergii* larvae reared at different densities (experiment 3).

4. Discussion

Larval development and survival of freshwater prawn are affected by several factors (Teshima and Kanazawa 1983). In the current study, larval growth and survival were strongly affected by larval stocking density and feeding regime (both in terms of feed ration and feeding frequency). However, the results of the different experiments also depended on other factors such as broodstock, quality of the newly-hatched larvae, the scale of the experimental system used, and the weather conditions during the experiment… Consequently, when possible, the results are also compared between experiments.

In experiment 1, when larval density was increased from 50 to 100 and 200 larvae L^{-1} without increasing the feed ration (number of *Artemia* nauplii ml^{-1} day^{-1}), both larval development rate and survival significantly decreased. Lim and Hirayama (1993) reported that larval stocking density is a crucial factor in larval production. The effect

of increasing larval density could however partly be counteracted by either increasing the feed ration (experiment 1) or increasing the feeding frequency (experiment 2). Larval growth and survival in treatments using a larval density of 100 l^{-1} clearly improved when the standard feed ration (a) was increased. Also in the treatments with a larval density of 200 l^{-1}, better results were obtained when the standard ration was increased. Interestingly, in experiment 2, larval development in treatments 150/6 and 200/6 was mostly the same as in treatments 50/2 and 100/2. This indicates that larval stocking density can be increased to 200 larvae l^{-1}, with no significant effect on development, using the same feed ration but merely increasing the feeding frequency to 6 times per day. The feeding frequency may affect larval development through differences in the energy content of the *Artemia* nauplii. At the high water temperatures during larval rearing, freshly hatched *Artemia* nauplii (instar I) develop into the second larval stage (instar II) within 6–8 h (Sorgeloos *et al.*, 2001). When *Artemia* nauplii molt from the instar I into the instar II–III stage their dry weight and energy contents decrease by 20% and 27% respectively (Benijts *et al.*, 1976). Therefore, larval growth may be reduced in the treatments that are fed only 2 times per day since part of the not immediately consumed *Artemia* nauplii reside in the culture tank for up to 10 hours. Cold storage of freshly-hatched nauplii at temperatures near 4 °C, in densities of up to eight million nauplii per liter for up to 24 h will greatly reduce their metabolic rate, i.e., only 2.5% drop in individual dry weight versus 30% at 25 ºC, and preclude molting to the second instar stage (Léger *et al.*, 1983). In addition, feeding several times per day ensured that the newly-hatched *Artemia* nauplii were always available in the rearing tank at appropriate densities. In contrast, both the quantity and quality of the *Artemia* nauplii in the treatments fed only twice a day varied greatly throughout the day. This can have negatively affected

larval food intake and triggered cannibalistic behaviour, in this way not only affecting larval development, but also larval survival. In this respect Teshima and Kanazawa (1983) reported that prawn larvae had higher survival when fed twice a day rather than once a day. Also Barros *et al.* (2003) concluded that suboptimal feeding during larval rearing may result in reduced growth rate and cannibalism. In experiment 2, larval survival was 56–66% in the treatments with a stocking density of 50 l^{-1}, but was only 11–16% in the treatments with a density of 200 l^{-1}. Mortality of the larvae most likely was due to cannibalism, which is characteristic for *M. rosenbergii*, especially in case of high larval densities. Coyle *et al.*, (2004) reported that survival is highly variable and may be related to the cannibalistic behaviour of juvenile freshwater prawn when cultured at high densities in the nursery. In addition, cannibalism of *M. rosenbergii* larvae depended on the feeding regime and/or feed ration. Ohs *et al.* (1998) showed different proportions of cannibalism, namely 35.0 and 9.3% in freshwater prawn larvae fed artificial diets and live *Artemia* nauplii, respectively. Diaz *et al.* 1990 compared survival of *M. rosenbergii* juveniles of which the tip of the propodus of both chelae was removed with untreated animals. Survival of the treated group was 92%, while that of the untreated group was only 25%.

In experiment 3, it was interesting to note that similar results in terms of larval growth and survival were obtained between treatments with different initial larval stocking density, but with the rearing volume gradually increased and hence rearing density gradually lowered during culture. This indicates that, in the first phase of the larval rearing cycle (first 7 days) larval density may be increased to 400 l^{-1} without negative effects on larval development or survival. The high larval density in the early stage of rearing may not only help saving hatchery space and water consumption, but may also result in more efficient use of costly live feed. Smaller rearing volumes in early larval

stages can reduce *Artemia* nauplii consumption which is a function of water volume and prey density requirements of the predator larvae. Subsequently, in a later stage, when the larvae become more efficient predators, but also cannibalism becomes more pronounced, the larval density can then be decreased by increasing the rearing volume. In this study, the water volume was increased in 3 intervals, based on four rearing periods. However, in practice, the water volume may also be gradually increased to reach the final rearing volume at the end of the rearing cycle, starting with an initial stocking density of 100 larvae per liter. It is expected that this rearing protocol would be helpful to reduce water and feed consumption and hence production cost.

The economical efficiency of seed production is based on the production efficiency (number of PL per rearing unit) and the production cost. Hatcheries strive at increasing production efficiency, with the aim to improve benefit. In a study on kuruma prawn (*Penaeus japonicus*), Lim and Hirayama (1993) showed that in the range of larval stocking densities of 8–48 l^{-1}, the maximum production efficiency was reached at 25 larvae l^{-1}. In Vietnam, a few studies were performed on stocking density in larval rearing of giant freshwater prawn, e.g. Tinh, *et al*. (1999), investigated different water exchange methods and reported survival of 40% at a stocking density of 80–100 larvae l^{-1}. Hung and Phuc (1999) showed a variation in survival from 24 to 53% at a stocking density of 100 larvae l^{-1} using an open water system. Unfortunately however, these studies did not link their results with efficiency of hatchery production. In the current study production efficiency in terms of water volume varied greatly between treatments and between experiments. The highest values were found for treatment 200/3X (48 PL l^{-1}) in experiment 1 and treatments 100/6 and 150/6 (38 PL l^{-1}) in experiment 2. However, very different results were obtained when

considering production efficiency in term of number of *Artemia* nauplii used per postlarva. For this, the best results were obtained in treatments 100/2X (experiment 1), 50/6 (experiment 2) and treatment D400 (experiment 3). In practice however, several other factors also affect production efficiency. For example, artificial diets are usually used to supplement the live feed from larval stage VII onwards (Barros *et al*., 2003). Depending on which factors are more decisive for the local situation (space availability, feed cost…) larval rearing protocols may change.

In current hatchery practices, larval quality is becoming of major concern. Unfortunately, to date limited information is available on standardised methods for accurate measurement of larval and postlarval quality (Cavalli *et al.*, 2000). A short–term ammonia toxicity test was used in this study to assess differences in larval quality between the different treatments. Cavalli *et al*. (2000) showed that ammonia tolerance (LC_{50}) of larvae fed enriched *Artemia* was higher than in larvae fed starved *Artemia*. In the current study, postlarval quality in terms of ammonia tolerance was significantly higher in the low density treatments (50 larvae l^{-1}) as compared to the high density treatments (200 larvae l^{-1}). This may be due to the better environmental and nutritional conditions in the low density treatments, resulting in stronger larvae.

The results of this study suggest that newly–hatched instar I *Artemia* nauplii should be applied several times per day to ensure sufficient quantities of fresh food throughout the day. It would be interesting to design an automatic drip–feeding system to control and maintain feed quantity and quality. The initial larval stocking density should be decided based on the local situation. 100 to 150 larvae l^{-1} seems appropriate when a constant rearing volume is used. However, higher densities (200 larvae l^{-1}) could be applied when feed cost is not decisive. Alternatively, the initial density may be as

high as 400 larvae l^{-1}, but should then gradually be lowered by increasing the rearing volume as the animals develop.

Acknowledgments

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CHAPTER 4

SECTION 2

Evaluation of different diets to replace *Artemia* nauplii for larval rearing of giant freshwater prawn (*Macrobrachium rosenbergii*)

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Abstract

A study was conducted on *Macrobrachium rosenbergii* larvae to evaluate the efficiency of different diets to replace *Artemia* nauplii in the feeding scheme. The study included two experiments performed at pilot scale in 12–l tanks using a recirculating system. Larval stocking density was 100 l^{-1}. After 7 days of feeding by *Artemia* nauplii, different diets, included wet and dry diets and decapsulated *Artemia* cysts, were tested to replace *Artemia* nauplii. An extra treatment using only decapsulated *Artemia* cysts throughout the complete larval rearing was also included. The results showed that feeding larvae exclusively decapsulated cysts for the complete rearing cycle was not appropriate. When gradually replacing up to 50% of the *Artemia* nauplii ration with wet or dry diets, good results in terms of growth, survival and quality of the larvae were obtained, similar to the control treatment receiving only *Artemia* nauplii. However, abruptly replacing 50% of the *Artemia* nauplii ration with artificial diets negatively affected larval development. Weaning could start from larval stage V, with about 25% of the *Artemia* nauplii replaced with artificial diet. Subsequently, the weaning ration could be increased up to 50% from stage IX to postlarva stage. Artificial diets should be provided in different particle size ranges based on the larval stage, gradually increasing from 250 to 1000 µm from stage V to postlarva stage. The results obtained in the present study may aid future research and serve as a baseline for further optimization of feeding strategies in prawn larviculture.

Keywords: *Macrobrachium rosenbergii*, *Artemia*, larval rearing, artificial diet, weaning.

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* is a commercially important species in freshwater aquaculture in Vietnam and other Southeast Asian countries. Freshwater prawn farming has been pinpointed as one of the major target species of the aquaculture sector. The Ministry of Fisheries of Vietnam has put forth that the annual production of *M. rosenbergii* must reach 60,000 tons utilizing 32,000 ha by the year 2010 (Government of Vietnam, 1999). Freshwater prawn culture has great potential for rural aquaculture, generating considerable employment and income, thereby bringing prosperity to rural poor. Freshwater prawn farming is environmentally sustainable, since it is practiced at lower grow–out density (New 1995). A majority of seed used in grow out farming of *M. rosenbergii* comes from hatcheries (Murthy *et al*. 2004, Phuong *et al*. 2006). Existing hatcheries in the country are however not producing up to their installed capacity due various constraints.

Artemia nauplii are the preferred live food source used in the larviculture of many crustaceans of commercial value. Several authors demonstrated that *Artemia* nauplii suffice to produce *M. rosenbergii* postlarvae (Devresse *et al*., 1990; Lavens *et al*., 2000). However, others showed that *Artemia* nauplii do not completely fulfil the nutritional requirements of larvae during the last larval stages and therefore recommend the use of supplemental diets (New, 1995; Valenti and Daniels, 2000). As a feed source, decapsulated *Artemia* cysts have a higher energy and nutritional value than live *Artemia* nauplii (Bengtson, *et al*., 1991). Leger *et al*. (1987) showed that decapsulated *Artemia* embryos have 30–50% more energy than newly–hatched nauplii (instar I). Sorgeloos *et al*. (1977) suggested the use of decapsulated cysts as a direct source for fish and crustacean larvae. Subsequent studies demonstrated that

decapsulated cysts are a good feed similar to freshly hatched *Artemia* nauplii for the larvae of marine shrimps and freshwater prawn, such as *Penaeus monodon* (Mock, *et al*., 1980), and *Macrobrachium rosenbergii* (Bruggeman, *et al*., 1980). For fish species, decapsulated cysts could be used as a substitute for *Artemia* nauplii or *Moina* in fish larviculture e.g. milkfish, *Chanos chanos* (De Los Santos *et al*., 1980), African catfish, *Clarias gariepinus* (Verreth *et al*., 1987) and in ornamental fish culture (Lim *et al*., 2002).

Although live food such as *Artemia* nauplii has proven successful for raising the larvae of many species, inherent problems remain such as the potential introduction of pathogens into the culture system or the high costs of labour and equipment required for preparation. In addition, the nutritional quality and physical properties of *Artemia* nauplii are depending on the source and time of harvest of cysts (Sorgeloos *et al*., 1983). Imported *Artemia* cysts are predominantly used, which are expensive and uncertain in availability. Dependence entirely on *Artemia* as feed not only makes hatchery operations expensive, but also unsustainable (Murthy *et al*. 2008). The dependence on *Artemia* is also a major constraint in the expansion of *Macrobrachium rosenbergii* hatcheries (New 1990). Hence, there is a need to look for acceptable alternative diets to replace *Artemia* and reduce the cost of prawn larval rearing. Several alternative foods, both live and inert, are being investigated as either supplement or replacement for *Artemia* nauplii in crustacean hatcheries. Ohs (1995) and Wan (1999) developed several semi–purified spray–dried diets and evaluated their performance with larval striped bass, *Morone saxatilis* and freshwater prawn *Macrobrachium rosenbergii*. Larvae of both species consumed the diets, but growth and survival were significantly less than that of *Artemia*–fed larvae. However, Kovalenko *et al*. (2002) reported that larval growth of freshwater prawn fed a

microbound diet was 90% of that achieved for larvae fed newly–hatched nauplii of *Artemia*. Survival of the larvae fed the microbound diet was not significantly different from that of *Artemia*–fed larvae. Several studies also investigated supplementation of *Artemia* with prepared feed in prawn larval rearing (Sick and Beaty 1975, Corbin *et al.*, 1983). However no standard substitute for *Artemia* has been developed for freshwater prawn hatcheries. Barros and Valenti (2003b) developed an ingestion rate model of *Artemia* nauplii for *M. rosenbergii* larvae based on the individual ingestion rate and prey density. However, this equation indicated that *Artemia* is not an adequate prey for later larval stages and that there is a necessity for a supplementary diet from stage IX onwards. Several studies indeed confirm this finding, however controversy still exist concerning the best timing to introduce formulated feeds in the feeding schedule. Aquacop (1983) and Daniels *et al*. (1992) recommend diet supplementation from stages V–VI. Barros and Valenti, (2003a) reported supplementation should start from stage VII onwards. The development of the larval digestive tract and the increase of enzyme activity from stage VI onwards (Jones *et al.*, 1993; Kamarudin *et al.*, 1994; Kumlu and Jones, 1995) may explain the acceptance of inert diets, since digestion processes become thoroughly functional. In order to further optimize the feeding schedule for *M. rosenbergii* larval rearing, a series of experiments were performed in the present study to evaluate the use of formulated larval diets to supplement or partially replace *Artemia* nauplii.

2. Materials and methods

2.1 Experimental animals

Two experiments were conducted at the experimental hatchery of the Faculty of Fisheries, Nong Lam University, Vietnam. *M. rosenbergii* breeders bearing yellow eggs were obtained from culture ponds in Ben Tre province, Southern Vietnam and acclimated to the hatchery conditions for egg incubation. 24h after hatching, larvae were collected and stocked into the experimental tanks.

2.2 Experimental design

Experiment 1 consisted of seven treatments, which originated from the combination of different diets (*Artemia* nauplii, decapsulated *Artemia* cysts, two commercial dry diets and a wet egg custard diet (Table 1). Experiment 1 was performed in pilot–scale 12–l cylindro–conical rearing tanks with three replicates per treatment. Three separate recirculation systems were installed, with one replicate of each treatment assigned to each system. Each recirculation system consisted of 120–l cylindro–conical reservoir tank connected to a 160–l submerged biological filter and a 60–l overhead tank. Water was continuously pumped from reservoir tank to the overhead tank and then forced back through the bottom of the rearing tanks by gravity at 0.3 l min^{-1}. An outlet screen (150 μm) at the surface of the rearing tank led the water back to the biological filter tank and at the same time retained the larvae and *Artemia* within the rearing tank. The filter screen was cleaned daily to avoid water overflow. Water with a salinity of 12 g l^{-1} was obtained through mixing deionised water and natural seawater. Aeration in the rearing tanks and filter tanks maintained the oxygen level above 5 mg l^{-1}. Ammonia, nitrite and nitrate were always below 0.1, 0.03 and 50 mg l^{-1}

respectively, while pH varied from 7.8 to 8.2. The waste and uneaten food in rearing tanks were removed every morning before feeding by siphoning. The same amount of prepared water was added into the system to keep the water volume constant. Light was supplied for 12h per day at 800–1000 lux (fluorescent lamp) at the water surface. Larvae were stocked at an initial density of 50 larvae l^{-1}. Experiment 2 consisted of four treatments. In three treatments 25–50% of the *Artemia* nauplii ration was replaced with different artificial diets based on the larval stage of the animals. A control treatment was fed 100% *Artemia* nauplii (Table 2). Experiment 2 was performed in pilot–scale 12–l cylindro–conical rearing tanks with three replicates per treatment at initial larval density of 50 l^{-1} using the same recirculation system as described in experiment 1.

2.3 Diet preparation and feeding

M. rosenbergii larvae in the two experiments were fed different diets including *Artemia franciscana* nauplii (Great Salt Lake strain, Crystal Brand, Ocean Star International, Inc. USA); a wet egg custard–like diet following the formulation of Hien *et al*. (2002); and two kinds of commercial shrimp larval diets (1) Brine Shrimp Flakes (Ocean Star International, Inc. USA) and (2) Gromate (Fantai company, Taiwan). The formulation of the wet diet and the proximate composition of the three different substitution diets are presented in Table 3.

Artemia naupllii were hatched according to standard techniques following Van Stappen (1996). *Artemia* nauplii were collected as instar I stage and kept in a refrigerator at 4–6 ºC with gentle aeration in order to maintain instar I stage nauplii for feeding throughout the day. Decapsulated *Artemia* cysts used in the experiment 1 were prepared following Tunsutapanich (1979). The ingredients of the wet diet were

weighed and blended. The resulting mixture was placed in a pan and cooked in a water bath to pudding consistency. After cooling, it was cut into small pieces, individually wrapped with polyethylene film and kept in a freezer for use the next 1–2 weeks. Before being fed to the larvae, the pieces were made into smaller particles, which were then sieved with different mesh screens to obtain three size classes of 250–500, 500–750 and 750–1000 µm for feeding based on the larval stages IV–VI, VII–IX and X–XII respectively. The Brine Shrimp Flake diet was also sieved into different size classes using mesh screens to obtain the desired sizes for feeding. The Gromate feed had a particle size from 150–500 µm and could directly be fed to the larvae. All supplemental or substitution diets were fed to the larvae from day 8 after hatching onwards (about larval stages V–VI). The artificial diets were fed several times daily following the feeding schemes in Tables 1 and 2. The different substitution and supplementation treatments were based on a standard *Artemia* ration of 6, 8 and 10 Artemia nauplii ml^{-1} day^{-1} for the periods from day 1–7; day 8–15 and day 16–PL stage respectively. The amount of formulated feeds given was based on visual observation of the larval tanks upon feeding. Special care was taken not to overfeed, as this may cause degradation of the water quality.

Table 1. Different diets and feeding schedules used in experiment 1

Treatment	Feeding scheme										
	Day 1–7		Day 8–PL								
	7h	17h	7h	9h	10h	11h	12h	13h	14h	15h	17h
100N	50N	50N	50N								50N
50N+50C	50N	50N	50C								50N
100C	50N	50N	50C								50C
75N+F	50N	50N	25N		F		F		F		50N
75N+W	50N	50N	25N		W		W		W		50N
50N+F	50N	50N	F	F		F		F		F	50N
50N+W	50N	50N	W	W		W		W		W	50N

N: *Artemia* nauplii; C: Decapsulated *Artemia* cysts F: Brine Shrimp Flakes; W: Wet diet. Values represent the percentage of the standard daily *Artemia* nauplii/cysts ration, which constitutes 6, 8 and 10 *Artemia* nauplii/cysts ml^{-1} for day 1–7; day 8–15 and day 16–PL stage respectively.

Table 2. Different artificial diets and feeding schedules used to supplement or substitute *Artemia* nauplii in experiment 2.

Treatment	Larval stage	Feeding scheme				
		7h00	10h00	12h00	14h00	17h00
Control (1) 100N	I–PL	50N				50N
Replaced *Artemia* nauplii	I–V	50N				50N
(2) N+W (3) N+F	VI–VIII	25N	x	x	x	50N
(4) N+G	IX–PL	x	x	x	x	50N

N: *Artemia* nauplii; W: Wet diet; F: Brine Shrimp Flake; G: Gromate; "x": time points when artificial diets were fed. Values represent the percentage of the standard daily *Artemia* nauplii ration, which constitutes 6, 8 and 10 *Artemia* nauplii ml^{-1} for day 1–7; day 8–15 and day 16–PL stage respectively.

Table 3. Formulation of the wet diet and proximate composition of the three formulated diets

Formulation of wet diet (%)		Proximate composition of formulated diets (% dry weight)			
			Wet diet	Flakes*	Gromate*
Milk powder	53.8	Protein	48.6±1.2	53	57
Chicken egg yolk	41.7	Lipid	25.5±0.7	9	8
Squid oil	3.0	Ash	5.8±0.1	4	13
Lecithin	1.5	Mineral	6.5±0.1	2	2
Vitamin C	200 mg kg^{-1}	Fiber	0.3±0.0	2	4
		Moisture	57.7±2.5	9	9

* Composition based on the product file

2.4 Evaluation parameters

At day 10 and 15, a larval stage index (LSI) was determined following Maddox and Manzi (1976) to assess larval development. For this at least 30 larvae were sampled from each treatment and the average larval stage determined. The larval stage was recorded based on the description by Uno and Kwon (1969). The duration of the rearing cycle (days) was determined for each rearing tank. For this the duration from larval stocking up to the time 90% of the larvae in the rearing tank had metamorphosed into postlarvae was recorded. At the same time the final larval survival in each treatment was recorded. Larvae were also subjected to a total ammonia nitrogen (TAN) toxicity test following the procedure described by Cavalli *et al*. (2000) in order to assess larval quality. The test was performed on postlarvae in a series of 1–l glass cones at 28±1 ^{o}C. Groups of 30 animals from each treatment were exposed during 24h to 4 increasing concentrations of total ammonia and a control (no ammonia added). As the toxicity of TAN is a function of temperature and pH, the pH

of the test solution was adjusted at 7.8–8.0. Based on the mortality, the mean lethal concentrations for 50% of the population (24h–LC_{50}) were estimated.

2.5 Statistical analyses

Larval stage index; duration of rearing cycle; survival and ammonia toxicity data were analyzed by analysis of variance (one–way ANOVA) and, if significant differences were found ($P<0.05$), the least significant differences (Weller–Duncan) test was applied for post hoc comparison. All percentage data were normalized by square root–arcsine, but only non–transformed means are presented.

3. Results

3.1 Experiment 1

Larval development rate in terms of larval stage index in experiment 1 showed significant differences between treatments. At day 10, three different groups had formed based on larval stage index ($P<0.05$). The lowest performance was observed in the treatments 50N+50C and 100C. In contrast the fastest growth was found for treatments 100N, 75N+F and 75N+W. Treatments 50N+F and 50N+W showed intermediate development rates. At day 15 of the experiment, the larval development rate in treatment 100C was significantly lower compared to all others treatments ($P<0.05$). The treatment 50N+50C had a significantly higher LSI than the treatment 100C but lower than treatment 75N+W (Fig. 1). Larval survival at the end of rearing cycle also showed significant differences. Three different groups could be distinguished. The lowest survival (30%) was observed in the treatments 100C and 50N+F. The highest survival (43–45%) was observed in the treatments 100N, 75N+F and 75N+W. Intermediate values around 35% were found in the treatments 50N+50C

and 50N+W (Fig. 2). Considering the duration of the rearing cycle, an opposite trend as for survival was noted. Larvae in the treatments 75N+F and 75N+W needed around 24–25 days of rearing to reach the postlarval stage, which was significantly shorter than for treatments 50N+50C and 100C, in which the duration of the rearing cycle was extended up to 28–29 days (Fig. 2). The results of the ammonia stress test showed differences in postlarval tolerance (LC_{50}) ($P<0.05$). The group containing treatments 100C and 75N+F presented the lowest values (136–138 mg l^{-1} TAN), intermediate tolerance levels were found in treatments 50N+50C and 50N+W (165–168 mg l^{-1} TAN), while the highest tolerance was found in treatments 75N+F and 75N+W (185–189 mg l^{-1} TAN) (Fig. 3). In general, the treatments 100N, 75N+W and 75N+F showed the best overall results in term of larval development, survival and larval quality. While the treatments 100C and 50N+F showed the lowest results.

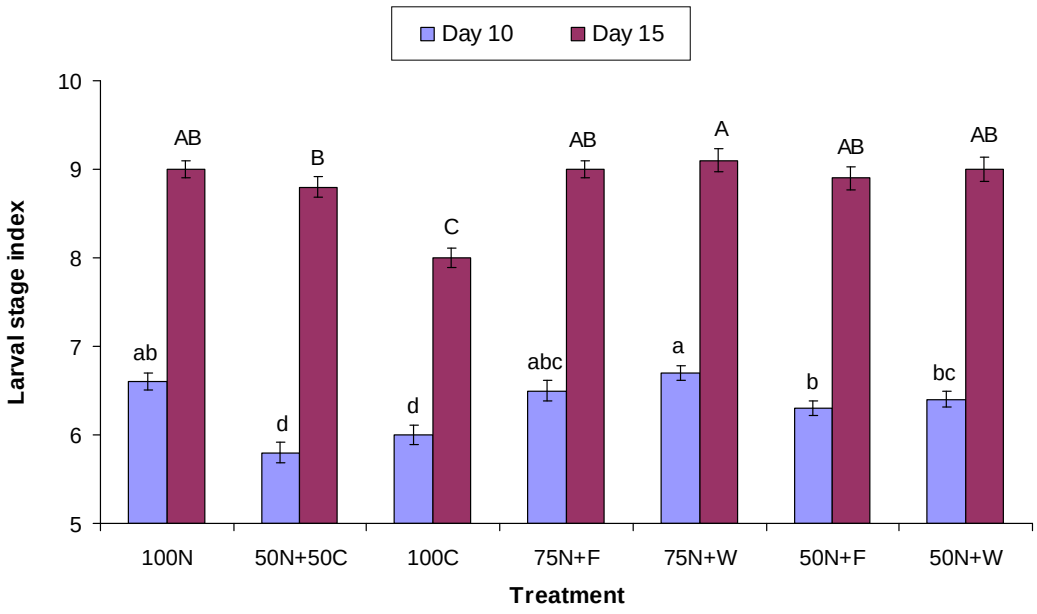

Fig. 1. Larval stage index at day 10 and 15 of *M. rosenbergii* larvae reared according to different feeding schedules in experiment 1. Different letters between treatments denote significant differences ($P<0.05$). For description of treatments refer to Table 1

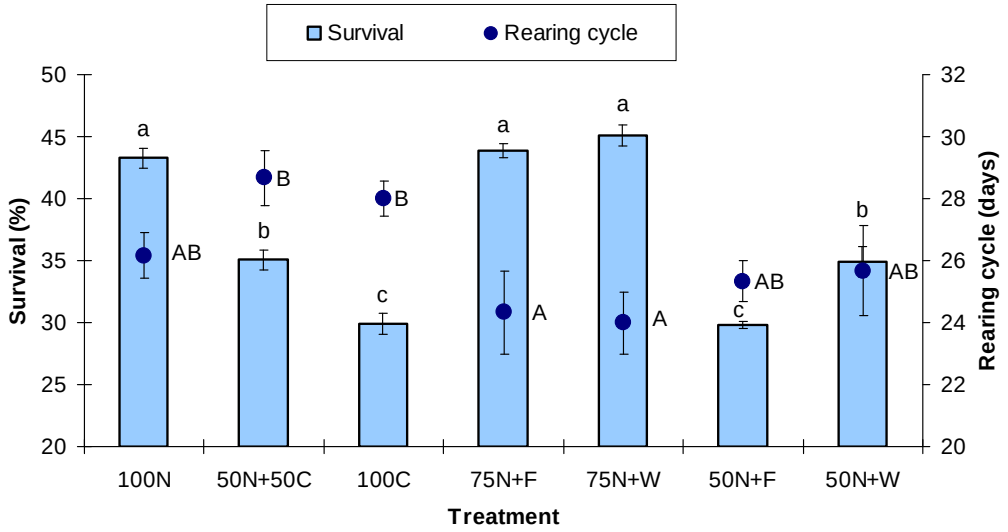

Fig. 3. Survival and duration of the rearing cycle of *M. rosenbergii* larvae reared according to different feeding schedules in experiment 1. Different letters between treatments denote significant differences (P<0.05). For treatment descriptions refer to Table 1.

