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Improved techniques for rearing mud crab *Scylla paramamosain* (Estampador 1949) larvae

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Abstract

A series of rearing trials in small 1 L cones and large tanks of 30–100 L were carried out to develop optimal rearing techniques for mud crab (*Scylla paramamosain*) larvae. Using water exchange (discontinuous partial water renewal or continuous treatment through biofiltration) and micro-algae (*Chlorella* or *Chaetoceros*) supplementation (daily supplementation at 0.1–0.2 million cells mL^{-1} or maintenance at 1–2 millions cells mL^{-1}), six different types of rearing systems were tried. The combination of a green-water batch system for early stages and a recirculating system with micro-algae supplementation for later stages resulted in the best overall performance of the crab larvae. No clear effects of crab stocking density (50–200 larvae L^{-1}) and rotifer (30–60 rotifers mL^{-1}) and *Artemia* density (10–20 L^{-1}) were observed. A stocking density of 100–150 zoea 1 (Z1) L^{-1}, combined with rotifer of 30–45 mL^{-1} for early stages and *Artemia* feeding at 10–15 nauplii mL^{-1} for Z3–Z5 seemed to produce the best performance of *S. paramamosain* larvae. Optimal rations for crab larvae should, however, be adjusted depending on the species, larval stage, larval status, prey size, rearing system and techniques. A practical feeding schedule could be to increase live food density from 30 to 45 rotifers mL^{-1} from Z1 to Z2 and increase the number of *Artemia* nauplii mL^{-1} from 10 to 15 from Z3 to Z5. Bacterial disease remains one of the key factors underlying the high mortality in the zoea stages. Further research to develop safe prophylactic treatments is therefore warranted. Combined with proper live food enrichment techniques, application of these findings has sustained a survival rate from Z1 to crab 1–2 stages in large rearing tanks of 10–15% (maximum 30%).

Keywords: *Scylla paramamosain*, rearing techniques, water exchange, micro-algae, larval density, life food density, prophylaxis

Introduction

Aquaculture of mud crabs, *Scylla* spp., contributes a large proportion to the world production of the genus (FAO 1999). Moreover, mud crabs represent a valuable component of small-scaled coastal fisheries in many countries in tropical and subtropical Asia, for which there has been a general trend of increased exploitation in recent years (Angell 1992; Keenan 1999a). In Vietnam, the mud crab *Scylla paramamosain* is the second most important marine species next to shrimp, being cultured widely in the coastal area. However, mud crab farming currently relies entirely on the wild for seed stock and the main obstacle for expansion is the unavailability of hatchery-reared seed (Liong 1992; Rattanachote & Dangwatanakul 1992; Keenan 1999a; Shelley & Field 1999; Mann, Asakawa, Pizzuto, Keenan & Brock 2001; Xuan 2001).

Rearing techniques, disease and nutrition are the three main areas of research that have supported commercial production of marine fish and crustacean larvae (Sorgeloos & Léger 1992). These three aspects are to a large extent interconnected and developing hatchery techniques for a 'new' species is not possible unless all the areas are addressed. The

design of rearing systems covers more than purely technical aspects. Sub-optimal rearing conditions (e.g. physical stress, lack of oxygen or sub-optimal water quality) affect larval health and can cause mass mortality due to disease outbreaks. Similarly, system design influences (live) food quality and its availability to the predator larvae.

There has been a great deal of progress in marine larval rearing technology since its beginning in the 1960s (Shelbourne 1964; Howell, Day, Ellis & Baynes 1998). Many of these technical improvements developed over the past decades could be applied for mud crab with some modifications. An overview of the rearing systems currently applied for larviculture of mud crabs was presented by Davis (2003). Although knowledge has been obtained from these systems, there is a need to further optimize rearing techniques in order to maximize larval survival and quality. Furthermore, techniques should be adapted for each *Scylla* species (Keenan, Davie & Mann 1998; Keenan 1999b) in relation to local conditions (seawater source, status of hatchery management, local resources). The aim of this research is to adapt the existing rearing systems for larviculture to mud crab species (*S. paramamosain* being the test case) and to improve other techniques in order to maximize larval survival and quality.