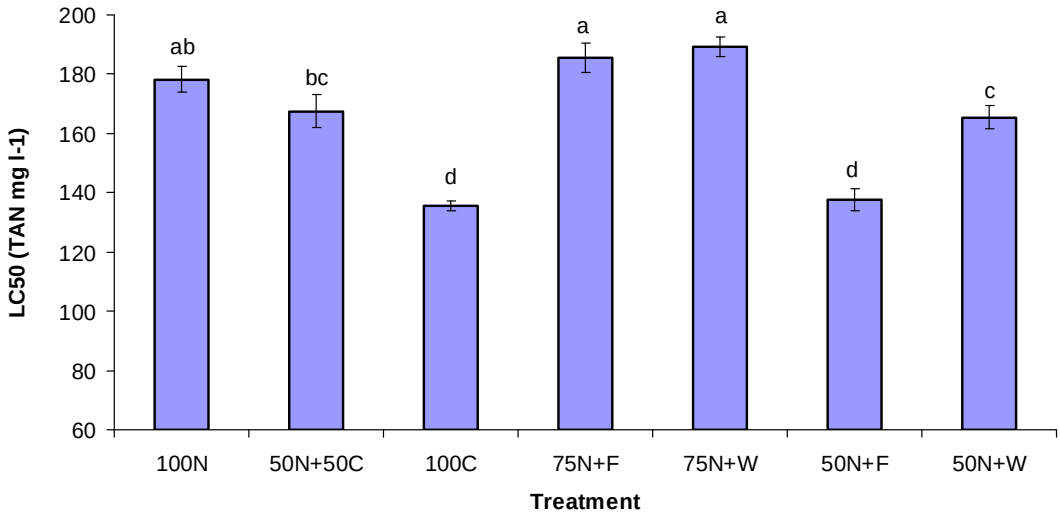

Fig. 4. Ammonia tolerance (expressed as 24hour LC_{50}–TAN) of *M. rosenbergii* larvae reared according to different feeding schedules in experiment 1. Different letters between treatments denote significant differences (P<0.05). For treatment descriptions refer to Table 1.

3.2 Experiment 2

At day 10 of the rearing period, the larvae in the different treatments showed the same development rate ($P>0.05$). However, larval development rate in treatments 100N and N+W became significantly higher compared to treatment N+G ($P<0.05$) by day 15 of the rearing cycle (Fig. 4). Larval survival results at the end of the experiment revealed a significantly higher survival in treatments 100N and N+W (53–54%) compared to treatment N+G, which had a survival of only 40% ($P<0.05$). Evaluation of the duration of rearing cycle showed that larvae in the treatment N+W completed the rearing cycle in 25 days, which was significantly shorter than in the treatments N+F and N+G which needed 28 and 29 days respectively (Fig. 5). Postlarval tolerance to total ammonia was significantly higher in treatments 100N and N+W (190 and 214 mg l^{-1} TAN respectively), compared to treatment N+G for which the LC50 was only 145 mg l^{-1} TAN ($P<0.05$) (Fig. 6). In general, the treatments 100N and N+W showed better results in terms of larval development, survival, rearing and larval quality compared to treatment N+G.

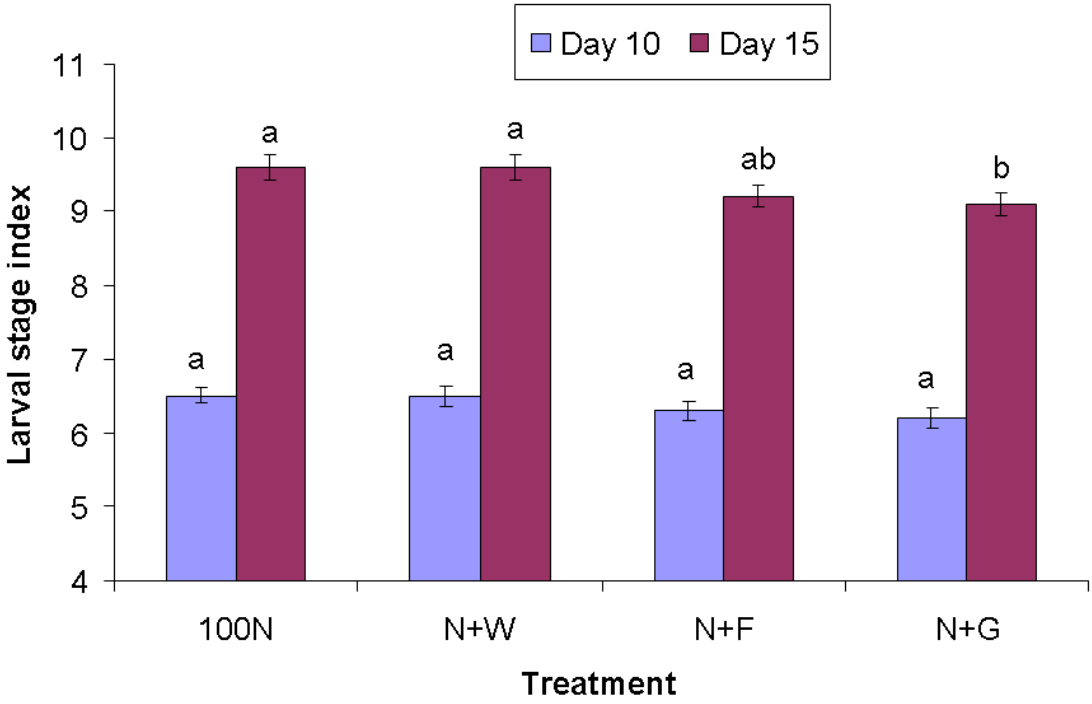

Fig. 4. Larval stage index at day 10 and 15 of *M. rosenbergii* larvae reared according to different feeding schedules in experiment 2. Different letters between treatments denote significant differences (P<0.05). For treatment descriptions refer to Tables 2 and 3.

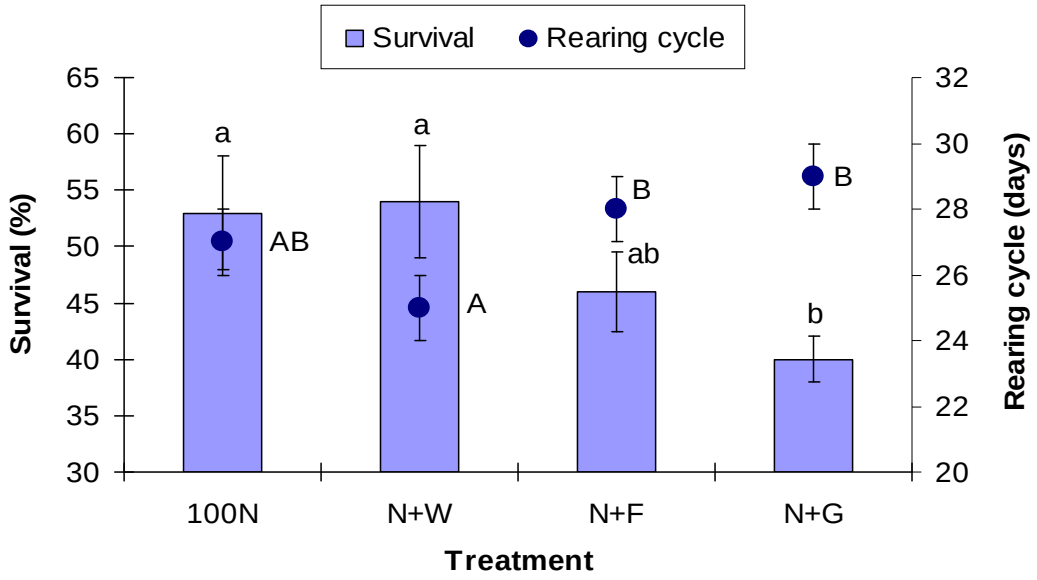

Fig. 5. Survival and rearing cycle of *M. rosenbergii* larvae reared according to different feeding schedules in the experiment 2. Different letters between treatments denote significant differences (P<0.05). For treatment descriptions refer to Tables 2 and 3.

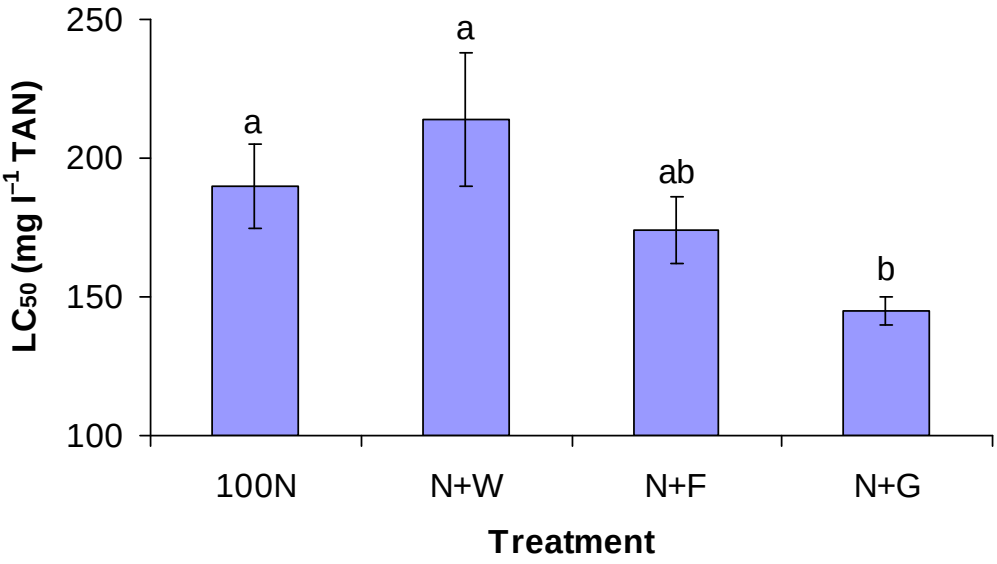

Fig. 6. Ammonia tolerance (expressed as 24hour LC_{50}–TAN) of *M. rosenbergii* larvae reared according to different feeding schedules in experiment 2. Different letters between treatments denote significant differences ($P<0.05$). For treatment descriptions refer to Tables 2 and 3.

4. Discussion

In experiment 1, the results of larval development, survival, duration of the rearing cycle and larval quality distributed the treatments into three distinct groups. The best group included the treatments fed exclusively *Artemia* nauplii and the treatments in which around 25% of the *Artemia* ration was replaced with artificial wet or dry diets. Consequently, the replacement of a part of the live food in the feeding schedule did not affect performance of the larvae. However, treatments in which 50% of the live feed was replaced from day 8 onwards reduced larval survival and larval quality. Especially, the use of an exclusive diet of decapsulated *Artemia* cysts seemed not appropriate for *M. rosenbergii* larval development. Although *Artemia* cysts are reported to contain higher energy and nutrient levels than *Artemia* nauplii (Sorgeloos *et al.*, 1977, Bengtson, *et al.*, 1991, Leger *et al.*, 1987), it was observed that they

rapidly sink to the bottom upon feeding, thus reducing their availability for the larvae to feed upon in the water column (Lavens and Sorgeloos, 1996). This while the behaviour of prawn larvae is rather to swim in the upper part of the water column or at the water surface. Increasing the aeration in the rearing containers may keep these particles better in suspension, however the increased turbulence may make it more difficult for the larvae to capture and ingest the prey. Decapods larvae do not specifically orientate towards a food source, they depend on chance encounter to capture food (Mooler, 1978; Meyers and Hagood, 1984; Kurmaly *et al*., 1990). In addition, *Artemia* cysts have a round shape, which may be difficult for the larvae to capture and hold on to during eating. In contrast, the mobility of *Artemia* nauplii allows its permanence in the water column, thus, increasing the chances of encounter (Barros and Valenti, 2003a). It therefore seems that the use of decapsulated cysts in larval rearing is rather suited for bottom feeders such as older *Penaeid* larvae or freshwater catfish (*Clarias gariepinus*) or carp (*Cyprinus carpio*) (Lavens and Sorgeloos, 1996). Using exclusively decapsulated cysts, which have a narrow size range (210–260 µm, Tackaert *et al*. 1987) may also not be appropriate for all larval stages during development. Barros and Valenti, (2003a) suggested that live food supplementation should start from stage VII onwards, using food particles increasing from 250 to 1190 µm. Therefore, the dimensions of decapsulated cysts may be appropriate for stage VII and VIII *M. rosenbergii* larvae only.

Replacing *Artemia* nauplii by artificial diets at a constant ratio of 50% from larval stage V–VI onwards (in experiment 1) negatively affected larval survival, but did not affect larval growth. This may be explaining by the drastic and sudden reduction of live feed in these treatments. In these treatments live feed was supplied only one time per day in the evening, and consequently the live feed density during the day time was

low. Especially in the early period of weaning, the larvae may not have been adapted yet to non–living feed, probably resulting in low survival due to increased cannibalism. Indeed, when the larvae were more gradually weaned from *Artemia* onto formulated feeds (experiment 2), better results were obtained. Therefore it is recommended to replace only 25% of the *Artemia* ration at the start of the weaning period to allow the larvae to adapt to the new diet. Subsequently, the weaning ration may be increased up to 50%, spread over several feedings per day. The replacement diets need to be offered with increasing particle sizes in function of the larval stage. In this respect, it was found that the Gromate feed, which had a rather narrow particle size range of 150–500 µm showed lower results compared to the wet and flake diets. Although the Gromate feed contained a higher protein level than the other diets, the narrow particle size range may have been a disadvantage for later *M. rosenbergii* larval stages. In contrast, the wet and flake diet could easily be sieved into the desired particle sizes using sieves with different mesh sizes.

In the present study, artificial diets were supplied from day 8 (stage V–VI) onwards. It was noticed that the larvae readily accepted the inert feeds. In this respect, the wet diet seemed to be more attractive to the larvae than the dry diets. Barros and Valenti, (2003a) stated that the larvae only accepted inert feed from stage VII onwards and suggested that the live feed could totally be replaced with wet or dry diets from stages VII and IX onwards respectively. However, it is necessary to evaluate final survival and productivity when applying total substitution of *Artemia* for commercial larviculture. Murthy *et al*, (2008) suggested that using wet diets which contain shrimp and clam meat fed to larvae in combination with *Artemia* nauplii showed larval survival of 40% in 150–l rearing tanks. Islam *et al*., (2000) reported that freshwater prawn larvae reared in a recirculation system with 140–l rearing tanks fed *Artemia*

nauplii supplemented with egg custard obtained a survival of 30%, which was higher than larvae fed exclusive *Artemia* (only 12%). However, Kamarudin *et al*., (2002) studied the use of artificial diets containing various ratios of cod liver and corn oil to replace 25-100% of the standard *Artemia* nauplii ration from stage III to XI. The results showed that there were no significant differences in survival between the substitution treatments and the control treatment fed solely *Artemia* nauplii. In the current study, a gradual replacement of up to 50% of the *Artemia* nauplii ration with wet and dry diets showed similar compared to a 100% *Artemia* control in terms of larval development, survival and larval quality. However, performance was impaired when the Artemia diet was abruptly replaced at a constant rate of 50% from day 8 onwards. In practice production efficiency depends on the production cost, which is based on the feed source and cost, labour cost, etc., Cost–effectiveness may therefore vary from one region to another. Therefore, the feeding strategy in *M. rosenbergii* larviculture cannot be standardized. The results obtained in the present work may however serve as a guideline for practical considerations of feeding strategies.

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CHAPTER 5

MICROBIAL CONTROL

CHAPTER 5

SECTION 1

Effect of N–acyl homoserine lactone–degrading enrichment cultures on *Macrobrachium rosenbergii* larviculture

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Abstract

N–acyl homoserine lactone molecules (AHL) are quorum sensing molecules known to be involved in the production of virulence factors. In this study, N–acyl homoserine lactone (AHL)–degrading enrichment cultures (ECs) as biocontrol agent in prawn larviculture was investigated. Enrichment cultures EC5(D) and EC5(L), originating from microbial communities of the fish gut of *Dicentrarchus labrax* and *Lates calcarifer*, were tested in the larval rearing of *Macrobrachium rosenbergii* through their addition to the rearing water and by bio–encapsulation in *Artemia* nauplii. The EC's were grown at the expense of glycerol released in the medium by hatching *Artemia*. The larval stage index of AHL–exposed larvae was 4.06 ± 0.07 in experiment 1 and 6.18 ± 0.10 in experiment 2 and 5.04 ± 0.07 and 6.50 ± 0.08 in the control treatment, respectively. Furthermore, a distinct difference in survival between AHL–exposed larvae (37.3% ± 10.6 and 64.5% ± 2.0) versus non–exposed larvae (77.7% ± 5.6 and 76.8% ± 3.2 in experiment 1 and 2, respectively) was observed. Both ECs were effective in improving prawn larvae survival under experimental conditions, *i.e.* when the survival of prawn larvae was compromised through a daily exposure to AHL molecules (1 mg l^{-1}). Through the addition of ECs, the negative effect of AHL could be counteracted, especially obtaining better larval quality, indicated by ammonia tolerance. These experiments demonstrated that AHL at a concentration of 1 mg l^{-1} could have a negative effect on prawn larvae, presumably through their effect on the prevailing opportunistic pathogenic microorganisms, while the selected ECs can counteract this. For this reason, using N–acyl homoserine lactone–degrading microbial communities might be a useful tool in prawn larviculture.

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879), is an important aquaculture. In recent years, the culture has expanded rapidly not only within Asia but also in regions far remote from the natural distribution of the species (FAO, 2000). However, the availability of healthy and high quality seeds has always been a major obstacle in the expansion of *M. rosenbergii* culture. One of the major factors that are hampering the quality of hatchery–reared seed, culminating in low yield, because of mass mortality, is diseases. Diseases caused by various opportunistic pathogenic bacteria such as *Vibrio* are often reported as a limiting factor not only in prawn hatcheries (Nayak and Mukherjee, 1997; Jayaprakash *et al*., 2006), but also in the intensive rearing of fish and molluscs, causing great losses, even up to 100% (Soto–Rodriguez *et al*., 2003). Although, antibiotics and disinfectants, the conventional approaches to control bacteria populations in prawn hatcheries, are quite popular, they have begun to be withdrawn from the market in many countries owing to concerns about public health and environmental safety (Schneider *et al*., 2003). Therefore, the search for novel, eco–friendly alternatives for maintaining a healthy microbial environment in the *Macrobrachium* larval rearing tanks has become increasingly important.

In recent years, it has become clear that diverse bacterial pathogens (including aquatic pathogens, Defoirdt *et al*., 2004) employ signal molecules to regulate the production of virulence factors. In Gram–negative bacteria, this type of regulation (termed quorum sensing, QS) is mediated, among others, by N–acyl–L–homoserine lactones, AHLs (Eberl, 1999), while Gram–positive bacteria use oligopeptides to communicate (Miller and Bassler, 2001; Whitehead *et al*., 2001). Examples of the phenotypes

regulated by AHL–based QS include luminescence, conjugation, nodulation, swarming, sporulation, biocorrosion, antibiotic production, biofilm formation, production of lytic enzymes, toxins, siderophores, and adhesion molecules (Defoirdt *et al*., 2004). However, dissemination disruption of these AHL signals can significantly decrease virulence factor production in bacteria without interfering with their growth (Finch *et al*., 1998; Hentzer *et al*., 2003; Rasch *et al*. 2004), and it may be a particularly useful method in aquaculture (Defoirdt *et al*., 2004, 2005). One of the approaches proposed for QS disruption is the isolation of bacteria that degrade signal molecules involved in quorum sensing. A number of bacteria utilize AHL molecules as sole sources of carbon and nitrogen; thus, they can be used as potential quenchers of QS–regulated functions in pathogenic bacteria. In our previous study in turbot larvae, a sharp reduction in the larval survival subsequent to the addition of AHL mixtures to the rearing water was observed. However, the negative effect of the AHL mixtures could be counteracted by introducing a microbial community able to degrade AHL molecules (Tinh *et al*., 2008b). In prawn larvae, we have elucidated the effect of AHLs on larval performance where the impaired growth and survival of the prawn larvae was to a great extent due to the interaction of several AHL molecules rather than individual molecules (Baruah *et al*., 2009). The evidence for AHL degrading bacterial isolates in the other aquatic animals is scarce, in spite of the progress made in the study of terrestrial species. Therefore, these AHL–degrading ECs need to be tested in other host–microbe systems, preferably where AHLs do have an effect on survival of the cultured organisms and thus in systems where AHL degradation would be a good strategy to protect animals.

Several Gram–negative bacteria were reported to be associated with *Macrobrachium* larvae in hatchery systems (Kennedy *et al*., 2006) and these types of bacteria release

several AHL molecules (Swift *et al*., 1999). These AHL molecules were reported (as observed in our previous study, Baruah *et al*., 2009) to interact and to negatively affect the growth and survival of *Macrobrachium* larvae, possibly by instigating the production of various virulence factors in microorganisms associated with the larvae.

In the present study, we wanted to confirm that AHL has a negative effect on prawn survival and development and that AHL–degrading ECs can counteract this negative effect. In addition, we want to verify whether it was possible to grow the EC at the expense of glycerol released by hatching *Artemia*.

2. Materials and methods

2.1 Enrichment cultures: source and preparation

Two enrichment cultures, EC5(D) and EC5(L) used in this study were mixtures of AHL–degrading bacteria able to grow on *Artemia*–released glycerol. EC5(D) and EC(L) were isolated from European sea bass, *Dicentrarchus labrax* at the Laboratory of Aquaculture & *Artemia* Reference Centre, Ghent University, Belgium and from Asian sea bass, *Lates calcarifer* at the Research Institute for Aquaculture No.2 (RIA2), Vietnam, respectively. Isolation of both the AHL–degrading ECs was done by using glycerol released by hatching *Artemia* and AHL molecules as the sole sources of carbon and nitrogen, respectively (Cam *et al*., 2009). The enrichment cultures (ECs) were made resistant to 100 mg l^{-1} rifampicin, by a procedure of sub–culturing them in a medium of Marine broth (MB, Difco Laboratories, Detroit, USA) with gradually increasing rifampicin concentrations: 1, 2, 8, 16, 50, and 100 mg l^{-1} to be able to distinguish them from other bacteria after their addition to prawn cultures, by plating on Marine agar (MA, Difco Laboratories, Detroit, USA) supplemented with 100 mg l^{-1} of rifampicin. The ECs were then preserved in 20% glycerol at –80

ºC. The determination of the nearest phylogenetic neighbours for the 16S rRNA gene sequences of the EC5(D) showed that EC5(D) was dominated by bacteria closely related to *Bacillus circulans*, *Bacillus* sp., and *Vibrio* sp. (accession numbers: FJ750276, FJ750277, FJ750280). The most dominant band of EC5(L) was closely related to a species belonging to the family of the Enterobacteriaceae (accession numbers: FJ997610, FJ997611, FJ997612, FJ997613). The result from the degradation assay shows that the rifampicin–sensitive (RS) and rifampicin–resistant (RR) enrichment cultures have the same AHL degradation rate (Fig. 1).

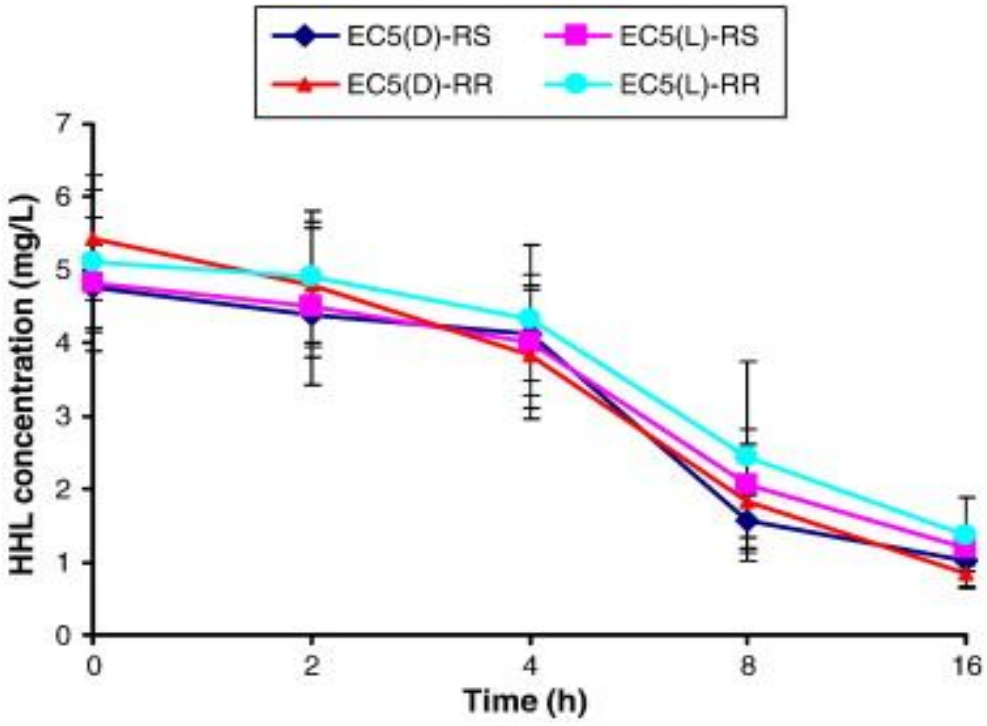

Fig. 1. Degradation of HHL by bacterial enrichment cultures used in this study

Values are average ± S.D., n = 6.

The stock cultures were inoculated into the axenic hatching medium of decapsulated *Artemia* cysts at a density of 10^5 CFU ml^{-1}. During the hatching process, the bacterial strains in the ECs grow by utilizing the released glycerol and limiting nitrogen (from the *Artemia*), reaching a final concentration of around 5 x 10^7 CFU ml^{-1}. The bacterial

density of this hatching medium was determined spectrophotometrically at 550 nm, assuming that an optical density of 1.0 corresponded to 1.2 x 10^9 CFU ml^{-1}. Part of this hatching medium was then used as source of enrichment cultures for daily addition to the larval rearing water at 10^6 CFU ml^{-1}. The rest was centrifuged at 6000 g for 10 min; the cells were re–suspended in autoclaved brackish water (12 g l^{-1}) at the concentration of 5 x 10^8 CFU ml^{-1} for *Artemia* enrichment.

2.2 Artemia and axenic hatching

Artemia franciscana originating from the Great Salt Lake, Utah, USA (EG® grade, batch 6940, INVE Aquaculture, Dendermonde, Belgium) was used as food for *Macrobrachium* larvae throughout the experiment. The required amount of *Artemia* cysts were axenically hatched according to the procedure described by Sorgeloos *et al*. (1986). After hatching, the axenity of the *Artemia* nauplii was verified following the method described by Marques *et al*. (2005). The newly hatched *Artemia* nauplii were kept in axenic conditions until instar II stage and then immersed in a suspension of ECs or LVS3 (an AHL non–degrading *Aeromonas* strain) (circa 5 x 10^8 CFU ml^{-1}) for enrichment. After 1 h, the enriched *Artemia* were rinsed and population density was adjusted to 500 ind. ml^{-1} by using autoclaved brackish water. Non–enriched *Artemia* (starved instar II) were used as a negative control.

2.3 Experimental animals

The first (experiment 1) was conducted at the Laboratory of Aquaculture and *Artemia* Reference Centre, Ghent University, Belgium, in a controlled temperature room, where adult *M. rosenbergii* (male and female) imported from Thailand and reared indoors at this lab were used as breeders. The second (experiment 2) was carried out at the Research Institute of Aquaculture No.2 (RIA2), Vietnam. Adult prawns

originating from the culture ponds in the south of Vietnam (An Giang) and transported to the hatchery of RIA2, were used as breeders. The water quality parameters of the broodstock tanks, photoperiod, and feeding were adjusted in accordance with the recommendations for prawn rearing (New, 2003). In both experiments, the larvae were obtained from a single oviparous female breeder (Cavalli *et al*., 1999, 2000; Baruah *et al*., 2009).