Materials and methods

Source of larvae

Gravid crabs were bought from local markets and transported to the hatchery. Before stocking in the hatchery, the crabs were subjected to a bath of 100 μL L^{-1} of a 40% formalin solution for 1 h. The crabs were stocked individually in 100 L compartments of a roofed 2 × 2 × 0.5 m cement tank, equipped with a biofilter. Rearing water of 30 ± 1 g L^{-1} salinity was diluted from brine (90–110 g L^{-1}) with tap water and chlorinated before use. Ambient temperature fluctuated slightly around 28 °C. Every crab was fed a daily ration of 10–15 g of fresh marine squid, bivalve or shrimp meat alternately.

After 3–5 days of acclimation, unilateral eyestalk ablation was applied to induce spawning. After spawning, berried crabs were again subjected to a 100 μL L^{-1} formalin bath for 1 h and transferred to a 70 L plastic tank connected to a biofilter for egg incubation. Daily management consisted of siphoning out waste materials and shedded eggs from the tank bottom and controlling the temperature (30 °C), salinity (30 g L^{-1}) and ammonia and nitrite levels. Every other day, the crabs were placed in a 50 μL L^{-1} formalin bath for 1 h to reduce or prevent infestation of the eggs with fungi and bacteria. During egg incubation, the crabs were not fed.

One to two days before hatching, the berried female was moved to a 500 L fibreglass tank. When the hatching process was completed, larvae were selected based on their phototactic behaviour. Aeration in the hatching tank was turned off for several minutes and the active larvae swimming up to the surface were collected by gentle scooping.

The larvae were then transferred to the rearing containers. Acclimation was performed by placing the larvae in a 50 L plastic mesh bucket and slowly rinsing them with water from the larval rearing containers for 20–30 min before release.

Food and feeding

Micro-algae culture

Starting cultures of the micro-algae *Chaetoceros calcitrans* and *Chlorella vulgaris* were maintained indoors with Walne solution in seawater of 30 g L^{-1} at 25 °C. Large-scale production was performed indoors in 500 L tanks under a transparent roof. A haemocytometer was used to count micro-algal densities.

Rotifer culture and enrichment

The same *Brachionus plicatilis* L-strain with a lorica length and width of 164 ± 22 and 120 ± 22 μm, respectively, was used in all the experiments. Rotifers were cultured indoors in 100 L fibreglass tanks operated in a batch mode, following the procedure described in Sorgeloos and Lavens (1996). Rotifers were initially grown on baker's yeast, but later on fed Culture Selco® (INVE Aquaculture, Dendermonde Belgium) before feeding to the larvae. Temperature and salinity were controlled at 25 °C and 25 g L^{-1} respectively. They were harvested through a 60 μm screen and rinsed thoroughly.

Rotifers were enriched with micro-algae or artificial enrichment media before being fed to the crab larvae. Enrichment with *Chlorella* was performed at a density of 5 × 10^6 cells mL^{-1} for 3 h (Dhert 1996). Rotifers were also enriched with Dry Immune Selco® (DIS®, INVE Aquaculture), using two separate doses of 0.05 g L^{-1} at a 3-h interval. Enrichment was performed at a density of 500 rotifers mL^{-1}. The water in the enrichment vessel was slowly heated to

Table 1 Overview of larval rearing systems applied in this study based on the method of water exchange and micro-algae supplementation

	Water exchange	
Algae supplementation	**Discontinuous manual partial water renewal**	**Continuous water treatment through the use of a biofilter**
No micro-algae supplemented (indoors)	Clear-Batch system	Clear-Recirc system
Micro-algae supplemented at low levels to provide extra food for live preys (indoors or outdoors)	Algae-Batch system	Algae-Recirc system
Micro-algae supplemented at a high concentration and self-sustainable under natural sunlight as an extra food for live prey and water conditioning (outdoors)	Green-Batch system	Green-Recirc system (Combination of Green-Batch and Algae-Recirc system at early and late larval stages respectively)

29–30 °C to avoid exposing the rotifers to thermal shock when they were added to the larval rearing tanks. Before being fed to the larvae, enriched rotifers were rinsed and re-suspended in clean seawater at the same temperature as the crab-rearing tanks.