2.4 Experimental design

Twenty four hours after hatching, the larvae were distributed to glass cones containing 800 ml autoclaved brackish water (12 g l^{-1} salinity), at a density of 100 larvae l^{-1} and were reared for 8 days (experiment 1) and 10 days (experiment 2). The pH of the brackish water was previously adjusted to 7.0 by using 3–(N–morpholino) propanesulfonic acid (MOPS, Sigma) to minimise the chemical breakdown of AHL. The glass cones were placed in a rectangular tank containing water maintained at 28 ± 1 ºC using a thermostatic heater. The cones were covered with perforated plastic lids to insert the aeration pipes. The larvae were fed twice daily from day 2 post hatch onwards with hatched *Artemia* (mostly instar II) at the density of 5 nauplii ml^{-1} day^{-1}. Subsequently, the feeding rate was increased with 1 nauplii ml^{-1} day^{-1} on each following day. From day 7 onwards, the feeding quantity was kept at 10 nauplii ml^{-1} day^{-1}. Water renewal was started from day 3 onwards, with 20–50% water renewed per day. This operation was carried out with great care to avoid the loss of larvae.

The treatments for both experiments are summarized in Tables 1 and 2. In experiment 1, the AHL–degrading microbial enrichment culture EC5(D), was tested for its putative probiotic effect by adding it directly to the larval rearing water and by bio–encapsulation in *Artemia*. In the second experiment, the effects of EC5(L) and

EC5(D) on *M. rosenbergii* larvae were compared. Each treatment was set up in 6 replicates. No bacteria were added in the control treatment. An AHL mixture (details in Table 3) at a concentration of 1 mg l^{-1} was added daily to the rearing water in the AHL treatment. A treatment with an AHL non–degrader *Aeromonas hydrophila* LVS3 (Verschuere *et al*., 1999) was used as a negative control. ECs and/or LVS3 were added daily to the larval rearing water at 10^6 CFU ml^{-1}, and bio–encapsulated in *Artemia* instar II at 5 x 10^8 CFU ml^{-1}.

Table 1. Experimental design (experiment 1) for testing putative probiotic effect of EC5(D) either in the absence or presence of AHL molecules.

	No bacteria addition	LVS3 addition	EC5(D) addition
No AHL addition	–/–	– /LVS3	–/EC5(D)
AHL addition	+/–	+ /LVS3	+/EC5(D)

(+): AHL addition.

(–): no AHL or bacteria addition.

Table 2. Experimental design (experiment 2) for testing putative probiotic effect of EC5(D) and EC5(L) either in the absence or presence of AHL molecules.

	No bacteria addition	LVS3 addition	EC5(D) Addition	EC5(L) Addition
No AHL addition	–/–	– /LVS3	–/EC5(D)	–/EC5(L)
AHL addition	+/–	+/LVS3	+/EC5(D)	+/EC5(L)

(+): AHL addition.

(–): no AHL or bacteria addition.

Table 3. Different AHL molecules used (at equal quantities) in the AHL mixture

AHL molecule	Abbreviation
N–Butyryl–DL–homoserine lactone	C4–HSL
N–Butyryl–DL–homocysteine thiolactone	C4–HST
N–Hexanoyl–DL–homoserine lactone	C6–HSL
N–Heptanoyl–DL–homoserine lactone	C7–HSL
N–Octanoyl–DL–homoserine lactone	C8–HSL

2.5 Sampling and analysis

Water samples were collected twice, *i.e.* three days after the first feeding and on the last day of each experiment. Prawn samples were collected on the last day of each experiment. The EC–treated prawn larvae were sampled to determine the ability of the EC to colonize the prawn gut. Three samples of ten larvae were taken randomly from all replicates and prawn larvae were collected on a sterile 500–µm sieve. Surface bacteria were removed according to a procedure described by Huys *et al*. (2001). The prawn sample was first immersed in a benzocaïne solution (Sigma, 0.1%) for 10 s, transferred to benzalkonium chloride solution (Sigma, 0.1%) for another 10 s, and rinsed three times in autoclaved nine salts solution (NSS) (Olsson *et al*., 1992) for 5 s each time. The larvae were then transferred to a sterile plastic bag containing 10 ml of NSS, and homogenized by means of a stomacher blender for 6 min. The water samples and the prawn homogenates were serially diluted in NSS. Fifty µl from each dilution was plated on MA, on MA supplemented with 100 mg l^{-1} of rifampicin and on Thiosulphate–Citrate–Bile Salt–sucrose (Biokar Diagnostics, France) for

enumerating the total culturable bacteria, ECs, and *Vibrio* density, respectively. The number of colonies was counted after incubation at 28 °C for 48 h.

2.6 Residual AHL concentration in larval rearing water

In experiment 2, the residual AHL concentration (RAC) in filter–sterilised larval rearing water was determined on day 3, 6, and 9. *Chromobacterium violaceum* strain CV026, a mini–Tn5 mutant derived from the *C. violaceum* strain ATCC31532 (McClean *et al*., 1997) was used as AHL–reporter for measuring the RAC in larval rearing water. This strain cannot produce AHL, but can detect and respond to a range of AHLs by inducing the synthesis of purple pigment violacein. An overnight–grown culture of CV026 strain was diluted in NSS to obtain an optical density of approximately 0.1. Fifty µl of that suspension was spread plated on Luria–Bertani (LB) agar, containing tryptone (BD, France, 1% w/v), yeast extract (Sigma, Germany, 0.5% w/v), and NaCl (BD, France, 0.4% w/v). This medium was supplemented with 20 mg l^{-1} of kanamycin. Ten µl of each water sample was subsequently dropped at the centre of the LB agar plate. The plates were incubated at 28 °C for 24 h, and then the diameter of the purple–pigmented halos produced by CV026 strain was measured. Before analyzing the water samples, a standard curve correlating the diameter (cm) of the purple–pigmented halo produced by CV026 strain with the concentration of AHL mixture was established. The RAC in the rearing water at each sampling time was calculated based on the standard curve: diameter of purple–pigmented halo = 1.1392 ln[AHL] + 0.7191 (regression coefficient R^2 = 0.9823).

2.7 Larval stage index (LSI)

LSI was estimated according to Maddox and Manzi (1976) by sampling 10 larvae from each of the six replicates per treatment. LSI was then calculated as:

LSI = $\sum$ Si/N, where Si is the stage of the larvae (i = 1 to 12); N is the number of larvae examined.

2.8 Larval survival study

The number of surviving larvae was counted on day 2 (experiment 1) and on the final day of the experiment.

The number of surviving larvae was counted on day 7, 15 (experiment 2) and on the final day of the experiment. Only those larvae presenting movement of appendages and responding to mechanical stimulus were considered alive. The survival was recorded as follows:

Larval survival (%) = (Total number of live larvae

/Initial number of larvae stocked) x 100

2.9 Ammonia toxicity test (LC_{50}–TAN–24h)

An ammonia stress test was performed in triplicate in a series of 500 ml glass cones at 28 ^{o}C and 12 g l^{-1} salinity. A total ammonium (TAN; $NH_4^+ + NH_3$) stock solution was prepared with reagent grade NH_4Cl and was added to the glass cones immediately before stocking test animals. Groups of 10 larvae from each treatment were exposed to five increasing concentrations of total ammonia (0, 20, 40, 60 and 80 mg l^{-1}) for 24 h. TAN concentrations were based on preliminary tests, identifying suitable ranges for each larval stage, and thus varied according to the age of the larvae. A control (no NH_4Cl added) was run to measure natural (background) mortality. Measurements of pH were carried out at the beginning (after adding the NH_4Cl solution) and at the end of the tests. There was no feeding, water exchange and aeration during the test.

Based on the mortality after 24 h exposure, the mean lethal concentration for 50% of the population (24h–LC_{50}) was estimated according to Finney (1978). The pH values during the exposure ranged from 7.6 to 7.8 and therefore the proportion of non–ionised ammonia (NH_3) was around 1.9–2.9% of the TAN (Armstrong *et al*., 1978).

2.10 Data analysis

Survival data (%) are arcsin transformed to satisfy normal distribution and homocedasticity requirements, but only non–transformed means are presented. Differences in LSI and survival of the larvae were analyzed using one–way analysis of variance (ANOVA) followed by Duncan's multiple range test (SPSS version 13.0 software). Significance level was set at $P<0.05$. Correlations were determined using linear regression analysis. Ammonia tolerance was expressed as LC_{50}–24 h calculated by probit analysis (Finney, 1978).

3. Results

3.1 Experiment 1

3.1.1 Bacterial density

The density of culturable bacteria and supplemented bacteria [EC5(D) or LVS3] in the rearing water and in the prawn gut were determined at the end of the experiment (day 8) (Fig. 2 and 3). In the control (–/–) and AHL (+/–) treatments, no rifampicin resistant bacteria could be detected.

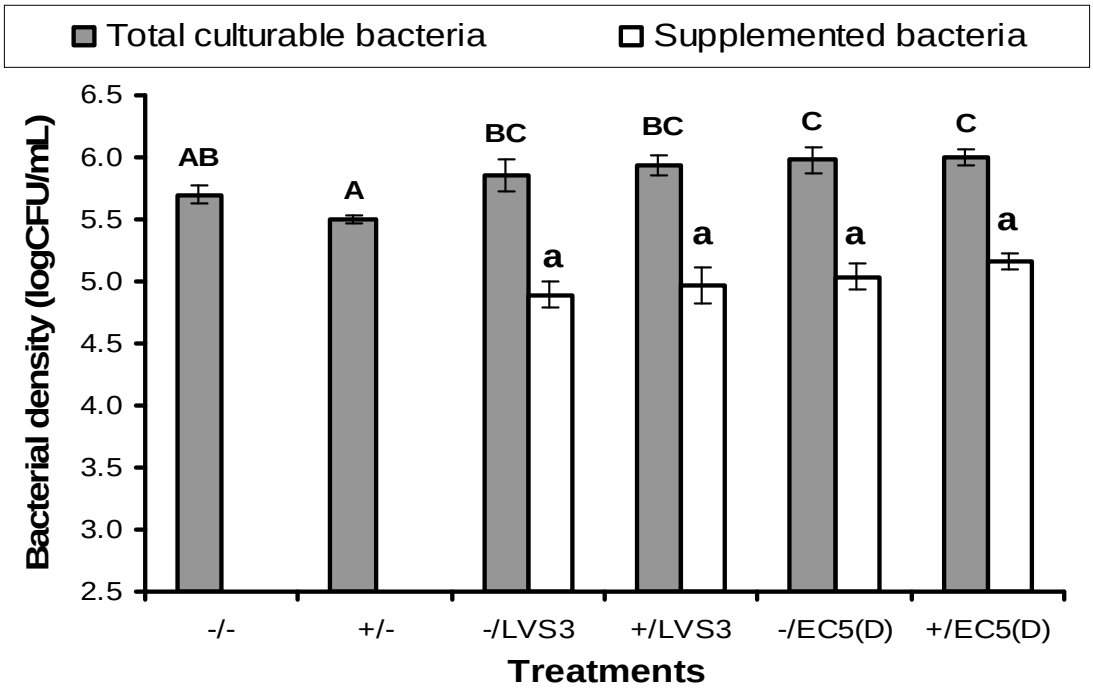

Fig. 2. Density of culturable bacteria and EC5(D) or LVS3 in prawn culture water on day 8, experiment 1. Values are average ± SE, n = 3. Different letters denote significant differences (P<0.05). Small and capital letters denote different comparisons. See Table 1 for information on the design of the experiment.

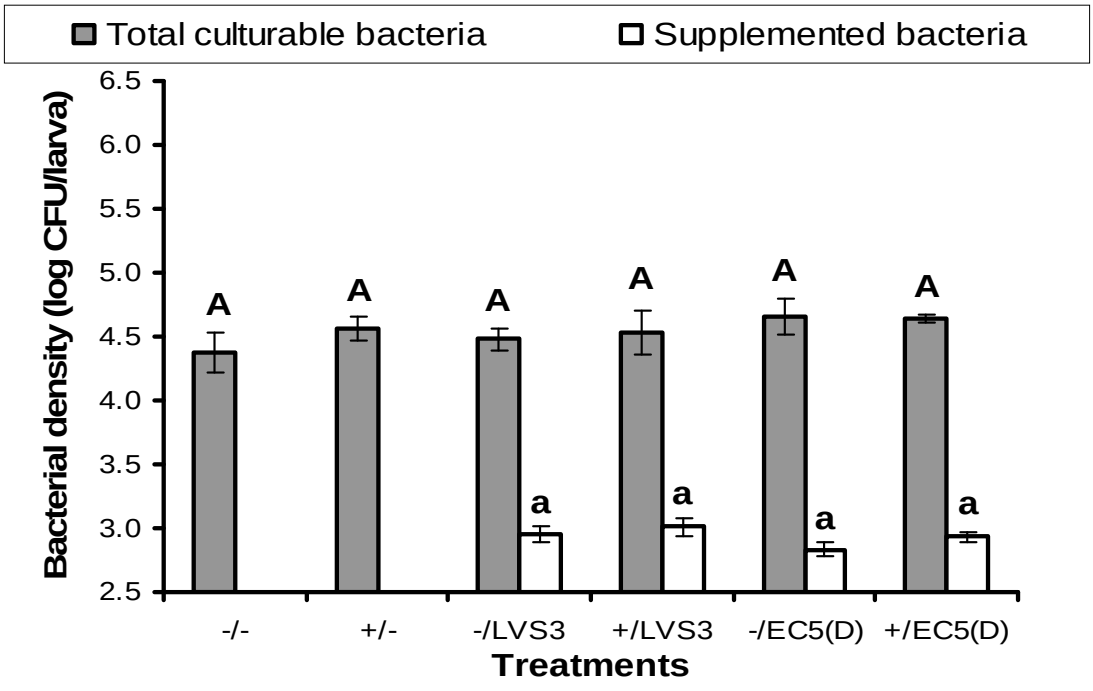

Fig. 3. Density of culturable bacteria and EC5(D) or LVS3 in prawn larval gut on day 8, experiment 1. Values are average ± SE, n = 3. Different letters denote significant differences (P<0.05). Small and capital letters denote different comparisons. See Table 1 for information on the design of the experiment.

EC5(D) and LVS3 remained in the culture water at around the added density (10^6CFU ml^{-1}). The density of culturable bacteria in the water in the last four treatments [–/EC5(D), +/EC5(D), –/EC5(L) and +/EC5(L)] slightly increased due to EC5(D) or LVS3 addition and reached approximately 10^6 CFU ml^{-1}. Both EC5(D) and LVS3 were found to be resident in the larval gut (circa 10^3 CFU $larva^{-1}$) (Fig. 3). The addition of AHLs did not stimulate the growth of the enrichment culture as the EC5(D) density in the water and in the larval gut were similar in the treatments, –/EC5(D) and +/EC5(D).

3.1.2 Larval stage index (LSI)

The addition of an AHL mixture significantly ($P<0.05$) retarded the larval developmental process [control (–/–) treatment versus AHL (+/–) treatment]. However, EC5(D was able to neutralise this negative effect [treatment –/EC5(D) versus treatment +/EC5(D)], while the *A. hydrophila*, LVS3 did not (treatment –/LVS3 versus treatment +/LVS3) (Fig. 4).

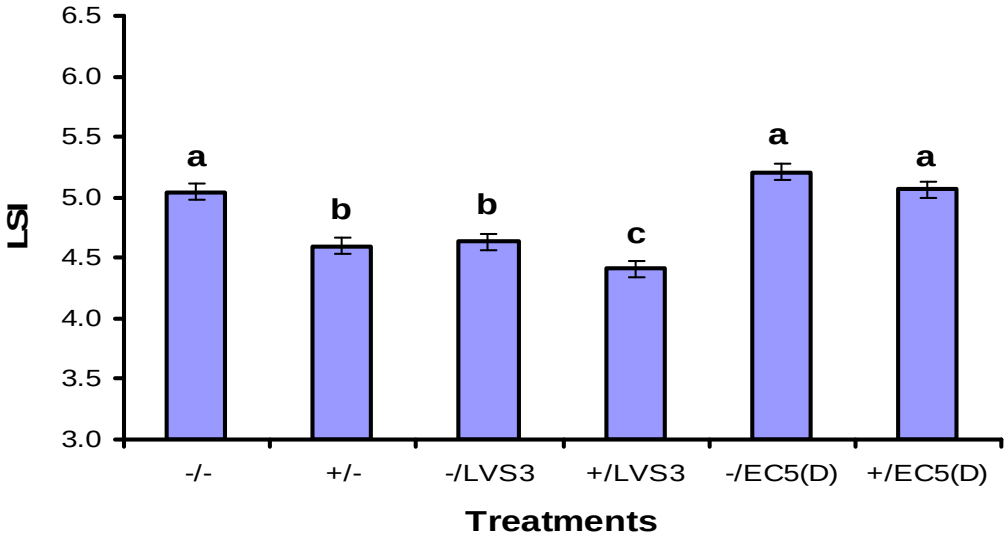

Fig. 4. Larval stage index of *M. rosenbergii* larvae on day 8, experiment 1. Values are average ± SE, n = 6. Different letters denote significant differences ($P<0.05$). See Table 1 for information on the design of the experiment.

3.1.3 Survival of prawn larvae

The addition of the AHL mixture significantly ($P<0.05$) affected the survival of larvae on day 2 indicated by a sharp decrease in the survival from 91.3% in the control to 57.0% in the AHL treatment (Fig. 5). Also on day 8, a clear significant difference between these treatments was observed (77.7% ± 5.6 versus 37.3% ± 10.6), indicating that AHL addition had a detrimental effect on larval survival (Fig. 5). Addition of LVS3 did not protect the larvae from the detrimental effect of AHL addition, as the survival in treatments –/LVS3 (89.6% ± 4.6 and 77.1% ± 3.5) and +/LVS3 (71.3% ± 5.3 and 48.5% ± 3.0) was significantly lower both on day 2 and day 8, respectively. In contrast, EC5(D) effectively protected the larvae, as the survival in treatments –/EC5(D) (91.7% ± 2.7 and 77.3% ± 7.3) and +/EC5(D) (84.2% ± 0.8 and 61.5% ± 4.8) was not significantly different on both day 2 and 8, respectively.

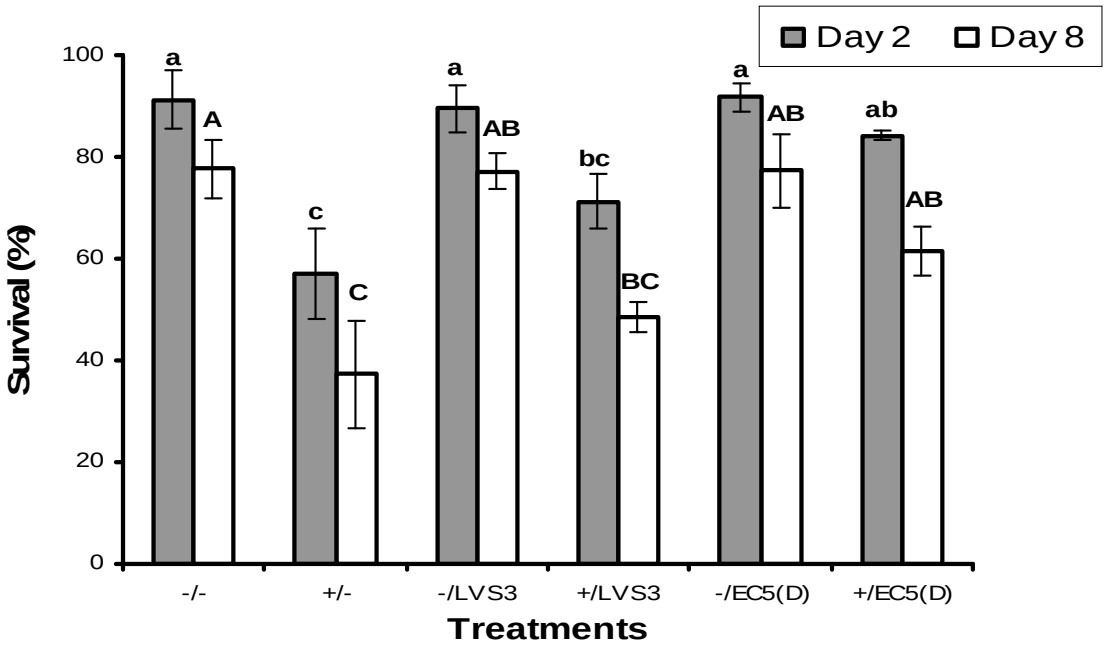

Fig. 5. Survival of *M. rosenbergii* larvae on day 2 and day 8, experiment 1

Values are average ± SE, n = 6. Different letters in survival at the same day denote significant differences ($P<0.05$). Small and capital letters denote different comparisons. See Table 1 for information on the design of the experiment.

3.2 Experiment 2

3.2.1 Bacterial density

The density of culturable bacteria, culturable *Vibrio* spp. As well as supplemented bacteria (ECs or LVS3) in the rearing water and in the prawn gut are presented in Fig. 6, 7 and 8. In the control (–/–) and AHL (+/–) treatments, no rifampicin resistant bacteria could be detected.

The density of EC5(D) in the culture water and in the larval gut in this experiment (circa 0.6 x 10^5 CFU ml^{-1} and 0.3 x 10^3 CFU $larva^{-1}$, respectively) was slightly lower than in the previous experiment. The ability of the enrichment culture EC5(L) to remain in the culture water (circa 0.7 x 10^5 CFU ml^{-1}) and to colonise the larval gut (circa 0.3 x 10^3 CFU $larva^{-1}$) was comparable with EC5(D). The addition of AHLs did not affect the growth of the enrichment culture as the EC5 densities in the water and in the larval gut were similar when AHLs were added or not [treatment –/EC5(D) versus treatment +/EC5(D) and treatment –/EC5(L) versus treatment +/EC5(L)]. The *Vibrio* density in the culture water in the control treatment and AHL treatment declined around 1 log unit from day 5 (10.8 x 10^4 and 6.4 x 10^4 CFU ml^{-1}) to day 10 (1.6 x 10^4 and 1.1 x 10^4 CFU ml^{-1}, respectively), while in the other treatments, this decline was not observed.

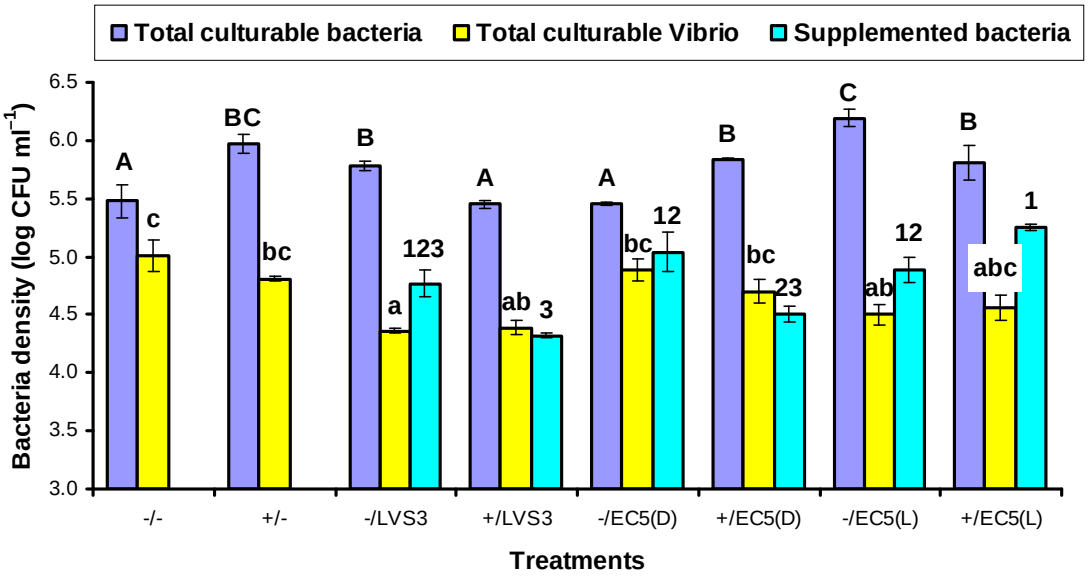

Fig. 6. Density of culturable bacteria, culturable *Vibrio* and EC5(D) or LVS3 in prawn culture water on day 5, experiment 2. Values are average ± SE, n = 3. Different letters denote significant differences (P<0.05). Small, capital letters and numbers denote different comparisons. See Table 2 for information on the design of the experiment.

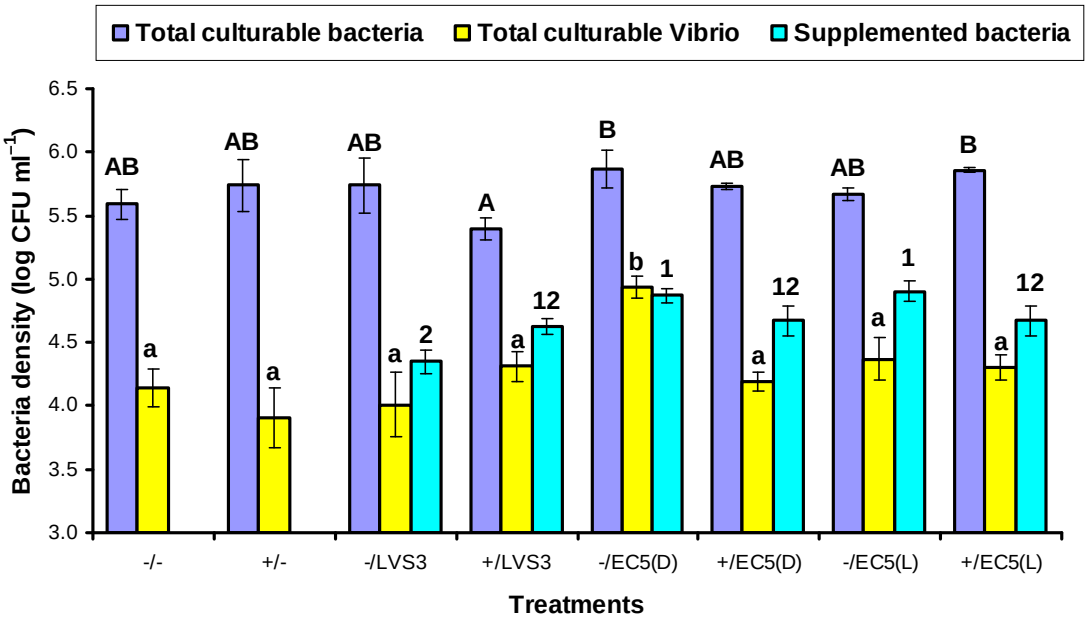

Fig. 7. Density of culturable bacteria, culturable *Vibrio* and EC5(D) or LVS3 in prawn culture water on day 10, experiment 2. Values are average ± SE, n = 3. Different letters denote significant differences (P<0.05). Small, capital letters and numbers denote different comparisons. See Table 2 for information on the design of the experiment.

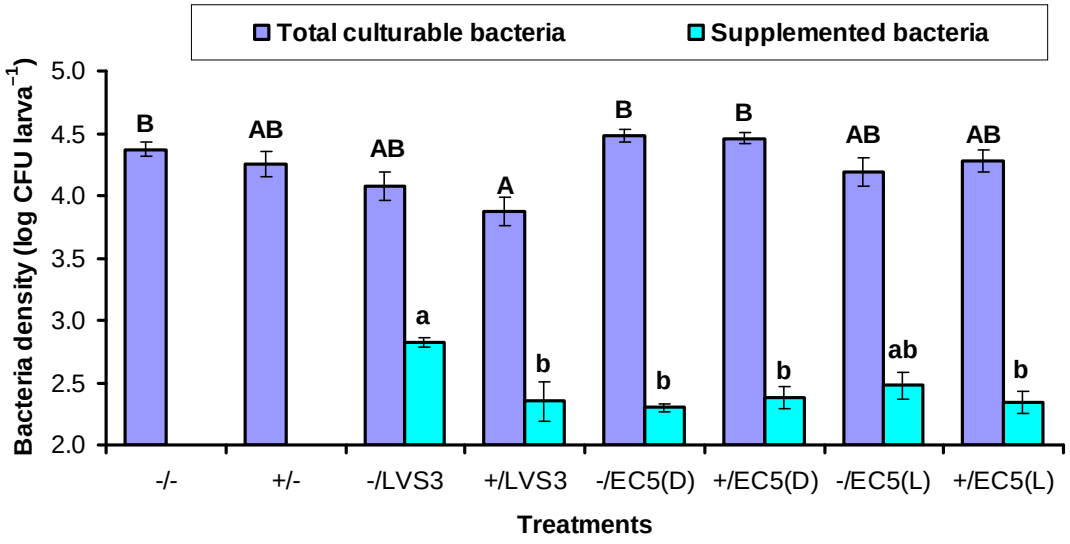

Fig. 8. Density of culturable bacteria and EC5(D) or LVS3 in the prawn larval gut on day 10, experiment 2. Values are average ± SE, n = 3. Different letters denote significant differences (P<0.05). Small and capital letters denote different comparisons. See Table 2 for information on the design of the experiment.

3.2.2 Residual AHL concentration

The residual AHL concentration (RAC) in the rearing water was monitored at 3–day intervals during experiment 2 (Fig. 9). In all treatments, the residual AHL on day 3 was below the detection limit of the AHL–reporter (0.1 mg l^{-1}). From day 6 onwards, a RAC was detected in all the treatments. It increased respectively from 2.3 ± 0.2 and 2.1 ± 0.4 mg l^{-1} on day 6 to 3.6 ± 0.6 and 3.5 ± 0.7 mg l^{-1} on day 9 in the treatments in which AHL and AHL + LVS3 were added, whereas it decreased from respectively 1.6 ± 0.3 mg l^{-1} and 1.6 ± 0.3 mg l^{-1} on day 6 to 0.6 ± 0.1 mg l^{-1} and 0.8 ± 0.1 mg l^{-1} on day 9 in the treatments due to the addition of respectively EC5(D) and EC5(L). These differences indicated a strong biological degradation of AHL by the ECs. The biological degradation by EC5(D) was comparable to that by EC5(L). Throughout the experiment, the pH fluctuated between 7.4 and 7.7.

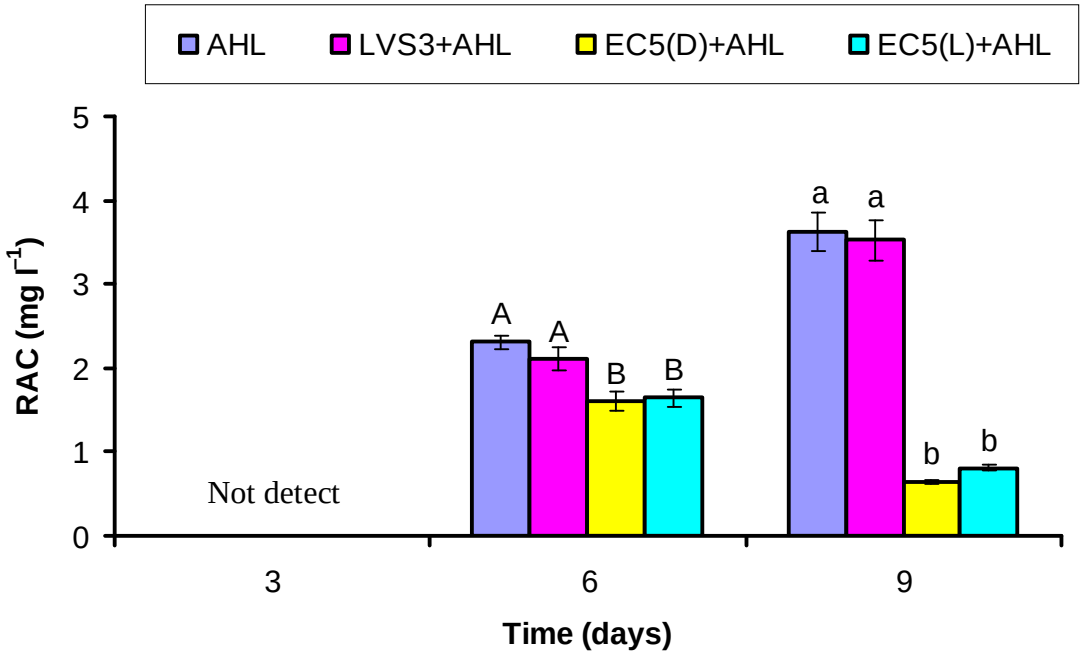

Fig. 9. Residual AHL concentration (RAC) in the rearing water of treatments exposed daily to AHL, experiment 2. Values are average ± SE, n = 3. Different letters denote significant differences (P<0.05). Small and capital letters denote different comparisons. See Table 2 for information on the design of the experiment.

3.2.3 Larval stage index (LSI)

The LSI results confirmed the results of experiment 1 (Fig. 10). The addition of the AHL mixture delayed the larval development (control treatment versus AHL treatment) and LVS3 could not neutralize this negative effect (treatment –/LVS3 versus treatment +/LVS3). Both enrichment cultures, EC5(D) and EC5(L), were not only able to neutralize the AHL effect, but also promoted the larval developmental process as the LSI in the last four treatments [–/EC5(D), +/EC5(D), –/EC5(L) and +/EC5(L)] was significantly higher than in the control treatment, basically indicating that the EC's could stimulate growth both with or without AHL addition.

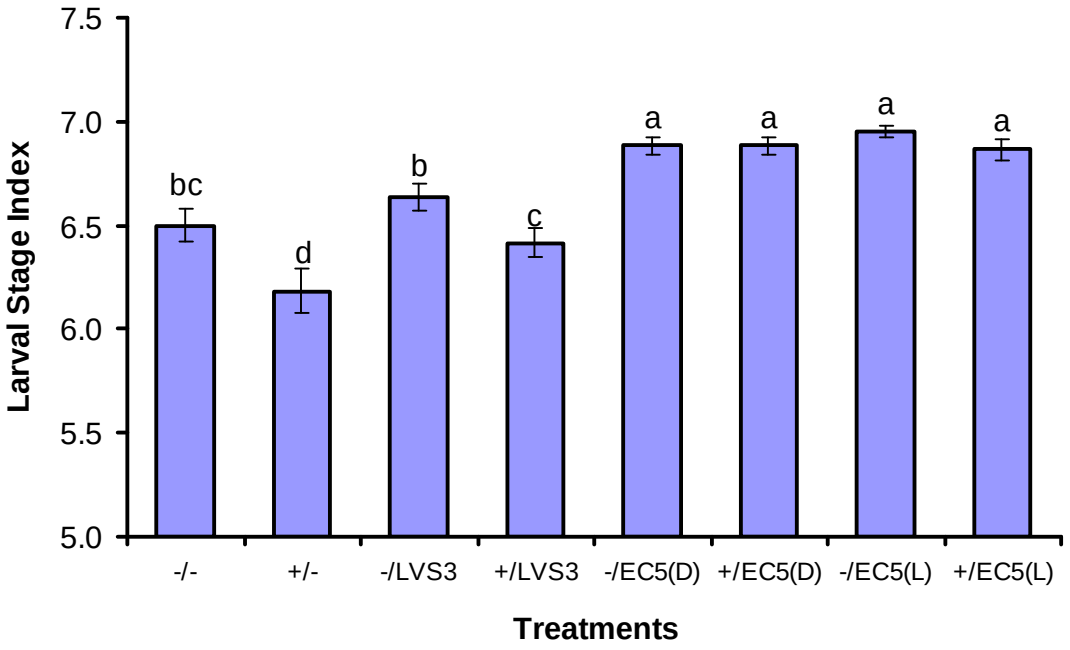

Fig. 10. Larval stage index of *M. rosenbergii* larvae on day 10, experiment 2. Values are average ± SE, n = 6. Different letters denote significant differences (P<0.05). See Table 2 for information on the design of the experiment.

3.2.4 Survival of prawn larvae

One–way ANOVA analysis showed a significant difference in the survival of larvae between the control treatment (76.8% ± 3.2) and AHL treatment (64.5% ± 2.0), confirming the negative influence of AHL on survival (Fig. 11). There was no significant difference between treatments, –/EC5(D) (82.0% ± 3.1) and +/EC5(D) (73.6% ± 0.6), and also between treatments, –/EC5(L) (74.8% ± 1.3) and +/EC5(L) (72.8% ± 5.2), suggesting that the EC can mitigate the negative effects of AHL.

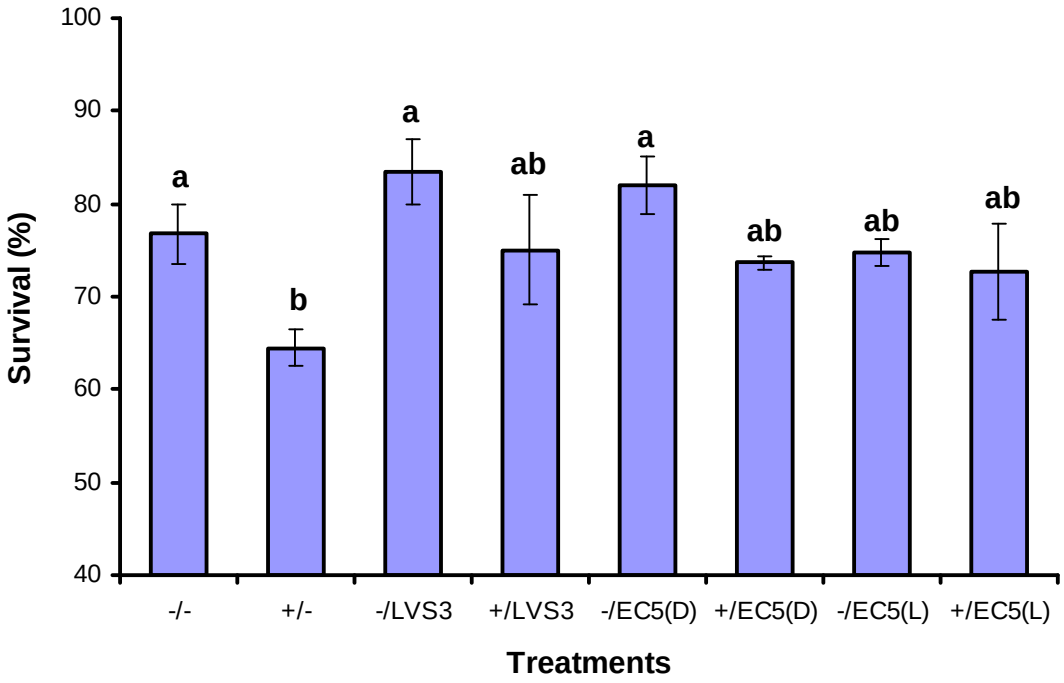

Fig. 11. Survival of *M. rosenbergii* larvae on day 10, experiment 2. Values are average ± SE, n = 6. Different letters in survival at the same day denote significant differences (P<0.05). See Table 2 for information on the design of the experiment.

3.2.5 Ammonia stress test (LC_{50})

Based on the mortality, the LC_{50} for 10–day old larvae was calculated (Fig. 12). LC_{50} values for the control and LVS3–added treatments were not significantly different (ranging from 70 to 78 mg l^{-1}) indicating that neither AHL nor LVS3 had an influence on ammonia sensitivity. In the presence of EC5(D) and EC5(L), but without addition of AHL (treatment –/EC5(D) and treatment –/EC5(L), respectively) the LC_{50} increased significantly to 108.2 ± 8.7 mg l^{-1} and 103.7 ± 12.7 mg l^{-1}, respectively. In the presence of AHL, the effect was not significant.

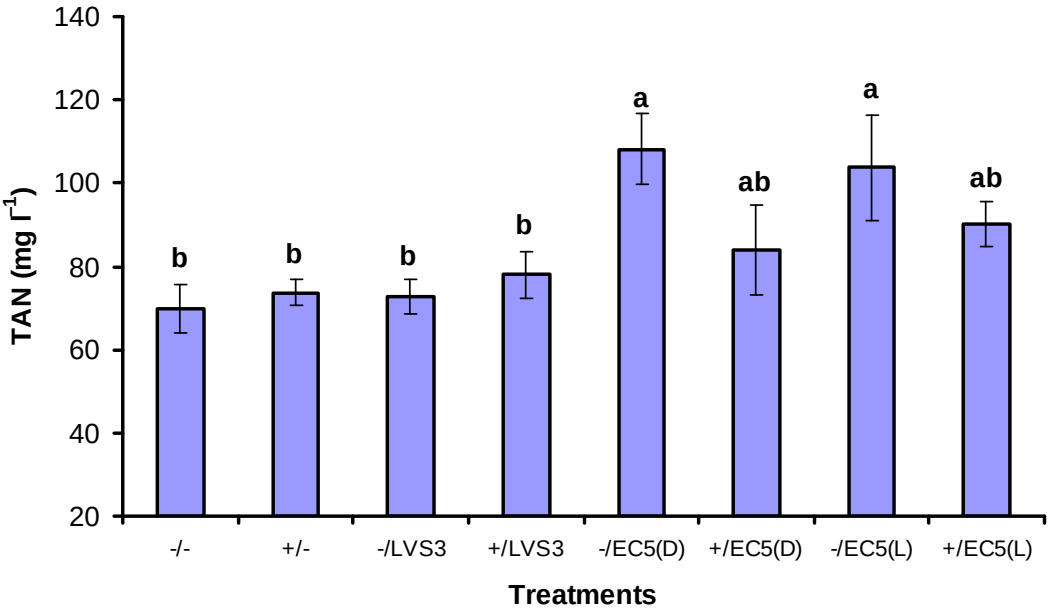

Fig. 12. Ammonia tolerance (LC_{50}–24h) of *M. rosenbergii* larvae on day 10, experiment 2. Values are average ± SE, n = 3. Different letters denote significant differences (P<0.05). See Table 2 for information on the design of the experiment.

4. Discussion

In our present study, enrichment cultures of AHL–degrading bacteria were used with the purpose of controlling putative opportunistic as well as pathogenic bacteria associated with the *Macrobrachium* larvae, whose virulence might be mediated by the AHL–dependent QS system (Eberl, 1999; Dong and Zhang, 2005; Juhas *et al*., 2005). The ECs were bio–encapsulated in *Artemia*, and gut analyses revealed their colonisation (data not shown). Our results are in agreement with the findings of Makridis *et al*. (2000), who quantified the accumulation of bacteria in *Artemia franciscana* during a short–term incubation by using immunocolony blot (ICB) and an enzyme–linked immunosorbent assay (ELISA) and found 4.5 x 10^4 bacteria per metanauplius after 20–60 min of grazing on high bacteria concentrations (≥ 5 x 10^7 bacteria ml^{-1}).

Results also showed that the daily addition of the AHL mixture (1 mg l^{-1}) caused a sharp reduction in the survival and development of the prawn larvae, in accordance with the findings of our previous study on *Macrobrachium* larvae (Baruah *et al*., 2009), where a significant reduction in the survival and LSI of the prawn larvae treated daily with an AHL mixture at a concentration of 1 mg l^{-1}. Similar to our results and those of the above–mentioned author, Tinh *et al*. (2008b) also observed a sharp reduction in the survival of turbot larvae subsequent to the addition of AHL mixtures (1 mg l^{-1}) to the rearing water. These authors demonstrated that AHLs are not toxic as such for turbot larvae, but that they have a negative influence on turbot larvae most probably by stimulating the production of virulence factors in microbial communities present in the culture water and in the fish gut. Because the newly–hatched larvae, collected from berried females from the wild, were not disinfected before starting up the present experiments, they were likely loaded with bacteria. We speculate that the low performance of prawn larvae in the presence of an AHL mixture in the present study could be due to the increased virulence of the microbial communities (regulated by quorum sensing) associated with the larvae, in a similar way as observed in our previous study on prawn larvae (Baruah *et al*., 2009).

In our present study, however, the survival of the prawn larvae treated with ECs was comparable with the control treatment regardless of the daily exposure to the AHL mixture. It should be emphasized that the ECs were only able to exhibit a probiotic effect when an exogenous source of AHL molecules was added, similar to the results of Tinh *et al*. (2008) with fish (turbot) larvae. Under normal conditions, on the other hand, the difference in prawn survival between the control treatment and the ECs treatment was not significant. However, larval development and ammonia tolerance in the ECs treatments were significantly enhanced in experiment 2. The effect of both

ECs on larval quality was detectable. Larvae cultured in the presence of the ECs with or without daily exposure to AHL molecules, had a higher tolerance to ammonia stress. This indicates the superior ability of the animals to cope with changing environmental conditions.

The EC5(D) and EC5(L) used in this study were isolated from the gut of *Dicentrarchus labrax* and *Lates calcarifer*, respectively and showed strong AHL–degrading properties *in vitro* (Cam *et al*., 2009). The ECs were made resistant to rifampicin through natural selection (for experimental purpose), so that its presence in shrimp culture could be detected by plating the water/shrimp samples on a growth medium containing the respective antibiotic. Our data demonstrated that there was no significant difference in density of total culturable bacteria between the treatments with and without AHL addition. Thus, the introduction of AHL molecules (at the used concentration) does not interfere with bacterial growth, but rather with the virulence of the opportunistic pathogenic bacteria. The bacterial density in the culture water in the present study ranged from about 0.23×10^5 to 0.92×10^5 CFU ml^{-1}, close to the results obtained by Phatarpekar *et al*. (2002) in a clear–water system on day 10 ($1.3 \pm 0.9 \times 10^6$ CFU ml^{-1}). We also investigated the capability of the ECs to reside inside the prawn gut. A long residence time of probiotics in the intestinal tract can prolong their potential beneficial effects (Lee *et al*., 2004). Attachment ability is not necessarily host/probiont species–specific, but rather dependent on the bacterial strain (Rinkinen *et al*., 2003). In our study, both EC5(D) and EC5(L) were able to live in the prawn larval gut, indicating a potential residence in the gut. The ratio of ECs to total culturable bacteria in the prawn gut was around 1 to 100. Similarly, Tinh *et al*. (2008b) investigated the effect of AHL–degrading communities on survival of first–

feeding turbot larvae, and found that the number of supplemented bacteria was 10^3 CFU $larva^{-1}$, while the number of total culturable bacteria was 10^5 CFU $larva^{-1}$.

There seems to be no correlation between the ability of AHL non–degrading bacteria to reside in the gut and its beneficial effect on larval growth, i.g. *Aeromonas hydrophila* (LVS3), a bacterial isolate selected by Verschuere *et al*. (1999) for its positive nutritional effect on *Artemia* culture under monoxenic conditions, was also able to reside in the prawn gut, but did not improve the survival of prawn larvae. The adhesion property of the ECs as potential probiotics needs to be studied in more depth, using more specific detection techniques such as immunohistochemistry. In our study, similar densities of culturable bacteria were observed in the shrimp gut, although the shrimp survival varied between treatments.

The fate of the daily added AHL molecules in the rearing water revealed interesting results: the residual AHL concentration (RAC) in the rearing water was below the detection limit of CV026 on day 3, but could be detected on day 6 and day 9. Results showed no significant differences upon LVS3 addition, indicating that LVS3 is not a potential quencher of QS molecules. However, both the ECs were shown to significantly reduce RAC. Reduction in RAC is most likely due to the quenching activity of the ECs. Leadbetter and Greenberg (2000) reported a strain of *Variovorax paradoxus* (VAI–C) capable of using AHL molecules as the sole sources of energy and nitrogen. Shiner *et al*. (2005) reviewed that there is evidence that host cells and bacterial cells produce “quorum quenching” enzymes that degrade autoinducers. Recently, Funke *et al*. (2008) demonstrated that the various N–acyl homoserine lactones indeed suffer rapid ring opening under the alkaline conditions of the insect gut due to the presence of a bacterial N–acyl–amino acid hydrolase (AAH), the ring–

opened products are rapidly further degraded into the amino acid and fatty acid building blocks.

In spite of being isolated from fish guts, the ECs showed to be effective in a prawn culture environment. This characteristic of the ECs may suggest its possible application in different fish/shrimp species. Furthermore, the ECs were apparently active even if they only constitute on average 1.86 and 1.27% of the bacterial population inside the prawn gut in experiment 1 and 2, respectively. In other bio augmentation experiments (Boon *et al.*, 2000), targeting the mineralization of 3–chloroaniline in soil, it has been demonstrated that the latter molecule is degraded despite the fact that the added strain could not be detected in the denaturing gradient gel electrophoresis (DGGE) profile of the resident microbial community. This seems to suggest that the supplemented bacteria do not need to become dominant in the microbial population in order to affect its activity, especially when specific molecules are targeted. Assuming that in intensive larviculture systems, the often observed high mortality is related to the induction of virulence factors by resident AHL concentrations in the gut, the addition of an AHL degrader, such as ECs, could be beneficial. However, such ECs may not be effective against those shrimp pathogens, whose virulence is not regulated by an AHL–mediated QS system. Hence, it will be interesting to investigate the effectiveness of ECs in the absence of AHL addition, under lab–scale as well as hatchery conditions.

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CHAPTER 5

SECTION 2

Application of N–acyl homoserine lactone–degrading bacteria in the larviculture of the giant freshwater prawn (*Macrobrachium rosenbergii*)

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Manuscript submitted to Aquaculture

Abstract

In this study, we investigated the effect of N–acyl homoserine lactone–degrading bacterial enrichment cultures on larviculture of the giant freshwater prawn *Macrobrachium rosenbergii*. The larval performance in terms of larval growth, larval survival, larval quality, duration of the larval rearing process and microflora levels in the rearing water as well as inside the prawn gut were investigated. The application of the enrichment culture bacteria were performed in two ways: by adding them directly into the larval rearing water and via enriched *Artemia* nauplii used for larval feeding. The results of the study demonstrated that both enrichment cultures that were tested had a similar positive effect on larval survival and larval quality, whereas they did not affect larval growth or the duration of the larval rearing process. Under normal hatchery conditions, the optimal enrichment culture densities were found to be 10^6 CFU ml^{-1} for adding into the rearing water and 5 x 10^8 CFU ml^{-1} for enrichment of *Artemia* nauplii used for feeding of the larvae. In the hatchery, the enrichment cultures can be grown on waste streams of *Artemia* hatching. Application of this kind of enrichment cultures could lead to a more sustainable aquaculture production, by replacing the use of antibiotics to control diseases.

Keywords: Enrichment cultures, bacteria, *Macrobrachium rosenbergii*, *Vibrio harveyi.*

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879), is an important aquaculture species, both as a food item for local consumption as well as a valuable export product. In recent years, the culture has expanded rapidly not only within Asia but also in regions far remote from the natural distribution of the species (FAO, 2000). However, one of the major obstacles for the further expansion of *M. rosenbergii* culture is the availability of healthy and high quality seed. Diseases caused by various opportunistic pathogenic bacteria such as *Vibrio* are often reported in prawn hatcheries (Thonguthai, 1997; Nayak and Mukherjee, 1997; Moriarty, 1998; Jayaprakash *et al.,* 2006; FAO, 2008). In the present situation, most pathogens are resistant to antibiotics and as a consequence it is difficult to control pathogenic bacteria in hatcheries. Moreover, antibiotics have become the subject of concerns about public health and environmental safety (Schneider *et al*., 2003). The use of antibiotics leads to undesirable environmental effects, such as the transfer of resistance to fish pathogens (Brown, 1989; Bruun *et al*., 2000; Agersø *et al*., 2007; Akinbowale *et al.,* 2007) and opportunistic human pathogens (Huys *et al.,* 2007). In addition to this, the chemicals themselves can remain as residues in human food (Willis, 2000). As a more sustainable alternative for antibiotics, the use of beneficial bacteria (probiotics) in aquatic seed production has become increasingly popular (Vine *et al*., 2006; Dierckens *et al*., 2009). The use of probiotics to displace pathogens by competitive processes is being used in the animal industry as a better remedy than administering antibiotics and is now also gaining acceptance for the control of pathogens in aquaculture (Havenaar *et al*., 1992). The search for novel, eco–friendly alternatives for maintaining a healthy microbial environment in *Macrobrachium* larval rearing tanks has become increasingly important. In the practice of aquatic seed

production, the major target is improvement of larval survival and larval quality in a cost–effective, safe and environmental-friendly manner.

The term "quorum sensing" refers to the process of bacterial cell–to–cell communication. The quorum sensing signal molecules, N–acyl homoserine lactone (AHL), were found to be involved in the regulation of virulence factors in many pathogenic Gram–negative bacteria, including fish (Federle and Bassler 2003; Morohoshi *et al*., 2004; Bruhn *et al*., 2005) and shrimp pathogens (Kennedy *et al*., 2006). Because many Gram–negative bacteria use quorum sensing systems for modulating a variety of physiological processes including the production of virulence determinants (Dunny and Winans, 1999; Swift *et al*., 1999), disruption of quorum sensing has been suggested as a new anti–infective strategy in aquaculture (Defoirdt *et al*., 2004). Disruption of AHL-mediated quorum sensing can significantly decrease virulence factor production in bacteria without interfering with their growth (Finch *et al*., 1998; Hentzer *et al*., 2003; Rasch *et al*. 2004), and it may be a particularly useful method for aquaculture (Defoirdt *et al*., 2004, 2005). Cam *et al*. (2009a) reported the isolation of two different mixtures of AHL–degrading enrichment cultures (ECs) from European sea bass (*Dicentrarchus labrax*, EC5(D)) and from Asian sea bass (*Lates calcarifer*, EC5(L)). Finding of these novel ECs, which act as quorum sensing–quenching and effect as probiotics in aquaculture system (Cam, 2009c). Consequently, in the current study, we investigated the effect of EC5(D) and EC5(L) on *Macrobrachium rosenbergii* larval performance and on the microflora in the rearing water as well as inside the prawn gut with and without *Vibrio harveyi* administration into the rearing water.

2. Materials and methods

2.1 Enrichment cultures: source and preparation

In this study, two bacterial enrichment cultures were used, EC5(D) and EC5(L) were isolated from European sea bass, *Dicentrarchus labrax* and from Asian sea bass, *Lates calcarifer,* respectively (Cam *et al*., 2009a). The enrichment cultures (ECs) were made resistant to rifampicin in order to be able to distinguish them from other bacteria after their addition to prawn cultures by plating on Marine agar (MA, Difco Laboratories, Detroit, USA) supplemented with 100 mg/L of rifampicin. The ECs were preserved in 20% glycerol at –80 ºC. EC5(D) was dominated by bacteria closely related to *Bacillus circulans*, *Bacillus* sp., and *Vibrio* sp. (accession numbers: FJ750276, FJ750277, FJ750280). EC5(L) contained bacteria that are closely related to bacteria belonging to the family of the Enterobacteriaceae (accession numbers: FJ997610, FJ997611, FJ997612, FJ997613) (Cam *et al*., 2009b).

The enrichment cultures were inoculated into the hatching medium of decapsulated *Artemia* cysts at a density of 10^5 CFU ml^{-1}. During the *Artemia* hatching process, the ECs grew by utilizing the released glycerol, reaching a final concentration of around 5 x 10^7 CFU ml^{-1}. Part of this hatching medium was used as source of enrichment cultures for daily addition to the larval rearing water at 10^6 CFU ml^{-1}. The rest of the cultures was centrifuged at 6000 g for 10 min; the cells were re–suspended in autoclaved brackish water (12 g/L) at a concentration of 5 x 10^8 CFU ml^{-1} for *Artemia* enrichment.

2.2 Preparation of the Vibrio harveyi inoculum for challenge tests

Vibrio harveyi BB120 (Bassler *et al*., 1997) was stored in 40% glycerol at –80°C. The stored culture was inoculated into fresh Marine Broth (Difco Laboratories, Detroit,

USA) and incubated for 24 h at 28°C under constant agitation (100 min^{-1}). The cell density of the grown culture was determined by spectrophotometry at 550 nm and the pathogen was added to the larval culture water at approximately 10^5 CFU ml^{-1} daily.

2.3 Experimental animals

The experiments were carried out at the Research Institute of Aquaculture No.2 (RIA2), Vietnam. Adult prawns originating from culture ponds in the south of Vietnam (An Giang province) were used as breeders and acclimatised to the hatchery conditions for egg incubation. The water quality parameters of the broodstock tanks, photoperiod, and feeding were adjusted in accordance to the recommendations for prawn rearing (New, 2003). For each experiment, larvae from a single oviparous female breeder were used. Broodstock management techniques were similar to the techniques described by Cavalli *et al*. (1999) and 2000 and Baruah *et al*. (2009). 24h after hatching, the larvae were collected and stocked into the experimental tanks.

2.4 Experimental design

The experiments were performed in cylindro–conical rearing tanks containing 10 l brackish water (12 g l^{-1} salinity) in a completely randomized design with four replicates per treatment. The experiments were run inside the hatchery building with the water temperature maintained at 28±1 ºC. A fluorescent lamp system was installed to provide around 900–1000 lux at the water surface for 12h day^{-1}. The rearing tanks were gently aerated to ensure dissolved oxygen in the rearing water was always over 5 mg l^{-1}. In all experiments, an open clear water system with a daily water exchange of 30–50% was used. During water exchange, the remaining *Artemia* from the previous day, and the waste were removed by siphoning. Feeding was done after water exchange. The larvae were exclusively fed *Artemia franciscana* (Great Salt Lake

strain) nauplii *ad libitum* twice a day (at 9h00 and 17h00) during the complete experimental period. Depending on the treatment, instar II *Artemia* nauplii were enriched with EC bacteria at 5 x 10^8 CFU ml^{-1} in the *Artemia* culture water for 2 hours. In the control treatments, *Artemia* nauplii were prepared in the same way, without enrichment.

Experiment 1 was performed to investigate the efficiency of the enrichment cultures to protect prawn larvae from *Vibrio harveyi* and their effect on the microflora in the culture water and inside the larval gut. This experiment consisted of four treatments (Table 1). Experiment 2 was conducted to evaluate the dose–effect of the ECs on larvae cultured under normal hatchery conditions and on the microflora in the culture water and inside the larval gut. This experiment consisted of five treatments (Table 1). In both experiments, newly hatched larvae were stocked at an initial density of 100 l^{-1}

Table 1. Experimental design in experiment 1 and 2

Abbreviation		Enrichment cultures	ECs addition dose (10^6 CFU ml^{-1})	Infection dose of *Vibrio harveyi* (10^5 CFU ml^{-1})
Experiment 1				
1	C–	Control–	–	–
2	C+	Control+	–	+
3	D+	EC5(D)	+	+
4	L+	EC5(L)	+	+
Experiment 2				
1	C	Control	–	–
2	D6	EC5(D)	10^6	–
3	D7	EC5(D)	10^7	–
4	L6	EC5(L)	10^6	–
5	L7	EC5(L)	10^7	–

2.5 Sampling and analyses

2.5.1 Larval stage index

The larval development rate was estimated at day 10, 15 and day 7, 16 in experiment 1 and 2 respectively, through determining the larval stage index (LSI) according to Maddox and Manzi (1976). The average larval stage of at least 30 larvae from each treatment was recorded based on the description by Uno and Kwon (1969).

2.5.2 Larval survival

Larval survival was checked at the end of each experiment when 90% of the larvae in each treatment had reached the postlarval stage. All live larvae and postlarvae were counted and the percentage survival was calculated based on the initial number of larvae stocked.

2.5.3 Metamorphosis process

The time points when the first postlarva appeared and when 90% of the larvae reached the postlarval stage were recorded as an estimate of the average duration of the metamorphosis process and the duration of the total rearing cycle in each treatment.

2.5.4 Ammonia toxicity test (LC_{50} –NH_3 – 24h)

An ammonia stress test was performed in triplicate in a series of 500 ml glass cones at 28 ^{o}C and 12 g/L salinity. A total ammonium (TAN; $NH_4^+ + NH_3$) stock solution was prepared with reagent grade NH_4Cl and was added to the glass cones immediately before stocking the animals. Groups of 10 larvae from each treatment were exposed to five increasing concentrations of total ammonia for 24 h. A control (no NH_4Cl added) was run to measure natural mortality. Measurements of pH were carried out at the

beginning (immediately after adding the NH_4Cl solution) and at the end of the tests. There was no feeding, water exchange or aeration applied during the test. Based on the mortality after 24 h exposure, the mean lethal concentration for 50% of the population (24h–LC_{50}) was estimated according to Finney (1978). The concentration of non–ionized ammonia (NH_3) was estimated according to the general formula for the mean values of pH, salinity and temperature as presented by Armstrong *et al*. (1978).

2.5.5 Bacteria in the rearing water and inside the prawn gut

Rearing water samples were collected at three time points during the culture cycle, at day 7, 14 and 21. Three water samples were taken randomly from each treatment. Prawn larvae samples were collected at the end of each experiment to determine the bacteria in the prawn gut. Three samples of ten animals were taken randomly from each treatment. Surface bacteria were removed according to a procedure described by Huys *et al.* (2001). The larvae were then transferred into a sterile plastic bag containing 10 ml of Nine Salts Solution (NSS), and homogenized with a stomacher blender for 6 min. The water samples and the prawn homogenates were serially diluted in NSS. Fifty µl from each dilution was plated on Marine Agar, on Marine Agar supplemented with 100 mg l^{-1} of rifampicin and on Thiosulphate–Citrate–Bile Salt–sucrose agar (Biokar Diagnostics, France) for enumerating the total culturable bacteria, ECs and *Vibrios*, respectively. The number of colonies was counted after incubation at 28 °C for 48 h.

2.6 Statistical analyses

Survival data (%) were arcsin transformed to satisfy normal distribution and homocedasticity requirements for statistical analyses (only non–transformed means

are presented). Differences in LSI and survival of the larvae were analyzed using one–way analysis of variance (ANOVA) followed by Duncan's multiple range test (SPSS version 13.0 software). The significance level was set at 5%. Correlations were determined using linear regression analysis. Ammonia tolerance (expressed as LC_{50}–24 h) was calculated by probit analysis (Finney, 1978).

3. Results

3.1 Experiment 1

The larval stage index (LSI) of larvae treated with the ECs was significantly higher when compared to both control treatments (C– and C+) at day 10 and 15. The LSI in the infected control (C+) was significantly higher than that in non–infected control at day 15 (Fig. 1). There were no differences between EC5(D) and EC5(L).

The treatments with the addition of enrichment cultures resulted in the highest survival at the end of the experiment (up to 39–41%), while the challenged control showed the lowest survival (11%). The larval survival in the control without infection was 22%, which was significantly higher than in the infected control (Fig. 2). The survival of the EC-treated challenged larvae was thus even higher than that of unchallenged larvae, with no significant difference between both ECs.

The ammonia tolerance, expressed as 24h–LC_{50}, was used as a criterion for the evaluation of the quality of the *M. rosenbergii* larvae. Differences in ammonia tolerance between the treatments followed the same trend as for survival. The treatments with the addition of the ECs showed the highest tolerance levels (with no significant difference between both ECs), while the infected control showed the lowest one. The uninfected control treatment demonstrated an intermediate tolerance level and was significantly different from the highest and lowest treatments (Fig. 3).

The time from stocking to first postlarva appearance was shortest in the treatment with EC5(L), while in the infected control it was the longest. In the EC5(D) and non–infected control treatment intermediate results were obtained. The duration of the total larval rearing cycle (to reach 90% postlarva stage) was shortest for both EC5 treatments, while the infected control treatment needed a significantly longer rearing period. The non–infected control treatment was intermediate and was not significantly different from the other treatments (Fig. 4). Again, there was no significant difference in both parameters between EC5(D) and EC5(L).

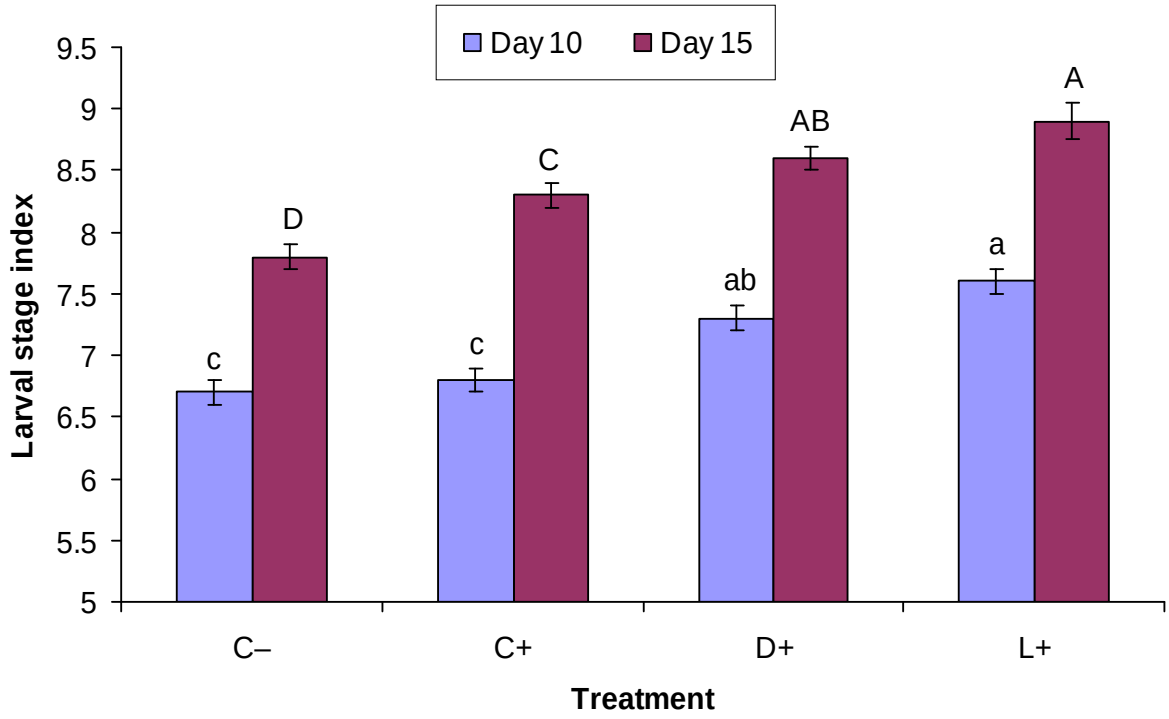

Fig. 1. Larval stage index of *M. rosenbergii* larvae in experiment 1 at day 10 and 15. Values are average ± SE, n = 4. Different letters denote significant differences (P<0.05). Small and capital letters denote different comparisons. C-: no challenge; C+: challenged with *V. harveyi* BB120; D+: challenge + EC5(D); L+: challenge + EC5(L)

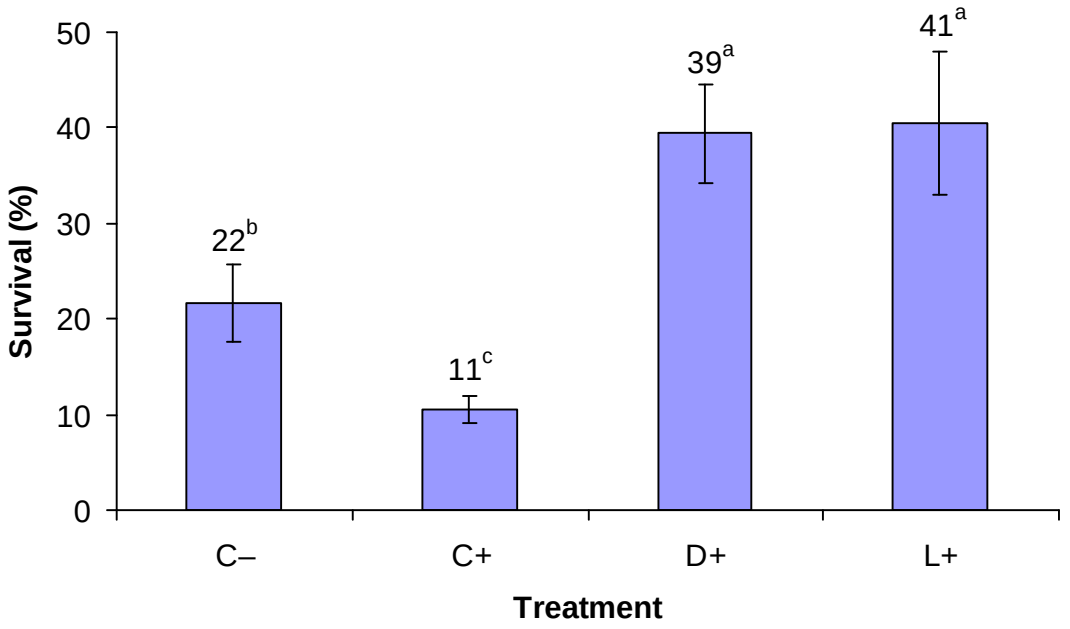

Fig. 2. Survival of *M. rosenbergii* larvae at the end of experiment 1. Values are average ± SE, n = 4. Different letters denote significant differences (P<0.05). C-: no challenge; C+: challenged with *V. harveyi* BB120; D+: challenge + EC5(D); L+: challenge + EC5(L)

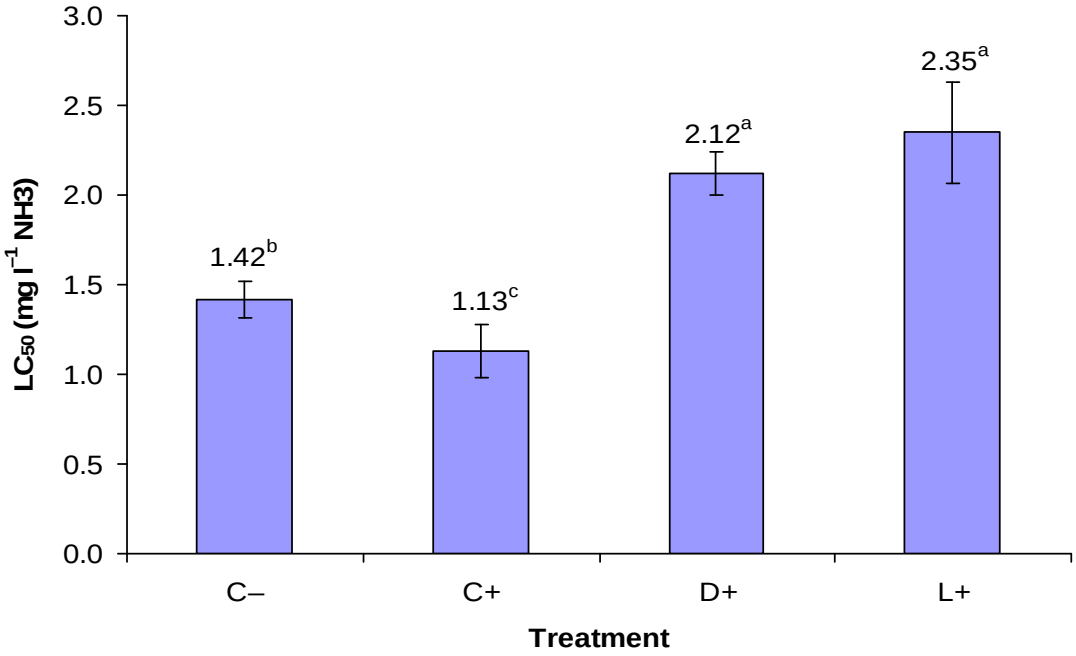

Fig. 3. 24h–LC_{50} (mg l^{-1} NH_3) of *M. rosenbergii* postlarvae in experiment 1. Values are average ± SE, n = 4. Different letters denote significant differences (P<0.05).C-: no challenge; C+: challenged with *V. harveyi* BB120; D+: challenge + EC5(D); L+: challenge + EC5(L)

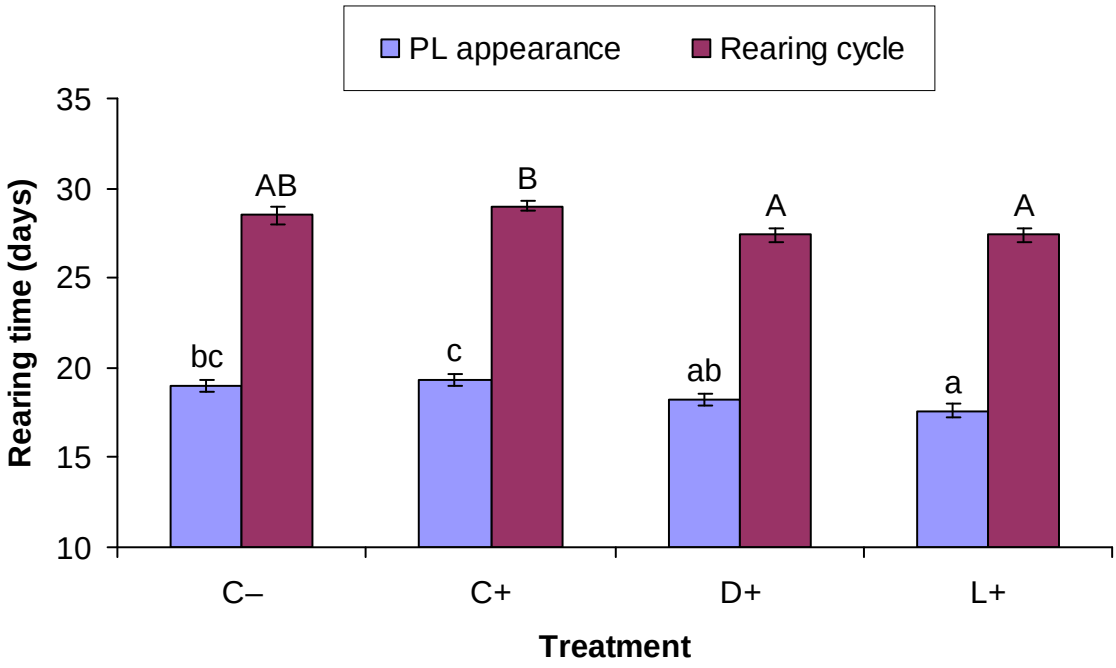

Fig. 4. Time of first postlarva appearance and duration of total larval rearing cycle in experiment 1. Values are average ± SE, n = 4. Different letters denote significant differences (P<0.05). Small and capital letters denote different comparisons. C-: no challenge; C+: challenged with *V. harveyi* BB120; D+: challenge + EC5(D); L+: challenge + EC5(L)

Total bacteria, enrichment culture bacteria and total *Vibrio* counts in the rearing water were checked at three different time points during the experiment (at day 7, 14 and 21). Total bacteria counts in the treatments with the addition of enrichment cultures and with *V. harveyi* infection (D+ and L+) were always significantly higher than in both control treatments. The total *Vibrio* counts in both treatments with addition of the ECs was always significantly lower than in the infected control treatment. The (infected) EC treatments showed similar *Vibrio* levels as the non–infected control treatment. Total *Vibrio* counts in the infected control showed an increase in time, while total *Vibrio* levels in the EC treatments were stable (Fig. 5).

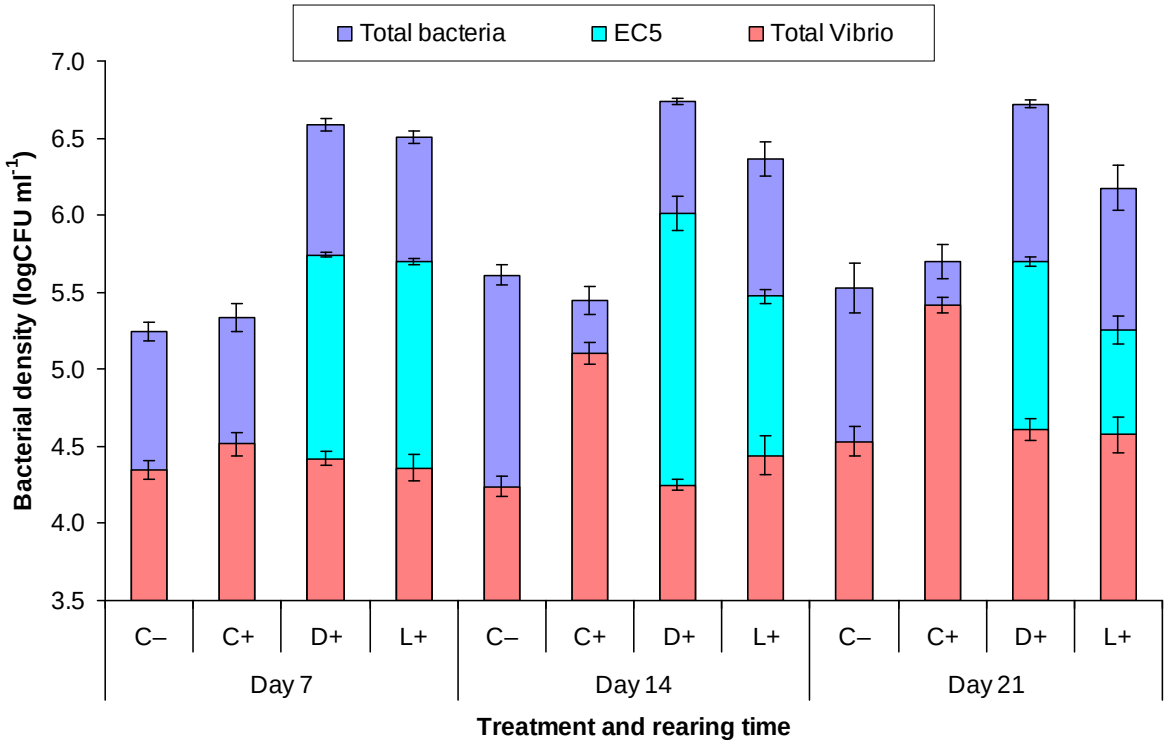

Fig. 5. Density of total bacteria, EC5 and total *Vibrio* in *M. rosenbergii* culture water on day 7, 14 and 21 in experiment 1. Values are average ± SE, n = 4. C-: no challenge; C+: challenged with *V. harveyi* BB120; D+: challenge + EC5(D); L+: challenge + EC5(L)

Total bacteria levels inside the prawn gut were also investigated at the end of the experiment and showed no significant differences between treatments; all were around 10^5 CFU postlarva^{-1}. The total *Vibrio* level in the infected control treatment was significantly higher than in the other treatments. The enrichment culture bacteria inside the prawn gut were similar in the D+ and L+ treatments, approximately 1.5 x 10^4 CFU postlarva^{-1}. They were not detected in the other treatments. (Fig. 6).

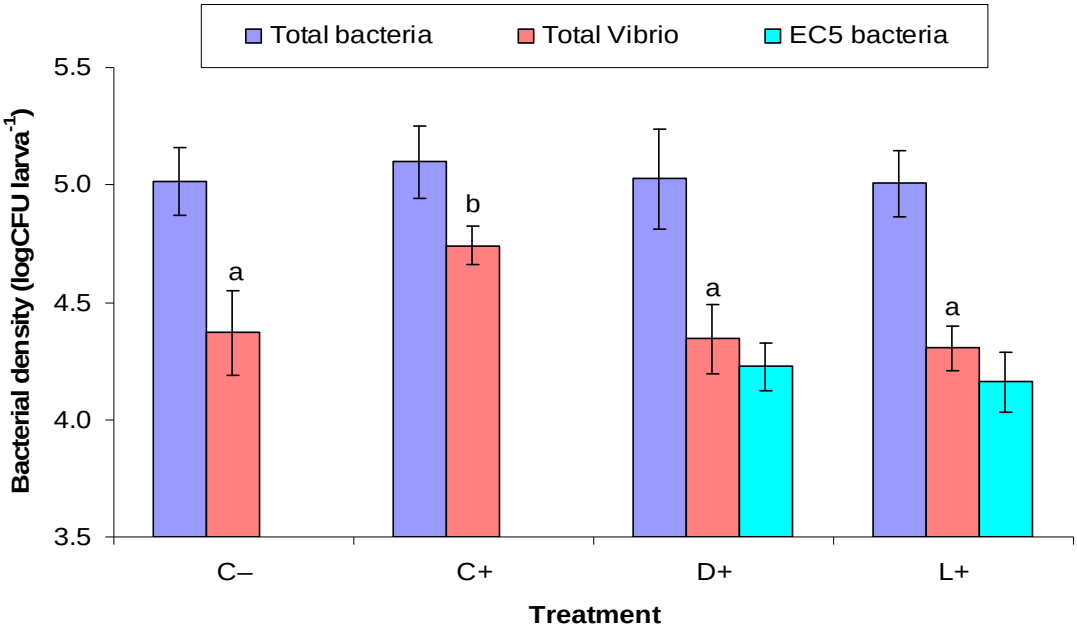

Fig. 6. Density of total bacteria, total *Vibrio* and EC5 inside the gut of *M. rosenbergii* at the end of experiment 1. Values are average ± SE, n = 4. Different letters denote significant differences (P<0.05). C-: no challenge; C+: challenged with *V. harveyi* BB120; D+: challenge + EC5(D); L+: challenge + EC5(L)

3.2 Experiment 2

The larval stage index was checked at two different time points (day 7 and 16) to evaluate larval growth. The results showed that the enrichment cultures did not affect larval growth (Fig. 7). However, at the end of the experiment, larval survival was significantly higher in the treatments with the addition of ECs when compared to the control treatment. Larval survival in all EC treatments was quite high (67–75%) and was not significantly different between the different EC treatments (Fig. 8). Assessment of larval tolerance to ammonia stress (24h LC_{50} NH_3) to evaluate larval quality in the different treatments showed that the larvae in the treatments with the addition of the ECs had a significantly higher tolerance than in the control treatment (Fig. 9). In this experiment the enrichment cultures did not affect the duration of the rearing cycle in terms of time of first postlarva appearance and total larval rearing cycle (Fig. 10)

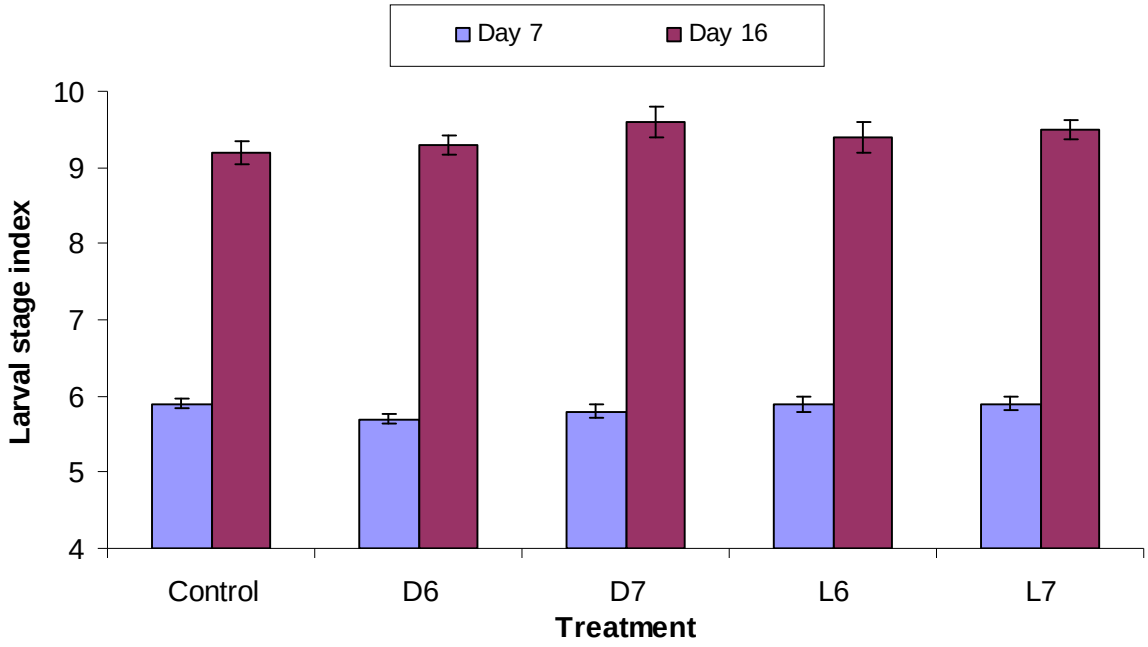

Fig. 7. Larval stage index of *M. rosenbergii* larvae at day 7 and 16 in experiment 2. Values are average ± SE, n = 4. D6: 10^6 CFU/ml EC5(D), D7: 10^7 CFU/ml EC5(D), L6: 10^6 CFU/ml EC5(L), L7: 10^7 CFU/ml EC5(L)

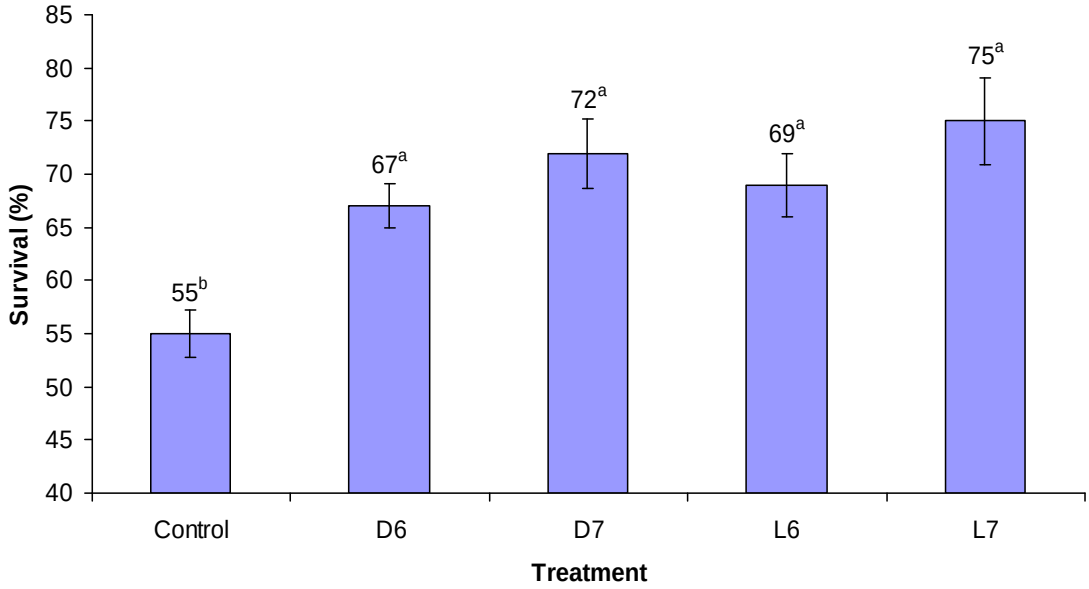

Fig. 8. Survival of *M. rosenbergii* larvae at the end of experiment 2.

Values are average ± SE, n = 4. Different letters denote significant differences (P<0.05). D6: 10^6 CFU/ml EC5(D), D7: 10^7 CFU/ml EC5(D), L6: 10^6 CFU/ml EC5(L), L7: 10^7 CFU/ml EC5(L)

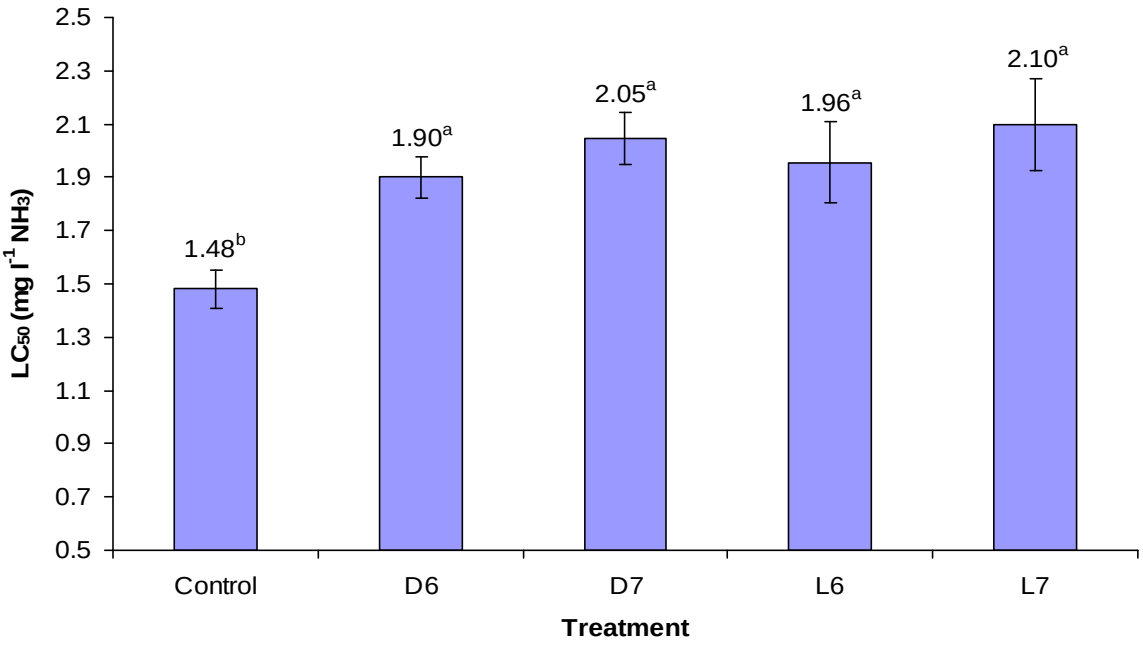

Fig. 9. 24h–LC_{50} (mg l^{-1} NH_3) of *M. rosenbergii* postlarvae in experiment 2.
Values are average ± SE, n = 4. Different letters denote significant differences (P<0.05). D6: 10^6 CFU/ml EC5(D), D7: 10^7 CFU/ml EC5(D), L6: 10^6 CFU/ml EC5(L), L7: 10^7 CFU/ml EC5(L)

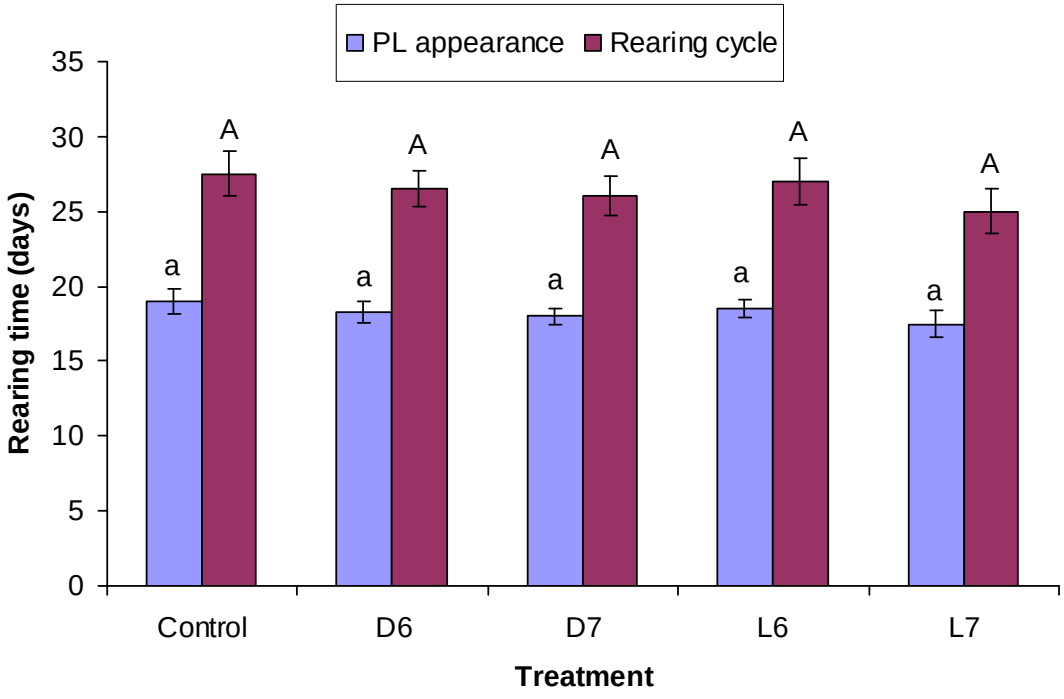

Fig. 10. Time of first postlarva appearance and duration of larval rearing cycle in experiment 2. Values are average ± SE, n = 4. D6: 10^6 CFU/ml EC5(D), D7: 10^7 CFU/ml EC5(D), L6: 10^6 CFU/ml EC5(L), L7: 10^7 CFU/ml EC5(L)

In this experiment, the microflora population density in terms of total bacteria, enrichment culture bacteria and total *Vibrio* were checked at three time points in the rearing water and at the end of the experiment in the prawn gut. The total bacterial population density in the rearing water in the EC treatments was always significantly higher than in the control treatment and tended to increase with time. In contrast, total *Vibrio* counts were decreasing in time in the EC treatments, while they increased in the control treatment. The enrichment culture bacteria were increasing in time and the addition of 10^7 CFU ml^{-1} always resulted in higher levels when compared to the addition of 10^6 CFU ml^{-1} (Fig. 11), with no differences between both EC types. Investigating the microflora density inside the prawn gut at the end of the experiment showed that total bacteria numbers in the treatments with 10^7 CFU ml^{-1} of the ECs were significantly higher than in the other treatments. The total *Vibrio* numbers were not significantly different between the different treatments (Fig. 12).

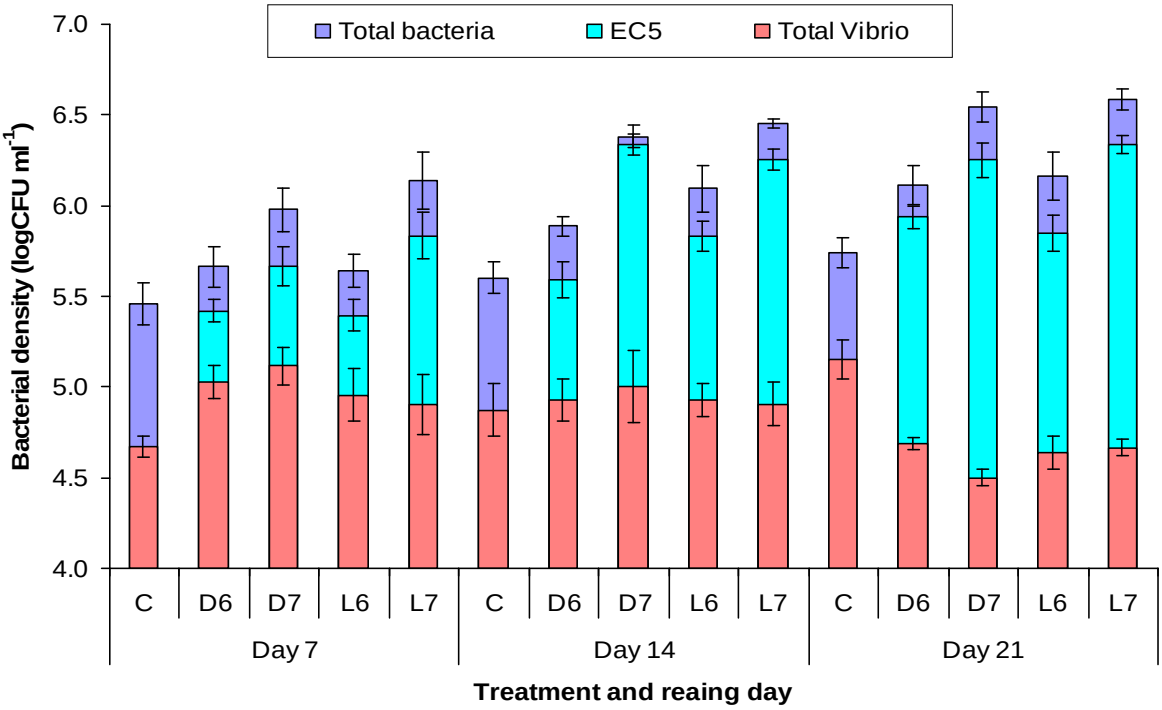

Fig. 11. Density of total bacteria, EC5 and total *Vibrio* in *M. rosenbergii* culture water on day 7, 14 and 21 in experiment 2. Values are average ± SE, n = 4. D6: 10^6 CFU/ml EC5(D), D7: 10^7 CFU/ml EC5(D), L6: 10^6 CFU/ml EC5(L), L7: 10^7 CFU/ml EC5(L)

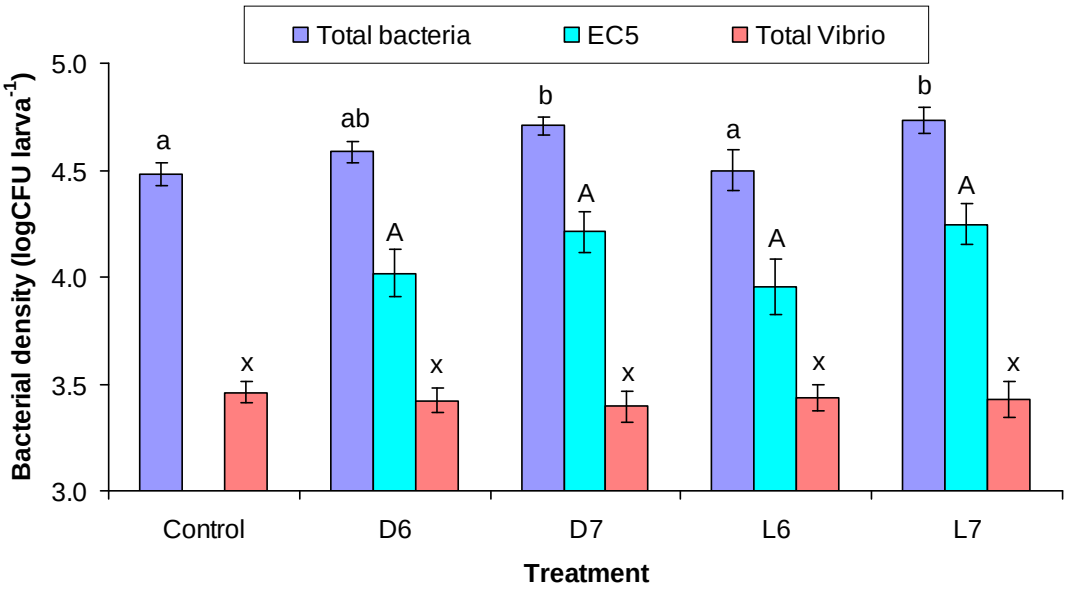

Fig. 12. Density of total bacteria, EC5 and total *Vibrio* inside the gut of *M. rosenbergii* at the end of experiment 2. Values are average ± SE, n = 4. Different letters denote significant differences (P<0.05). D6: 10^6 CFU/ml EC5(D), D7: 10^7 CFU/ml EC5(D), L6: 10^6 CFU/ml EC5(L), L7: 10^7 CFU/ml EC5(L)

4. Discussion

In this study, the use of enrichment cultures, which were isolated from European sea bass (*Dicentrarchus labrax:* EC5(D)) and from Asian sea bass (*Lates calcarifer:* EC5(L)) based on their AHL–degrading characteristics, was investigated with the aim to improve *Macrobrachium rosenbergii* larval performance and to protect against *Vibrio harveyi.* The ECs were added both to the larval rearing water and through the live food (*Artemia* nauplii). Our preliminary experiments indicated that EC dosages should not be lower than 10^6 CFU ml^{-1} for addition into the rearing water and 10^8 CFU ml^{-1} for *Artemia* enrichment in order to obtain the best results (data not shown). In experiment 1, the addition of ECs improved larval survival and larval quality. They also affected the density of the microflora populations both in the culture water and the prawn gut. The *Vibrio* level in the infected control was always higher than in the EC treatments (which were challenged daily with *Vibrio harveyi* at 10^5 CFU ml^{-1}). In the non–infected control treatment, the *Vibrio* level was similar to the one in the EC treatments, but larval survival was lower. This might be explained by the N–acyl homocerine lactone (AHL)–degrading capacity of the ECs, resulting in protection from vibriosis (Cam *et al*., 2009a). Indeed, the virulence of *Vibrios* might be mediated by AHL–dependent quorum sensing systems (Eberl, 1999; Dong and Zhang, 2005; Juhas *et al*., 2005). These results are in accordance with our previous study (Cam *et al*., 2009b) in which we found that ECs were able to degrade AHLs when they were added daily to the prawn rearing water. The addition of AHLs into the rearing water was shown before to negatively affect survival of *Macrobrachium* (Baruah *et al*., 2009) and turbot larvae (Tinh *et al*., 2008). In our study, the larval survival in the infected control treatment (no ECs added) was very low (11%), which might be due to the presence of unidentified deleterious bacteria. The addition of the ECs resulted in a

significantly higher larval survival, which was even higher than in the non–infected control. The addition of ECs into the larval gut via *Artemia* enrichment did not increase the total bacterial level inside the prawn gut. However, the presence of the ECs in the gut resulted in decreased *Vibrio* levels (Fig. 6). In addition, when compared with the non–infected control, the *Vibrio* levels inside the prawn gut of EC-treated larvae were similar, but the survival of the larvae was significantly higher. This can be explained by disruption of AHL-regulated virulence, both in deleterious bacteria that are naturally associated with *Macrobrachium* larvae (and caused mortality in uninfected larvae) and in the pathogenic *Vibrio harveyi*, which was added to challenge the larvae. In this experiment, EC5(D) and EC5(L) were isolated from different sources of sea bass, but their effects on protecting the larvae from pathogenic bacteria were similar.

In experiment 2, the larvae were cultured without addition of a pathogen to confirm the efficiency of the ECs under normal hatchery conditions and to determine the optimal concentration of the ECs in the rearing water of *Macrobrachium*. The results showed that the addition of the ECs into the rearing water and through *Artemia* enrichment did not affect larval growth and larval development. However, they significantly improved larval survival and larval quality in terms of larval tolerance in an ammonia stress test. In this experiment, larval survival in the control treatment was 55%, which is quite high when compared with what is normally obtained in practice, where survival is normally around 30–50% (Murthy and Satheesha 1998, Murthy *et al*. 2004, Phuong *et al*., 2006). Moreover, the larval survival of EC-treated larvae in this study was as high as 67–75%. These survival are remarkably high for freshwater prawn rearing. The EC bacteria could reduce the *Vibrio* level in the rearing water (Fig. 11), whereas they did not decrease *Vibrio* counts inside the larval gut (Fig. 12).

This could be expected because the ECs were isolated based on their quorum sensing-disrupting capacity and not on antibacterial properties. Baruah *et al*. (2009) reported AHL-induced mortality in *Macrobrachium* cultures, which was explained by the induction of quorum sensing-regulated virulence in unidentified deleterious bacteria. In this study, the ECs were shown to have a major impact on survival of the *Macrobrachium* larvae, most probably by disrupting AHL-regulated virulence expression of deleterious bacteria present in the cultures. Total bacteria and EC bacteria counts in the treatments with the addition of 10^7 CFU ml^{-1} EC bacteria were significantly higher than in the treatments with 10^6 CFU ml^{-1} EC bacteria addition. However, larval survival in all EC treatments was similar and was not different between different EC sources (European and Asian sea bass). Maeda (1994) suggested that aquaculture systems could not support bacterial densities higher than 10^6 cells ml^{-1}. Gomez–Gil *et al*. (2000) reported that the density of added bacteria rarely exceeds 10^7 cells ml^{-1} in culture water. Based on the results obtained in this study, the most cost-effective dosage of ECs for direct addition into the rearing water is 10^6 CFU ml^{-1}.

In conclusion, the use of ECs in freshwater prawn rearing proved to be highly efficient with respect to improving larval survival and larval quality. These parameters are very important for aquatic seed production in general and prawn larval rearing in particular. The application of AHL-degrading enrichment cultures fits in a new strategy in aquaculture, which focuses on sustainable production. However the development of a bacterial product to be used as commercial probiotics is a complex task and requires appropriate monitoring tools. The ECs can be cultured by using carbon sources which are readily available in hatcheries, and EC biomass production can be increased through the addition of cheap nutrients (Cam *et al* 2009a). Improving

prawn larval survival and quality is important with respect to seed production as it can contribute to increasing the cost–efficiency of aquaculture production.

Acknowledgments

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CHAPTER 5

SECTION 3

The effect of poly β–hydroxybutyrate on larviculture of the giant freshwater prawn (*Macrobrachium rosenbergii*)

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Abstract

In this study, we investigated the effect of poly β–hydroxybutyrate (PHB) on the culture performance of larvae of the giant freshwater prawn *Macrobrachium rosenbergii* and on the bacterial levels inside the larval gut. Instar II *Artemia* nauplii were cultured with or without PHB (5 g l^{-1}) and/or a lipid emulsion rich in highly unsaturated fatty acids (HUFA) for 24 hours. The effect of feeding PHB and/or HUFA enriched *Artemia* nauplii on the performance of *Macrobrachium* larvae was investigated. Feeding larvae of the giant freshwater prawn with PHB–containing *Artemia* nauplii significantly increased survival and growth of the larvae. Moreover, total bacterial counts and *Vibrio* counts were found to be significantly lower in PHB–fed larvae when compared to control larvae, indicating that the PHB addition had a growth–inhibitory effect towards these potentially pathogenic microorganisms. Finally, a combination of PHB addition and lipid enrichment resulted in the best overall culture performance since it synergistically improved larval survival. The optimal PHB concentration and formulation for bio-encapsulation into *Artemia* should be investigated further to increase the economical efficiency of *Macrobrachium* larval production.

Keywords: poly β–hydroxybutyrate, *Macrobrachium rosenbergii*, larval survival, larval growth.

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879), is a commercially important aquaculture species. In recent years, its culture has expanded rapidly not only within Asia but also in regions far remote from the natural distribution of the species (FAO, 2000). However, availability of healthy and high-quality seeds has always been a major obstacle in the expansion of *M. rosenbergii* farming. One of the major factors that is hampering the quality of hatchery–reared seed, culminating in low yield because of mass mortality, is disease. Outbreaks of disease in prawns are often attributed to bacterial infections (Sung *et al.*, 2000; Phatarpekar *et al.*, 2002; Al–Harbi, 2003). Mass mortality of larvae in the hatcheries restricts the regular production of high quality post–larvae. Such mortality is often attributed to opportunistic pathogenic bacteria (Skjermo and Vadstein, 1999). Vibrios have been reported as the causative agents for numerous disease outbreaks (Alavandi *et al.*, 2004; Kennedy *et al.*, 2006) and are often reported as a major problem in prawn hatcheries (Nayak and Mukherjee, 1997; Jayaprakash *et al.*, 2006). Antibiotics and disinfectants, the conventional approaches to control bacterial populations in prawn hatcheries, are quite popular. However, they are bring withdrawn from the market in many countries owing to concerns about public health and environmental safety (Schneider *et al.*, 2003). Moreover, the use of low doses of antibiotics as prophylactics has resulted in the development of antibiotic resistance (Teo *et al.*, 2000; Teo *et al.*, 2002), which makes antibiotic treatments ineffective in controlling diseases (Karunasagar *et al.*, 1994). Therefore, there is an urgent need to search for eco–friendly alternative control techniques which help to maintain the animals' health.

With the perception that antibiotics should no longer be used as animal growth promoters, there is currently a significant interest in short-chain fatty acids (SCFAs) as biocontrol agents in animal production (Defoirdt *et al.*, 2006). Several studies have shown that SCFAs inhibit the growth of yeasts and enterobacteria such as *Salmonella typhimurium, Escherichia coli* and *Shigella flexneri* (Bergeim, 1940; Wolin, 1969; Cherrington, 1991; Bearson *et al.*, 1997; Sun *et al.*, 1998; Van Immerseel *et al.*, 2003). SCFAs have previously been shown to inhibit or decrease the growth of *Salmonella* in chickens (Waldroup *et al.*, 1995; Van Der Wielen *et al.*, 2000; Van Immerseel *et al.*, 2005) and of pathogenic luminescent vibrios in *in vitro* tests (Defoirdt *et al.*, 2006). Moreover, SCFAs were shown to significantly increase the survival of challenged brine shrimp (*Artemia*) nauplii. In addition to their bacteriostatic effects, SCFAs are also known to provide mammals (and especially the mammalian colonic mucosa) with energy (Topping and Clifton, 2001), and this might also be the case for shrimp (Defoirdt *et al.*, 2006).

Effective fatty acid concentrations are rather high and consequently, it would economically not be feasible to dose fatty acids in the culture water of an aquaculture system in order to protect the animals. Moreover, the addition of high levels of organic carbon in the water would give rise to an excessive growth of heterotrophic bacteria which might have a negative effect on the health of the animals (because of oxygen depletion and/or because the growing bacteria could be pathogenic). Polyhydroxyalkanoates (PHAs) are water–insoluble polymers of β–hydroxy short–chain fatty acids that are produced as a reserve material by numerous bacteria (Anderson and Dawes, 1990). Interestingly, different studies provided some evidence that PHAs can also be degraded upon passage through the gastrointestinal tract of animals and consequently, adding these compounds to the feed might result in

biocontrol effects similar to those described for SCFAs (Defoirdt *et al*., 2009). Defoirdt *et al*. (2007) reported that the SCFA β–hydroxybutyrate was able to inhibit the growth of a virulent *Vibrio campbellii* strain *in vitro*. Based on this result, the authors investigated whether the polymer of this SCFA, the well–known bacterial storage compound poly–β–hydroxybutyrate (PHB), could be used to protect aquatic animals from pathogenic bacteria. The addition of commercial PHB particles (average diameter of 30 µm) at a concentration of 1000 mg l^{-1} to the culture water resulted in a complete protection of brine shrimp from the pathogenic *V. campbellii* (Defoirdt *et al*., 2007).

In this study, we investigated the effect of PHB (administered via the live food) on the survival and growth of larvae of the giant freshwater prawn *Macrobrachium rosenbergii* and on the microbiota (total bacteria and vibrios) associated with the larvae. To demonstrate the application potential, loading of the *Artemia* nauplii with PHB was combined with lipid enrichment, a technique which is routinely applied in many fish and crustacean hatcheries.

2. Materials and methods

2.1 Experimental animals

The study included 2 experiments on *Macrobrachium rosenbergii* larval rearing performed in 1–l glass bottles. Adult *M. rosenbergii* imported from Thailand were used as brooders. The water quality parameters of the broodstock tanks, the photoperiod, and the feeding regime were adjusted in accordance to the recommendations for prawn rearing (New, 2003). Larvae were obtained from a single ovigerous female breeder (Cavalli *et al*., 1999, 2000; Baruah *et al*., 2009). The newly–hatched larvae were ongrown for 10 days before being stocked into the experiments.

Twenty–four hours after hatching, the larvae were transferred into 10–l cylindro–conical jars connected to a single recirculation system as was described by Cavalli *et al*. (2001). Newly–hatched *Artemia franciscana* nauplii (EG® type, Batch 041004, INVE Aquaculture, Baasrode, Belgium) were used as live food and dosed to the culture water *ad libitum* (density always over 6 nauplii ml^{-1}) from day 2 to day 10. The *Artemia* dosage was split over two feedings at 9.00h and 17.00h.

2.2 Experimental design

The experiments were performed in glass cones containing 1000 ml brackish water (12 g l^{-1} salinity). The glass cones were placed in an aquarium tank containing water maintained at 29±1 ºC using a thermostatic heater. A fluorescent lamp system was installed to provide around 900–1000 lux at the water surface for 12h day^{-1}. The cones were supplied with gentle aeration to ensure dissolved oxygen in the rearing water was always above 5 mg l^{-1}. In all experiments, an open clear water system was used with a daily water exchange of 50%. During water exchange, the remaining *Artemia* and waste from the previous day were removed by siphoning. This operation was carried out with great care to avoid loss of larvae. Feeding was done after water exchange. The larvae were exclusively fed *Artemia franciscana* (Great Salt Lake strain) nauplii *ad libitum* twice a day at 9h00 and 17h00 during the complete experimental period. Depending on the treatment, instar II *Artemia* nauplii were enriched with PHB particles (average diameter 30 µm, Lot: S68924–488, Sigma–Aldrich, Bornem, Belgium) at a concentration of 5 g l^{-1} *Artemia* culture and/or a lipid emulsion (ICES 30/0.6/C, containing 30% total n–3 HUFA with a DHA/EPA ratio of 0.6, Lot: 903003.01, Han *et al*., 2000) at a concentration of 0.6 g l^{-1} *Artemia* culture

water during 24 h before feeding them to the *Macrobrachium* larvae. In the control treatment, *Artemia* nauplii were treated in the same way, without enrichment.

Experiment 1 consisted of two treatments of feeding the *Macrobrachium* larvae with PHB enriched *Artemia* nauplii or control nauplii (without enrichment). At the start of the experiment, 10 days–old larvae were stocked at an initial density of 50 l^{-1}. Experiment 2 consisted of 4 treatments with the addition of PHB and/or a lipid emulsion (Table 1). In this experiment, 4 day–old larvae were stocked at an initial density of 100 l^{-1}. The experimental duration was 15 days for experiments 1, and 28 days for experiment 2. In both experiments, the treatments were performed in six replicates.

Table 1. Experimental design in experiment 2 based on prepared *Artemia* for larval feeding

Treatment	PHB addition	Lipid enrichment
+P+L	+	+
+P–L	+	–
–P+L	–	+
–P–L	–	–

"P": PHB; "L": Lipid enrichment; "+": addition/enrichment; "–": no addition/enrichment.

2.3 Analyses

2.3.1 Detection of PHB in Artemia nauplii used to feed Macrobrachium

Instar II *Artemia* nauplii were enriched with PHB particles (average diameter 30 µm, Lot: S68924–488, Sigma–Aldrich, Bornem, Belgium), which were added to the culture water at a concentration of 5 g l^{-1}. PHB was detected in the nauplii following

the methodology described in Defoirdt *et al*. (2007). Briefly, after 15 min of incubation, 10 nauplii were killed with absolute ethanol and stained with the fluorescent dye Nile Blue A (Ostle and Holt, 1982) in order to detect the presented PHB under epifluorescence microscopy. The nauplii were examined with an Axioskop II microscope (Carl Zeiss, Jena, Germany) equipped with a Peltier–cooled single–chip digital camera (Orca Illm; Hamamatsu, Massay, France) connected to a computer.

2.3.2 Larval stage index

At day 10 and 15, larval development in each treatment was estimated through determining the larval stage index (LSI) according to Maddox and Manzi (1976). The average larval stage of at least 30 larvae from each treatment was recorded based on the description by Uno and Kwon (1969).

2.3.3 Larval survival

Larval survival was checked at day 5, 10 and 15 in experiment 1. In experiment 2, larval survival was checked at day 10, 15, 20 and 28. Counting the larvae was carried out with great care to avoid stress of the larvae.

2.3.4 Bacteria in the larval gut

Prawn larvae samples were collected at the beginning and on the last day of each experiment. Three samples of ten larvae were taken randomly from all replicates for analysis of bacterial counts in the prawn gut. Surface bacteria were inactivated according to a procedure described by Huys *et al.* (2001). The prawn sample was first immersed in a benzocaine solution (Sigma, 0.1%) for 10 s, transferred to a benzalkonium chloride solution (Sigma, 0.1%) for another 10 s, and rinsed three times

with autoclaved nine salts solution (NSS) (Olsson *et al.*, 1992) for 5 s each time. The larvae were then transferred to a sterile plastic bag containing 10 ml of NSS, and homogenized with a stomacher blender for 6 min. The water samples and the prawn homogenates were serially diluted in NSS. Fifty µL from each dilution was plated on Marine Agar, and on Thiosulphate–Citrate–Bile Salt–sucrose (Biokar Diagnostics, France) for enumerating the total culturable bacteria, and *vibrios*, respectively. The number of colonies was counted after incubation at 28 °C for 48 h.

2.4 Statistical analysis

Larval stage index, larval survival and countable bacterial density were analyzed by analysis of variance (one–way ANOVA) and, if significant differences were found ($P<0.05$), the least significant differences (Duncan, SPSS version 13.0 software) test was applied. All percentage data were normalized by arcsine transformation for statistical analysis, but only non–transformed means are presented. Two–way ANOVA was performed to determine the interaction between the experimental factors in experiment 2.

3. Results

3.1 Experiment 1

In a first experiment, the effect of feeding PHB to *Macrobrachium* larvae by enriching it into the live food was studied. Instar II *Artemia* nauplii used as live food for the *Macrobrachium* larvae were cultured with and without PHB (5 g l^{-1}). Microscopic analysis showed that the gut of the *Artemia* nauplii in the treatment without PHB was empty (Fig. 1), whereas in the treatment with PHB, the upper part of the gut was almost completely filled. Epifluorescence images of Nile Blue A

stained nauplii showed that the gut content of PHB–treated nauplii was brightly fluorescent and could clearly be distinguished from the (auto) fluorescence of the nauplii, which indicates that the nauplii had ingested the PHB particles. The effect of adding PHB to the live food on the performance of *Macrobrachium* larvae was studied by determining the larval survival and development over a culture period of 15 days. Evaluation of the larval development based on the larval stage index showed that larvae in the PHB treatment had grown significantly better than those in the control treatment (no PHB addition) after 10 days ($P<0.05$) (Fig. 2). Larval survival was determined at days 5, 10 and 15 (end of experiment) and was always significantly higher in the PHB treatment than in the control treatment ($P<0.05$). The difference in survival became more and more pronounced towards the end of the experiment (Fig. 3).

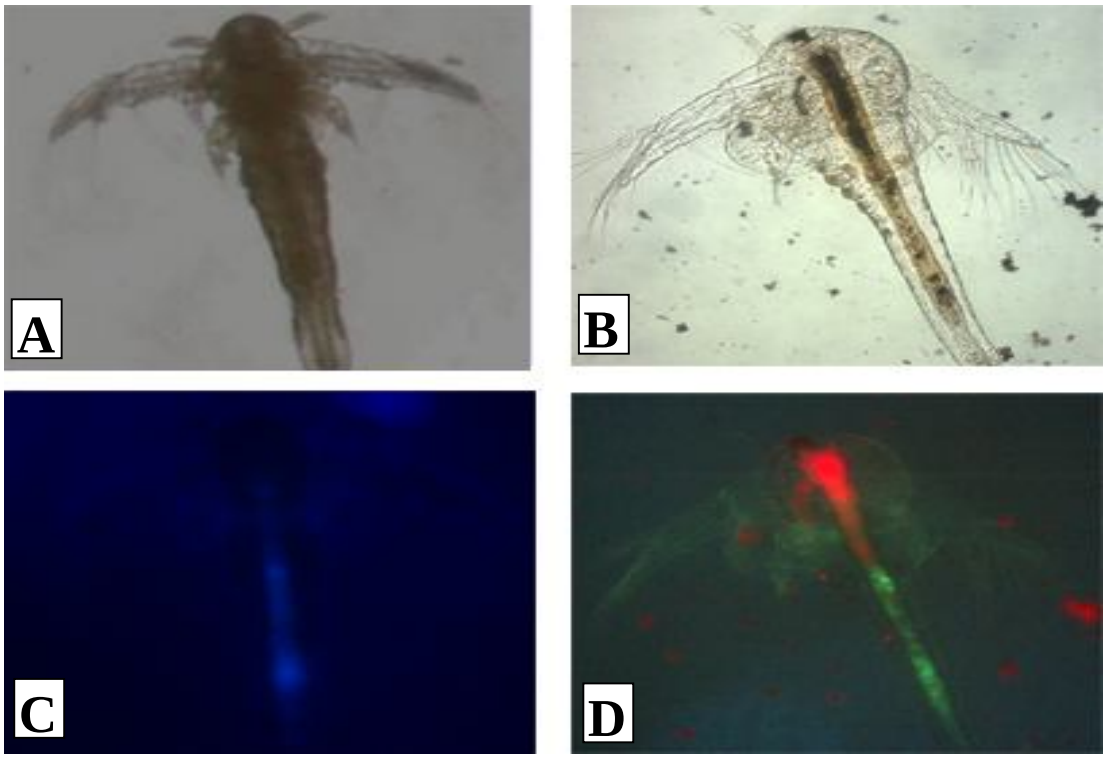

Fig. 1. Representative light (upper row) **and epifluorescence microscopy** (lower row) **images of Nile Blue A stained** *Artemia* **nauplii after 15 min without** (panels A and C) **and with** (panels B and D) **PHB added to culture water at 5 g l^{-1}**

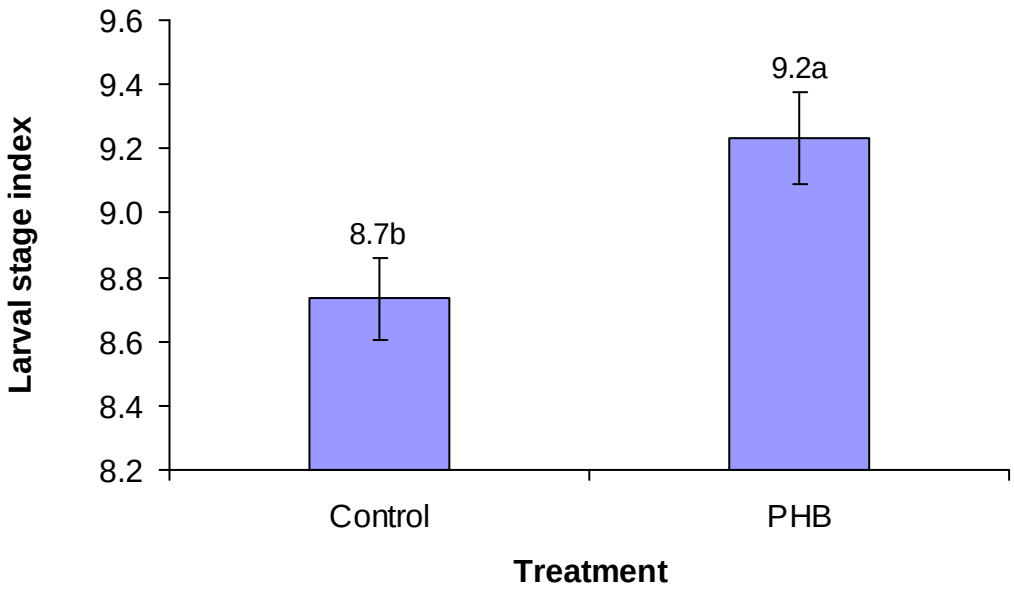

Fig. 2. Larval stage index at day 10 of the test (20 days after hatching) **of *Macrobrachium rosenbergii* larvae fed *Artemia* nauplii with or without PHB enrichment in experiment 1.** Values are average ± SE, n = 6. Different superscript letters denote significant differences (P<0.05).

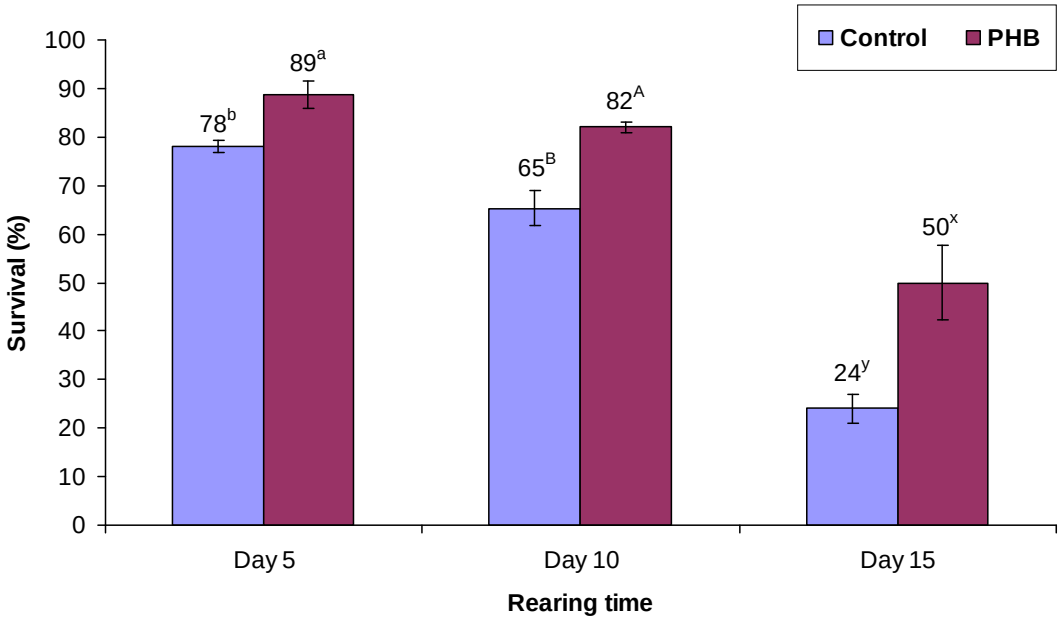

Fig. 3. Larval survival of *Macrobrachium rosenbergii* larvae fed *Artemia* nauplii with or without PHB enrichment after 5, 10 and 15 days of testing in experiment 1. Values are average ± SE, n = 6. Different letters denote significant differences (P<0.05). Different type of superscript denotes different comparisons.

3.2 Experiment 2

In this experiment, the effect of a combination of PHB and a lipid emulsion (both administered through the live food) on the performance of *Macrobrachium* larvae was investigated. The treatments with lipid enrichment showed the highest value of LSI, while the control treatment had the lowest larval growth. The LSI in the treatment with PHB was significantly higher than in the control treatment, but it was lower than in the treatments with lipid enrichment (Fig. 4). There was a significant interaction of feeding PHB and lipid enrichment with respect to larval stage index at day 10 and 15 ($P<0.05$). The larval survival was checked at four different time intervals during the experiment. The differences between the treatments were similar at all time points. The treatment with both PHB and lipid enrichment resulted in the highest survival (56% at the end of the experiment), while the survival in the control treatment was the lowest (12% at the end of the experiment). The treatments with either PHB or lipid enrichment alone were intermediate and were not significantly different from each other (Fig. 5). After 28 days of the experiment (32 days after hatching), 90% of the larvae in the treatments with lipid enrichment had reached the postlarval stage, while in the treatment with PHB only around 50% had metamorphosed, and in the control treatment only 10% of the animals were postlarvae. There was no interaction between PHB and lipid enrichment with respect to larval survival.

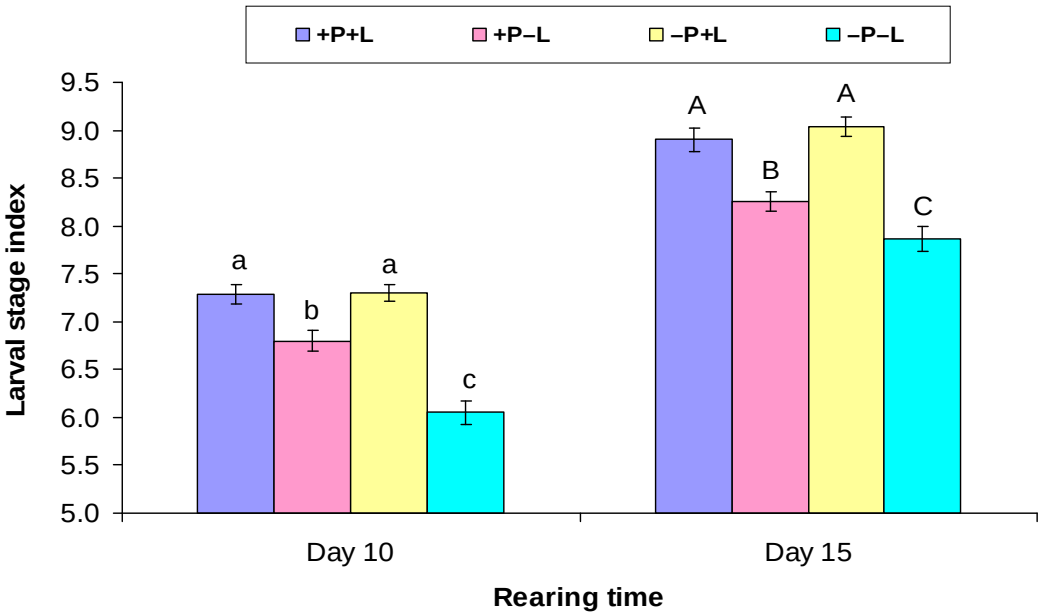

Fig. 4. Larval stage index at day 10 and 15 of *Macrobrachium rosenbergii* larvae fed *Artemia* nauplii with or without PHB and/or lipid enrichment in experiment 2. Values are average ± SE, n = 6. Different letters denote significant differences (P<0.05). Small and capital letters denote different comparisons.

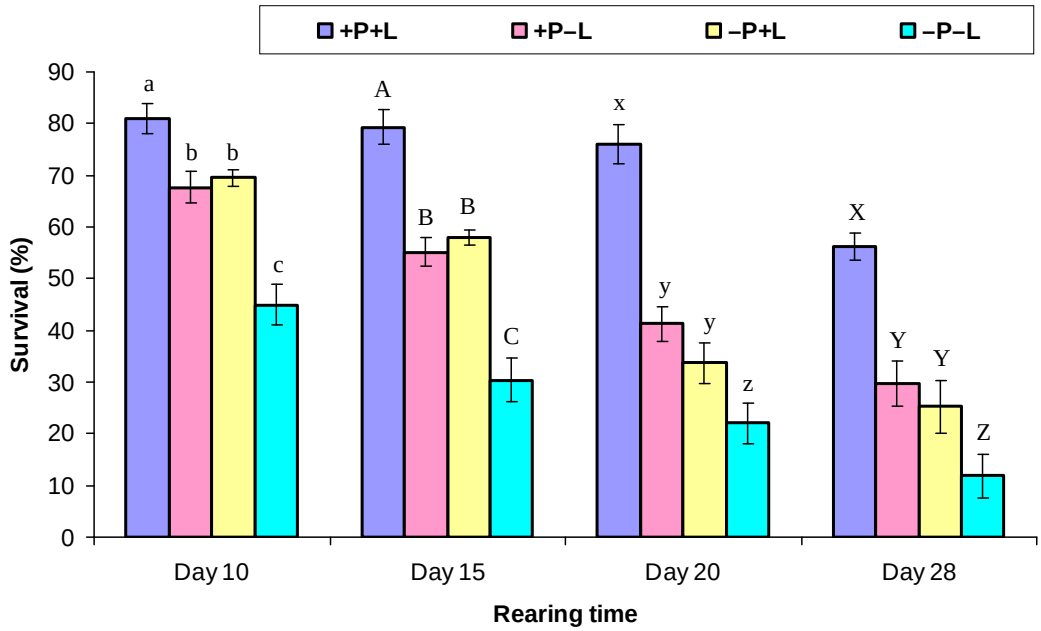

Fig. 5. Larval survival of *Macrobrachium rosenbergii* larvae fed *Artemia* nauplii with or without PHB and/or enrichment in experiment 2. Values are average ± SE, n = 6. Different letters denote significant differences (P<0.05). Different type of letters denotes different comparisons.

Total bacteria and *Vibrio* levels in the prawn larval gut were determined at the beginning and at the end of the experiment by plate counting on Marine Agar and TCBS Agar, respectively. At the end of the experiment, the total bacterial number in the larval gut was significantly higher in the treatment with lipid enrichment only (reaching up to $28x10^4$ CFU $larva^{-1}$) when compared to the other treatments. The *Vibrio* numbers in the larval gut at the end of the experiment were significantly higher in the treatments without PHB when compared to the treatments with PHB addition. The number of *Vibrio* spp. in the larval gut was initially 21±6 CFU $larva^{-1}$, and increased to 1.3–2.2 x 10^3 CFU $larva^{-1}$ at the end of the experiment in the treatments without PHB (Table 2). There was a significant interaction between feeding with PHB and lipid emulsion with respect to the microflora population density inside the larval gut ($P<0.05$), suggesting that the effect of PHB was stronger when combined with lipid emulsion.

Table 2. Microflora in the gut of *Macrobrachium rosenbergii* larvae fed *Artemia* nauplii with or without PHB and/or lipid enrichment in experiment 2

Treatment	Total bacteria (10^4 $larva^{-1}$)		Total *Vibrio*	
	Initial	Final	Initial (per larva)	Final (10^2 $larva^{-1}$)
+P+L	3.2±0.2	5.6±1.1[a]	21±6	0.3±0.1[a]
+P–L		5.4±0.8[a]		1.6±0.7[a]
–P+L		28.0±2.5[b]		22.0±5.3[b]
–P–L		6.8±1.0[a]		13.3±1.8[b]
Interaction between PHB and nutrient emulsion		P=0.002		P=0.008

Values are average ± SE, n = 3. Different letters within column denote significant differences ($P<0.05$).

4. Discussion

In this study, we investigated the effect of PHB on the culture performance of larvae of the giant freshwater prawn *Macrobrachium rosenbergii* and on the bacterial levels inside the larval gut. *Artemia* nauplii are commonly used as live feed for *Macrobrachium* larvae (Lavens *et al*., 2000) and because Defoirdt *et al*. (2007) reported before that PHB can be accumulated by *Artemia* when added to the culture water, we delivered PHB to the prawn larvae via *Artemia*. The accumulation of PHB particles in the *Artemia* gut was confirmed by using fluorescent microscopy (Fig. 1). It was not possible to detect PHB in the gut of the *Macrobrachium* larvae because the larvae were not sufficiently transparent for fluorescence microscopy and may be because PHB content was much too small to allow detection by chromatography. The results presented in this paper showed that feeding *Macrobrachium* larvae with PHB–containing *Artemia* nauplii significantly improved larval survival (Fig. 3 and 5). In experiment 2 where the combination of PHB and a lipid emulsion rich in highly unsaturated fatty acids was studied, the highest larval survival was noted in the treatment with addition of both PHB and the emulsion (Fig. 3 and 5). The treatments with PHB only and lipid emulsion only also significantly increased the survival of the larvae, though less pronounced than in the treatment with both additives. These results are in accordance to the report of Defoirdt *et al*. (2007), who found that PHB significantly increased the survival of brine shrimp larvae challenged with pathogenic vibrios. The low survival of prawn larvae in the control treatment in this study was probably due to the presence of opportunistic bacteria that are associated with the larvae (as also reported by Baruah *et al*., 2009). In our study, we found that feeding *Macrobrachium* larvae with PHB–containing *Artemia* nauplii resulted in significantly lower levels of total bacteria and vibrios (Table 2), which are frequently associated

with disease of aquatic organisms (Lightner, 1996; Otta *et al*., 2001). Phatarpekar *et al*., (2002) investigated the bacterial flora on prawn larvae and found that *Vibrio* spp. were detected in eggs and water but were conspicuously absent in larvae. However, the total viable count in larvae varied from $2.5x10^4$ to $1.6x10^8$ CFU g^{-1}. In the current study, total bacteria and total *Vibrio* counts in larvae of treatments without PHB addition varied from 15.9 to 28.0 x 10^4 and 0.4 to 22.0 x 10^2 CFU $larva^{-1}$, respectively, while in the PHB treatments they were only 1.7 to 5.6 x 10^4 and 0.3 to 1.6 x 10^2 CFU $larva^{-1}$, respectively.

Defoirdt *et al*. (2007) found that PHB particles protected brine shrimp larvae from pathogenic *Vibrio campbellii* and hypothesised that PHB particles were (partially) degraded into β–hydroxybutyrate in the shrimp gut and that the release of this fatty acid protected the shrimp from the pathogen in two ways, i.e. by providing them with energy (resulting in a gut epithelium that is more resistant to infection) and by inhibiting the growth of the pathogen. This can also explain why feeding the *Macrobrachium* larvae with PHB–containing *Artemia* nauplii did increase survival and growth of the larvae when compared with the control. However, administration of a lipid emulsion significantly increased growth of the larvae when compared with addition of only PHB. Indeed, the larvae could have used breakdown products of the PHB particles as an energy source, but they may have lacked essential nutrients required for growth, which were present in the lipid emulsion. Lipids are known to play several essential roles in crustacean larvae (Teshima 1972, 1997; Middleditch *et al*., 1980; Teshima and Kanazawa, 1983; Harrison, 1990). Apart from being a major source of metabolic energy and the main form of energy storage, lipids also supply essential fatty acids needed for the maintenance and integrity of cellular membranes, and serve as precursors of steroid and moulting hormones (Teshima, 1972; Harrison,

1990). Fatty acids released from PHB are short chain fatty acids, that may provide an extra energy source, but do not provide long chain fatty acids necessary for optimal metabolic functioning. Several studies investigated the lipid metabolism of larval and juvenile stages of the freshwater prawn (Devresse *et al*., 1990; Sheen and D'Abramo, 1991; Teshima *et al*., 1992, 1997; D'Abramo and Sheen, 1993; Querijero *et al*., 1997; Roustaian *et al*., 1999). Feeding the larvae with a lipid emulsion rich in highly unsaturated fatty acids (HUFA) both provided the larvae with an excellent source of energy (Tidwell *et al*., 1998), and the necessary structural components for tissue growth and hormone synthesis, resulting in better growth. This result was in accordance with several previous studies, for example Romdhane *et al*. (1995) who demonstrated that longer periods of feeding (n-3) HUFA-enriched *Artemia* nauplii, improves the results in terms of growth, metamorphosis rate, survival and stress resistance of prawn larvae. Similarly, Sorgeloos and Leger (1992) and Alam *et al*. (1995) reported that the application of live food enriched with marine oils rich in n-3 fatty acids improved the larval growth of *M. rosenbergii*.

Degradation of PHB can occur via several mechanisms, including chemical decomposition or hydrolysis and enzymatic hydrolysis (Defoirdt *et al*., 2009). However, the exact mechanism by which the PHB polymers are broken down inside the intestinal tract of animals, i.e. whether it is mainly driven by physico–chemical properties of the gut environment or by the release of digestive enzymes by the host and/or by microorganisms present in the gut, is not yet known. Defoirdt *et al*. (2007) reported that PHB–degradation in brine shrimp larvae was most probably physico–chemical or mediated by enzyme activity of the brine shrimp and not microbial since the larvae were axenic. The *Macrobrachium* larvae used in this study, in contrast, were not axenic and consequently, the microorganisms associated with the larvae

might also have contributed to PHB breakdown. Indeed, enzymatic degradation of PHB by PHB– depolymerase producing microorganisms is well–documented (Doi *et al*, 1990; Yoshie *et al*., 1999; Quinteros *et al*., 1999; Choi *et al*., 2004; Khanna and Srivastava, 2004; Jendrossek and Handrick, 2002), although as far as we know no intestinal PHB–degrading microorganisms have been reported yet in *Macrobrachium*.

The application of PHB in aquaculture, larval rearing or more specifically in prawn larval production may be constrained due to the currently high cost of commercial PHB products. However, PHB can be produced relatively easily by *Bacillus* and *Lactobacillus* spp. (Anderson and Dawes, 1990; Aslim *et al*., 1998; Yilmaz *et al*., 2005) from inexpensive substrates, such as molasses, potentially making PHB administration cost–effective and sustainable (Kim, 2000). Furthermore, Defoirdt *et al*. (2007) believe that it should be possible to produce PHB *in situ* in the culture water by adding C–rich compounds or by increasing the C/N ratio of the feed.

In conclusion, the results obtained in this study showed that feeding larvae of the giant freshwater prawn *Macrobrachium rosenbergii* with PHB–containing *Artemia* nauplii significantly increased larval survival and growth. Moreover, total bacterial counts and *Vibrio* counts were found to be significantly lower in PHB–fed larvae when compared to control larvae, indicating that the PHB addition had a growth–inhibitory effect towards these potentially pathogenic microorganisms. Finally, a combination of PHB addition and a lipid emulsion resulted via synaptic manna in the best overall culture performance. The optimal PHB concentration and formulation for encapsulation into *Artemia* should be investigated further to increase the economical efficiency of larval production.

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CHAPTER 6

GENERAL DISCUSSION

CONCLUSIONS AND FUTURE RESEARCH

1. General discussion

1.1 Giant freshwater prawn seed production: current status

Current techniques used for giant freshwater prawn seed production have evolved from the techniques developed by Takuji Fujimura and his team at the Anuenue Fisheries Research Centre in Hawaii in the 1960s and 1970s. The first type of prawn hatchery used a flow-through system, which is effectively used in many parts of the world. The second type was recirculation systems, involving the use of biological filtration developed to conserve water and energy usage, reduce the demand for seawater or brine, and facilitate the establishment of inland hatcheries. Recently, there are many variant systems that were modified from the two original types of prawn hatcheries such as green–water systems, modified stagnant green–water systems, dynamic closed systems, … However, production efficiency and larval quality are largely variable based on the type of system and the local situation. Therefore, no standard hatchery protocol for freshwater prawn would suit every local situation.

There has been a very rapid global expansion of freshwater prawn farming since 1995. Prawn seed demand for farming is very much increasing. Existing hatcheries are not producing up to their installed capacity due to various constraints. Availability of healthy and high–quality seeds has always been a major obstacle in the expansion of *M. rosenbergii* culture. The low survival or mass mortality of prawn larvae are not only due to disease problems or suboptimal larviculture techniques but are also affected by broodstock source and domestication issues. Success of freshwater prawn larviculture is large variable between locations or even between different batches of larvae, with average survival fluctuating from 20 to 70%. In general, to maximise larval survival and larval quality multiple inter-connected factors related to seed

production, including broodstock quality, larviculture zootechniques and pathogen control strategy, need to be addressed. In the current study, we investigated and developed improved hatchery protocols for freshwater prawn larviculture in Vietnam based on the current technology used in prawn hatcheries in order to maximise larval survival and larval quality. A series of experiments were carried out aiming to identify the optimal conditions with respect to broodstock sourcing, larval rearing techniques in terms of feeds and feeding regime and larval stocking density. In addition, the application of probiotics and a novel product (poly-hydroxy-butyrate) to control bacterial pathogens to replace the use of antibiotics was carried out. These factors showed strong effects on larval quality and production efficiency. A detailed discussion on each factor separately is provided in the following sections.

1.2 Broodstock

In aquaculture in general, and in aquatic seed production especially, selection of broodstock or breeders has always been an important factor to consider in the production process. Significant productivity improvements have been achieved via selective breeding programs in aquaculture over the last 10 years, particularly in finfish species where improvements of up to 10 to 20% generation^{-1} have been achieved (Quinton *et al*., 2005; Neira *et al*., 2006; Ponzoni *et al*., 2005; Eknath *et al*., 2007); but also in a few commercially important crustacean species (Hetzel *et al*., 2000; Argue *et al*., 2002; Goyard *et al*., 2002; Preston *et al*., 2004; De Donato *et al*., 2005; Jerry *et al*., 2005). However, until recently, very little progress has been made in the genetic improvement of *Macrobrachium,* although this topic has long been recognized as an area of research that could be expected to yield significant improvements. Despite over four decades of domestication of the giant freshwater

prawn (Ling & Merican 1961; New 2000a,b), little information is published concerning the effects of many generations of domestication on cultured stocks. While Thailand and Malaysia currently use domesticated stocks for commercial seed production, countries such as Vietnam and Bangladesh still rely to a large extent on wild seed (New 2000b, Phuong *et al*. 2006). Amrit and Yen (2003) demonstrated that there were significant differences in development and survival between offspring originating from two different broodstock sources (Thailand and Vietnam). The cause for these differences is probably due to genetic differences owing to the different origin of the strains and/or differences due to domestication. These authors suggested that Vietnamese seed producers could consider either domestication of their own stocks or import already domesticated stocks from Thailand to improve survival sufficiently high enough for commercial production. Several studies compared reproductive performance of wild and pond–reared penaeid shrimp broodstock. However, there are no previous studies that compared reproductive parameters of different strains of giant freshwater prawns or prawns from different geographical origin. Thanh *et al*. (2009) evaluated the growth performance of three strains of giant freshwater prawn. Two strains were from wild Vietnamese origin (two separated locations) and one was introduced from Hawaii. Growth performance of the Hawaiian *M. rosenbergii* strain was better than the two different strains from Vietnamese origin. Crossbreeding among giant freshwater prawn strains developed from geographically discrete populations in Vietnam resulted in offspring that grew at significantly different rates. Specific cross combinations showed evidence for heterosis and the direction of reciprocal crosses can apparently influence mean growth rates (Thanh *et al*., 2009).

In Vietnam, some hatcheries still prefer to use wild berried females from natural waters based on the belief that these produce better quality larvae than pond-reared ones. However, collecting ovigerous females from the wild often results in considerable egg loss and degradation during capture and transport. Therefore many hatcheries prefer to use adjacent rearing ponds for their supplies. In the current study it was shown that reproductive performance between *M. rosenbergii* breeders from four very different sources was largely the same in terms of reproductive capacity and fecundity. However, in contrast many indicators showed differences in terms of offspring quality between the different broodstock sources. In general, the pond–reared Chinese and Vietnamese breeders resulted in better offspring quality than the broodstock source from Hawaii and the wild Vietnamese breeders. Possible reasons for the better results with the former two broodstock sources may lay in the fact that these animals are domesticated and/or the fact that they originate from the same geographical area as where the current experiment was performed. The better performance of pond–reared animals may be results of some selection due to the domestication process, making the animals more adapted to the culture environment. This suggests that, despite earlier claims, wild *M. rosenbergii* are not necessarily a better source of breeders than pond–reared animals. Broodstock development in ponds adjacent to a hatchery therefore seems a feasible and efficient option. It allows the hatchery easy access to suitable sized berried prawns throughout the year and also reduces transportation stress and cost of using wild stocks. Wild berried animals on the other hand are usually captured during the beginning of the rainy season only. Pond–reared animals generally are available year round and also provide a more readily available source in regions where breeders do not occur in the wild.

1.3 Larval rearing zootechniques

There are many factors that affect larval rearing efficiency of prawns. These include feeds, feeding regime, larval density, husbandry, microbial management (Barros *et al*., 2003; Phuong *et al*., 2006). Suboptimal conditions or lack of control of any of these factors may negatively affect performance and result in low survival or poor offspring quality. There are numerous studies on larval rearing of giant freshwater prawn aiming to improve larval survival through investigating different rearing methods, feeds and feeding regimes, larval stocking density, … However, it seems difficult to define a standard larval rearing protocol.

Optimal larval stocking density, feed rations and feeding frequencies are of utmost importance in prawn larval rearing. Especially since *M. rosenbergii* is regarded as a very cannibalistic species. Most hatcheries use a larval rearing density which remains constant throughout the rearing cycle. An alternative may be to start at higher densities, but gradually reduce this by increasing the water volume in the tank as the animals develop. It is anticipated that this could make feeding and hence production more economical. The results of the current study suggested that an initial larval rearing density of 100 to 150 larvae l^{-1} is most effective for open water systems with a constant rearing volume. Lower (50 larvae l^{-1}) or higher densities (200 larvae l^{-1}) resulted in a similar production efficiency (number of PL produced per liter of rearing volume), however high stocking densities (over 150 larvae l^{-1}) reduced cost–efficiency of production and the resulting larval quality (Chapter 4.1, Figures 4,5,6). In an other experiment using a rearing system in which the water volume increased during the rearing cycle, it was found that the initial larval density could be as high as 400 larvae l^{-1}. Afterwards, the density should be gradually lowered by increasing the

rearing volume as the animals develop. In practice, larval stocking density will be chosen based on the larval rearing system, water source and condition of the hatchery facilities, so that production efficiency can be maximised from an economic point of view, but also in terms of product quality. In the case of Vietnam, prawn larval rearing techniques are very variable based on the experience of the culturist. Stocking rates in this respect range from 40 to 125 l^{-1} but are typically 40–60 l^{-1} in green–water system and 80–100 l^{-1} in open and recirculation systems. The choice of rearing system is largely dictated by the location of the hatchery, e.g. green–water or recirculation systems are preferentially used in locations far from a source of saline water, while open systems are normally applied for locations close to the sea.

Feeding frequency also strongly affects larval survival especially with carnivorous species. A low feeding frequency may lead to high mortality due to the cannibalism. In the current study it was found that newly–hatched instar I *Artemia* nauplii should be applied several times per day to ensure sufficient quantities of fresh food throughout the day. Performance of the larvae was significantly better when fed 6 times per day compared to only 2 times. It would be interesting to design an automatic drip–feeding system to control and maintain feed quantity and quality in prawn larval rearing systems.

Feed cost is one of the single most important factors in cost–efficiency of hatchery operations. Several alternative foods both live and inert are being investigated as either supplement or replacement for *Artemia* nauplii in crustacean hatcheries to reduce production cost (FAO, 1985, 2005). In the current study, the use of wet (egg custard) and dry (commercial food) diets to gradually replace *Artemia* up to 50% represented good results in term of growth, survival and quality of the larvae. For the

diets tested in this study, replacement should however only start from larval stage V onwards, with an initial replacement level of about 25% of the total ration. Subsequently, replacement could be increased up to 50% from stage IX onwards. It was noted that most of the formulated food only remained suspended in the water column for a short time and sank to the bottom after being saturated with water. Therefore, artificial diets should be fed several times daily, but avoiding overfeeding which may negatively affect water quality. Decapsulated *Artemia* cysts may also be used to replace *Artemia* nauplii as a direct source for fish and crustacean larvae (Sorgeloos *et al*. (1977). However, *M. rosenbergii* larvae have the behaviour to concentrate at the water surface or the top parts of the water column while the decapsulated cysts tended to sink to the bottom soon after rehydration. Therefore, complete replacement of *Artemia* nauplii with decapsulated cysts was not successful. Several studies investigated replacement of *Artemia* with artificial feeds. Amrit and Yen (2003) started to feed 5 day old larvae with supplemental formulated feeds.

1.4 Microbial control

The increasing demand for postlarvae, higher stocking densities, higher rearing temperatures, and other factors have lead to increased problems related to the maintenance of proper bacterial community stability. A number of microbial agents may be involved in causing mortalities in shrimp and prawn larvae. Bacteria, particularly *Vibrio* species, have been reported to cause larval mortalities in hatcheries. *Vibrio harveyi*, a luminous species, has been implicated in a number of such cases (Otta *et al.*, 2001). Antibiotics have been widely used in the past, not only to control disease but also to promote growth. Nowadays there is however huge pressure on the industry to find suitable alternatives and the application of antibiotics

in aquaculture and particularly in seed production is drastically reduced due to more severe legislation. During the past two decades, the use of probiotics as an alternative to antibiotics has shown to be promising in aquaculture, particularly in fish and shellfish larviculture. These alternatives must therefore offer both protection against diseases as enhance performance.

Enrichment cultures (ECs) of AHL–degrading bacteria were used in the current study with the purpose of controlling putative opportunistic as well as pathogenic bacteria associated with the *Macrobrachium* larvae. Daily addition of an AHL mixture (1 mg l^{-1}) caused a sharp reduction in the survival and development of the prawn larvae. This is in accordance with previous studies on *Macrobrachium* larvae (Baruah *et al*. 2009) and turbot larvae (Tinh *et al*. 2008b) which studied the effect of AHL addition (1 mg l^{-1}) to the rearing water. We speculate that the low performance of prawn larvae in the presence of an AHL mixture in the present study could be due to the increased virulence of the microbial communities (regulated by quorum sensing) associated with the larvae. The survival of the prawn larvae treated with ECs was comparable with a control treatment regardless of the daily exposure to the AHL mixture. In another experiment, application of the ECs through both the larval rearing water and through the live food (*Artemia* nauplii) showed significant improvement of *Macrobrachium rosenbergii* larval performance and protected the larvae against *Vibrio harveyi*. Microbial techniques revealed that the presence of the ECs also resulted in decreased *Vibrio* levels in the prawn gut. In an other experiment the larval survival of EC–treated larvae was significantly higher than in the control treatment while in contrast the *Vibrio* levels inside the prawn gut larvae were similar. Also under normal hatchery conditions (no *Vibrio* challenge) larval performance was significant improvement with addition of ECs. It was confirmed in this study that both

ECs showed strong disruption of AHL–regulated virulence, both in deleterious bacteria and in the pathogenic *Vibrio harveyi*, in accordance with Cam *et al*. (2009). Although the ECs were isolated from fish guts, they showed to be effective in the prawn culture environment. This characteristic of the ECs may suggest they could be applied in different fish and crustacean species.

In a second study aimed at controlling the microbial community in prawn larval rearing larvae of the giant freshwater prawn were fed poly β–hydroxybutyrate (PHB)–containing *Artemia* nauplii. This resulted in significantly increased survival and growth of the larvae. Defoirdt *et al.* (2007) found that PHB particles protected brine shrimp *Artemia* larvae from pathogenic *Vibrio campbellii* and hypothesised that PHB particles were (partially) degraded into β–hydroxybutyrate in the shrimp gut and that the release of this fatty acid protected the shrimp from the pathogen in two ways, i.e. by providing them with energy (resulting in a gut epithelium that is more resistant to infection) and by inhibiting the growth of the pathogen. The low survival of prawn larvae in the control treatment (without any addition) in this study was probably due to the presence of opportunistic bacteria that are associated with the larvae (as also reported by Baruah *et al*., 2009). Total bacterial counts and *Vibrio* counts were found to be significantly lower in PHB–fed larvae when compared to control larvae, indicating that the PHB addition had a growth–inhibitory effect towards these potentially pathogenic microorganisms. A second experiment investigated the combination of PHB and lipid enrichment of the live feed on prawn larval performance. Development rate was enhanced in larvae fed PHB–containing *Artemia*, however not to the extent of larvae receiving lipid enriched *Artemia*. It was speculated that the larvae could have used breakdown products from the PHB particles as an energy source, but they may have lacked essential nutrients required for growth,

which were present in the lipid emulsion. Fatty acids released from PHB are short chain fatty acids, that may provide an extra energy source, but do not provide long chain fatty acids necessary for optimal metabolic functioning. Finally, a combination of PHB addition and lipid enrichment resulted in the best overall culture performance since it significantly improved both larval survival as well as larval growth. The application of PHB in aquaculture may be constrained due to the currently high cost of commercial PHB products. However, PHB can be produced relatively easily by *Bacillus* and *Lactobacillus* spp. (Anderson and Dawes, 1990; Aslim *et al.*, 1998; Yilmaz *et al.*, 2005) from inexpensive substrates, such as molasses. It might also be possible to produce PHB *in situ* in the culture water by adding C–rich compounds or by increasing the C/N ratio of the feed (Defoirdt *et al.*, 2007). All these concepts may open new opportunities to produce PHB at a lower cost, opening possibilities for application in aquatic seed production in the future. Combining the addition of PHB with lipid enrichment of *Artemia* nauplii, which is a commonly used technique in larval rearing of different fish and shellfish species, demonstrates that this technique could readily be applied in hatcheries.

2. Conclusions

In summary, the following conclusions can be draw from this study:

– Comparison of four different sources of *M. rosenbergii* broodstock (Vietnam wild, Vietnam pond–reared, Hawaii pond–reared and China pond–reared) showed that, although reproductive performance was largely the same, offspring quality showed considerable differences. Larval quality of Chinese and Vietnamese pond–reared breeders was markedly better than that of Vietnamese wild and Hawaiian pond–reared animals in terms of larval development, survival and ammonia tolerance. The results demonstrate that pond–reared broodstock may be an equally good or even better broodstock source than wild animals. Performance and fitness should be carefully checked under the locally prevailing conditions when introducing broodstock from different geographical locations.

– For prawn larval rearing, optimal larval stocking density is 50–150 l^{-1} when using a constant water volume. Initial larval density could however be increased up to 400 l^{-1} in case a rearing system with increasing water volume is used. A feeding frequency of 6 times per day by first–stage *Artemia* nauplii proved better than feeding only twice daily.

– Wet or dry artificial diets can replace 25% of the *Artemia* ration from larval stage V onwards. Subsequently, this could be increased up to 50% from stage IX to the postlarval stage. This feeding schedule resulted in good growth, survival and quality of the prawn larvae.

– The use of enrichment cultures (ECs) of N–acyl homoserine lactone (AHL)–degrading microbial communities proved effective to control potential pathogens in

prawn larviculture. Furthermore, the addition of ECs into the larval rearing water and via enriched *Artemia* nauplii used for larval feeding may protect the larvae from *Vibrio harveyi* and/or opportunities pathogens, and have a positive effect on larval survival and larval quality.

– Feeding larvae of the giant freshwater prawn with poly–β–hydroxybutyrate (PHB)–containing *Artemia* nauplii significantly increased survival and growth of the larvae.

3. Future research

– In the current study, performance of broodstock from four different sources was investigated in terms of reproductive output and offspring quality. However, it would be interesting to verify and validate the observed differences between these different sources by using genetic techniques.

– Grow–out performance of these different prawn strains and their tolerance towards a number of selected environmental conditions (ammonia, salinity, temperature…) could be investigated in order to select the best strain for local aquaculture operations.

– Larviculture of *M. rosenbergii* could be performed to find out the optimization of environmental conditions in larval rearing.

– ECs could be isolated from the gut of freshwater prawns and their efficiency could be compared with the ones originating from seabass.

– Identification of the form and quantity of PHB inside the larval gut needs to be studied in the future. The exact mode of action of PHB and its break down products inside the larval gut should be elucidated.

– Further research is needed to explore the use of short chain fatty acid (SCFA) in order by investigating methods to deliver the SCFA into the larval gut. This approach could open new strategies for pathogen control in aquaculture.

CHAPTER 7

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SUMMARY

The giant freshwater prawn is commercially one of the most important crustaceans, being widely fished and reared in ponds and rice fields throughout its natural distribution and beyond. Freshwater prawn farming can be conducted by unskilled rural people on small establishments and prawns are consumed domestically by all social classes. It is an activity that can play a role in poverty reduction and the empowerment of women. It has aided in increasing job opportunities for women and increasing their contribution to household income in developing countries. However, a major constraint in the large scale aquaculture of this species is the adequate supply of seed at required quantities and in all locations where **t**he majority of seed used in grow out farming comes from hatcheries. Existing hatcheries are not producing up to their installed capacity due to various constraints. Availability of healthy and high–quality seedlings has always been a major obstacle in the expansion of *M. rosenbergii* culture. The low yield because of mass mortality of hatchery–reared seed may be affected by several factors such as broodstock management, larviculture zootechniques, microbial control, … Therefore, the current study investigated several factors which affect larval performance in terms of survival and larval quality in order to improve larviculture production and optimize the efficiency of larval rearing.

A first study investigated performance of *M. rosenbergii* broodstock from different sources. The broodstock origin had significant effects on the offspring quality. Larval quality of Chinese and Vietnamese pond–reared breeders was markedly better than that of Vietnamese wild and Hawaiian pond–reared animals in terms of larval development rate, survival and ammonia tolerance.

In prawn larval rearing techniques, the best initial larval stocking density was 100–150 l^{-1} when using a constant water volume, while this could be increased up to 400 l^{-1} in case a rearing method with increasing water volume is used. A feeding frequency of 6 times per day by first–stage *Artemia* nauplii proved better than feeding only twice daily. Wet or dry artificial diets can replace 25% of the *Artemia* nauplii ration from larval stage V onwards. Subsequently, this could be increased up to 50% from stage IX, without any negative effects on growth, survival and quality of the prawn larvae compared to a live food control. The use of enrichment cultures (ECs) of N–acyl homoserine lactone (AHL)–degrading microbial communities proved a promising tool to control potential pathogens in prawn larviculture. The addition of ECs into the larval rearing water and via enriched *Artemia* nauplii used for larval feeding protected the larvae from *Vibrio harveyi* and/or opportunitistic bacteria and had a positive effect on larval survival and larval quality. Feeding larvae of the giant freshwater prawn with β–hydroxybutyrate (PHB)–containing *Artemia* nauplii significantly increased survival and growth of the larvae. It proved possible to combine delivery of PHB with bio-encapsulation of *Artemia* nauplii with a lipid emulsion, which is a commonly used technique in the larval rearing of many fish and crustacean species.

In conclusion, several factors need to be addressed in order to optimize the larval rearing techniques of *M. rosenbergii* and to improve larval survival and larval quality These include amongst others broodstock source, larval rearing zootechniques and microbial control. However, the optimization of the hatchery process should also be based on the specific conditions of the location and therefore it is not possible to draft standard protocols for all locations. The results of the present study may be applied in freshwater prawn seed production, however many remaining challenges still require further research.

SAMENVATTING

De reuze zoetwatergarnaal is commercieel één van de meest belangrijke crustacea soorten die intensief bevist en gekweekt wordt in vijvers en rijstvelden doorheen zijn natuurlijke verspreidingsgebied en daarbuiten. De teelt van zoetwatergarnalen kan gedaan worden door de ongeschoolde plattelandsbevolking in kleine faciliteiten en zoetwatergarnalen worden lokaal geconsumeerd door alle lagen van de bevolking. Het is een activiteit die een rol kan spelen in armoedebestrijding en het verbeteren van de rol van de vrouw. Het heeft een positief effect gehad in het creëren van jobs voor vrouwen en heeft hun contributie in het huishoudbudget in ontwikkelingslanden doen toenemen. Een van de belangrijkste problemen in de grootschalige aquakultuur van de soort is echter de voorziening van voldoende pootgoed in alle plaatsen waar het merendeel van het pootgoed voor de vetmesterij van broedhuizen komt. Bestaande broedhuizen produceren niet volgens hun maximale capaciteit wegens verscheidene problemen. Beschikbaarheid van gezond en kwaliteitsvol pootgoed is altijd een belangrijk obstakel geweest in de expansie van de kweek van *M. rosenbergii*. De lage opbrengst wegens hoge mortaliteiten van pootgoed geproduceerd in broedhuizen kan te wijten zijn aan verschillende factoren zoals de kweektechnieken voor de ouderdieren, zootechnische factoren tijdens de larvale kweek, microbiologie, … De huidige studie onderzocht daarom verscheidene factoren die een effect hebben op de overleving, ontwikkeling en kwaliteit van de larven met het doel de productie van larven te verbeteren en de efficiëntie ervan te verbeteren.

Een eerste studie onderzocht de performantie van *M. rosenbergii* ouderdieren van verschillende oorsprong. De oorsprong van de ouderdieren had een significant effect op de kwaliteit van de nakomelingen. De larvale kwaliteit van Chinese en Vietnamese

ouderdieren die in vijvers gekweekt werden was duidelijk beter in vergelijking met wilde Vietnamese of gekweekte dieren uit Hawaii voor wat betreft hun ontwikkelingssnelheid, overleving en tolerantie tegen ammonium.

Wat betreft de larvale kweektechnieken, was de beste larvale stockeringsdensiteit 100–150 larven l^{-1} wanneer een constant kweekvolume gebruikt wordt. Dit kan echter stijgen tot 400 l^{-1} in het geval een kweekmethode met toenemend kweekvolume gebruikt wordt. Een voederfrekwentie van 6 keer per dag met pas-ontloken *Artemia* nauplii bleek beter dan slechts 2 maal per dag te voederen. Natte of droge artificiële voeders kunnen 25% van het *Artemia* nauplii rantsoen vervangen vanaf het larvaal stadium V. Vervolgens kan dit percentage stijgen tot 50% vanaf stadium IX, zonder enig negatief effect op groei, overleving en kwaliteit in vergelijking met een controle met levend voedsel. Het gebruik van aanrijkingskulturen (ECs) van N-acyl homoserine lactone (AHL)-degraderende microbiële gemeenschappen bleek een veelbelovende techniek voor de controle van potentiële pathogenen in de larvikultuur van zoetwatergarnalen. Het toedienen van ECs via het kweekwater en via aanrijking van Artemia gebruikt als voedsel voor de larven beschermde de larven tegen Vibrio harveyi en/of opportunistische bacteriën en hadden een positief effect op overleving en kwaliteit van de larven. Het voederen van de larven van de reuze zoetwatergarnaal met Artemia nauplii die β-hydroxybutyraat (PHB) bevatten, verhoogde significant de overleving en groei van de larven. Het bleek mogelijk de aanlevering van PHB te combineren met bio-encapsulatie van Artemia met een lipide-emulsie, hetgeen een veelgebruikte techniek is in de larvale kweek van vele vis- en crustacea-soorten.

Als conclusie kan gesteld worden dat verscheidene factoren bekeken dienen te worden voor het optimaliseren van de larvale kweektechnieken van *M. rosenbergii* en de

overleving en kwaliteit van de larven te verbeteren. Deze omvatten onder andere de oorsprong van de ouderdieren, zootechnische aspecten van de larvale kweek en technieken voor de controle van de microbiële omgeving. De optimalisatie van het kweekproces hangt echter ook af van de lokale omstandigheden en hierdoor is het niet mogelijk om algemeen geldende protocollen op te stellen. De resultaten van deze studie kunnen toegepast worden in de productie van pootgoed van de zoetwatergarnaal; er blijven echter nog vele uitdagingen die verder onderzoek vereisen.

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- Participant in research project on surveying of wild fish in Tay Ninh and Long An Provinces (1997–1998)
- Leader of study on subject of culturing live food of *Tubifex* and *Moina* (1998)
- Associate lecturer and preparing lecture notes on subjects of aquaculture engineering and general aquaculture aspects (1998–1999)
- Supervisor of 4 undergraduate students for their graduation thesis
- Award of scholarship by Danish International Development Agency (DANIDA) for M.Sc. (Aquaculture) study in Asian Institute of Technology (AIT), Thailand.

2002–present: Lecturer in Faculty of Fisheries, Nong Lam University

- Lecturer on the subject of "aquaculture engineering" for undergraduate students (Bachelor).

- Lecturer on the second subject of "general aquaculture aspects" for undergraduate students (Bachelor).
- Promoter or co–promoter of over 20 undergraduate students for their graduation thesis on many subjects related to aquatic seed production, larviculture, fish disease, water quality, …
- Leader of research on "Red Tilapia cage culture in the reservoir: the effects of cage culture to reservoir environment". This research is part of a Collaborative Research Support Project (CRSP-2003).
- Consultant for research project on planning for aquaculture development in Thac Mo reservoir (2004)
- Consultant on aquaculture and fish and shrimp health management for some companies, hatcheries and fish farming. Coordinator of many farmer workshops in aquaculture.
- Participant in the project of the "Australian Centre for International Agriculture Research" (ACIAR) for studying on management and improving of reservoir fishery in Vietnam (2003–2005).
- Participant in training course of "Participatory approaches and facilitation skills" (at Ca Mau, 11–15 Sept, 2002).
- Participant in training course "Fish nutrition and fish feeding" (at Szarvas, Hungary, 07–20 September, 2004).
- Participant in training courses "Biological treatment of intensive fish farm effluents" (at Szarvas, Hungary 21–29 September, 2004).
- Awards of Asian Institute of Technology as "General Assembly Member of AIT Student Community". Bangkok, Thailand, 2001.

- Doctoral research with Prof. Dr. Patrick Sorgeloos (Laboratory of Aquaculture and *Artemia* Reference Center, Ghent University) and Prof. Dr. Le Thanh Hung (Faculty of Fisheries, Nong Lam University) as scientific promoters. Research was supported by a BTC scholarship (2005–2009).

Publications

- Nhan, D.T., 1999. Investigating on some Biological Characteristics and Trial Culturing of *Tubifex tubifex*. Journal of Agriculture and Forestry. No 11/1999, p55–58, (in Vietnamese).
- Nhan, D.T., 1999. Status and potential of fish production of Thac Mo reservoir 2003–2004. Journal of Agriculture and Forestry. No 1/2004, p30–38, (in Vietnamese).
- Nhan, D.T., Yakupitiyake, A., 2003. Status of Fisheries Production in Tri An Reservoir, Vietnam. AARM Newsletter. Asian Institute of Technology (AIT). Bangkok, Thailand. Vol. 8, No.1.
- Hung, L.T., Nhan, D.T., Yi, Y., Diana, J.S., Lin, C.K., 2004. Mitigating environmental impact of cage culture through integrated cage-cum-cove culture system in Tri An reservoir of Vietnam. Aquaculture CRSP 22nd annual technical report.
- Nhan D.T., Wille, M., Hung, L.T., Sorgeloos, P., 2007. Comparison of reproductive performance and offspring quality of *Macrobrachium* rosenbergii broodstock from different sources. Proceeding of The Asian–Pacific Aquaculture. Hanoi, Vietnam.
- Cam, D.T.V, Nhan, D.T., Ceuppens, S., Hao, N.V., Dierckens, K., Wille, M., Sorgeloos, P., Bossier, P. Effect of N–acyl homoserine lactone–

degrading enrichment cultures on *Macrobrachium* rosenbergii larviculture. Aquaculture 294, 5–13.

- Nhan, D.T., Wille, M., Hung, L.T., Sorgeloos, P., 2009. Comparison of reproductive performance and offspring quality of giant freshwater prawn (*Macrobrachium rosenbergii*) broodstock from different regions. Aquaculture (in press)

Participation in National and International Conferences

- Workshop on aquaculture research 2005, Research Institute for Aquaculture No. 1, Bac Ninh, Hanoi, Vietnam.
- Asian Aqua–Feeds 2005 conference, Kuala Lumpur, Malaysia 12-13 April 2005.
- Larvi'05–Fish and Shellfish Larviculture Symposium. Ghent University, Belgium, September 5–8, 2005.
- The Asian–Pacific Aquaculture Conference. Hanoi, Vietnam, August 5-8, 2007.
- Larvi'09–Fish and Shellfish Larviculture Symposium. Ghent University, Belgium, September 7–10, 2009.