Artemia culture and enrichment

Artemia nauplii (Vinh Chau strain) were hatched as described by Van Stappen (1996). Both newly hatched or enriched *Artemia* nauplii were used in the experiments of this study. *Artemia* were enriched with *Chaetoceros* in the same micro-algal density as for rotifer enrichment. The nauplii were also enriched with DIS® (using two separate doses of 0.3 g mL^{-1} at a 6-h interval). Water temperature and salinity were maintained at 30 °C and 30 g L^{-1}, respectively, during *Artemia* enrichment. The density of *Artemia* during enrichment was 200 mL^{-1}. Before feeding to the crab larvae, the *Artemia* were rinsed with disinfected seawater and suspended at a known density in seawater.

Feeding

Rotifers were fed to the crab larvae from 0 to 6 days after hatch (DAH 0–6) [roughly corresponding to zoea 1 (Z1)–Z2 stages]. Newly hatched *Artemia* or *Artemia* meta-nauplii were offered from DAH 6 (Z3 stage) onwards. Rotifers and *Artemia* were added daily at 30–45 and 5–10 mL^{-1} to the rearing tank respectively (experiments 1, 2, 3, 4, 7 and 8). For experiments 5 and 6, live feed were fed at the required prey densities based on the planned treatments. Whenever the crab larvae were fed enriched live feed, algae- or DIS®-enriched live feed were used on alternate days.

Larval rearing experiments: objectives and experimental design

In experiments 1, 2 and 3, the effect of different water exchange schemes and the addition of micro-algae on larval survival and development were evaluated. In experiments 4–8, other culture aspects such as Z1 stocking density, live food density and the effect of different prophylactic treatments were investigated. The water quality management schemes tested in experiments 1–3 are summarized in Table 1. An overview of the experimental design and culture conditions of all the experiments is presented in Table 2. The small-scale experiments (1–30 L) were carried out in a temperature-controlled room (28–30 °C). The experiments in 100 L tanks were performed outdoors at ambient temperature (27–31 °C). The source and the disinfection procedure of the seawater for larval rearing were similar to those used for broodstock rearing. Formalin at a concentration of 20 µL L^{-1} was applied every other day as a prophylactic treatment in experiments 1–6.

Experiment 1

Larval survival and growth in a clear water system with daily partial water exchange (Clear-Batch) was compared with those in a clear water recirculating system (Clear-Recirc). In the first rearing system, 30–50% of the water was replaced daily. In the recirculating system, all rearing tanks were connected to a central biofilter. Water was recirculated at a rate of approximately 100% of the tank volume every 3–4 h. Live food and crab larvae were retained in the rearing tanks with by a mesh screen of 70 and 300 µm during the rotifer and *Artemia* feeding stage respectively. Larger mesh screens (250 and 500–1000 µm for rotifer and *Artemia* stage respectively) and higher flow

Table 2 Overview of the experimental conditions and water quality parameters (mean ± standard deviation) in experiments 1–8

Experiments	Factor	Rearing system	Container volume* (L)	No. of replicates	Stocking density (Z1 L^{-1})	Ammonium (mg L^{-1})†	Nitrite (mg L^{-1})†
1	Rearing system	Clear-Batch	30	5	50	0.35 ± 0.14^{a}	0.14 ± 0.11^{a}
		Clear-Recirc				0.03 ± 0.07^{b}	0.11 ± 0.09^{a}
2	Rearing system	Clear-Recirc	100	8	100	0.02 ± 0.04^{b}	0.04 ± 0.01^{b}
		Algae-Recirc				0.07 ± 0.08^{b}	0.10 ± 0.08^{b}
		Green-Batch				1.72 ± 1.30^{a}	0.57 ± 0.33^{a}
3	Rearing system	Green-Batch	100	4	100	1.54 ± 1.50^{a}	0.43 ± 0.13^{a}
		Green-Recirc				0.11 ± 0.07^{b}	0.15 ± 0.10^{b}
4	Z1 density	Green-Batch	100	3	50	1.50 ± 1.09^{a}	0.53 ± 0.39^{a}
					100	1.07 ± 0.62^{a}	0.43 ± 0.35^{a}
					150	0.98 ± 0.68^{a}	0.43 ± 0.29^{a}
					200	0.73 ± 0.48^{a}	0.34 ± 0.26^{a}
5	Rotifer density (30, 45 and 50 rotifers mL^{-1})	Clear-Recir	30	5	50	0.06 ± 0.05	0.05 ± 0.06
6	*Artemia* density (10, 15 and 20 *Artemia* mL^{-1})	Clear-Recir	30	5	100	0.04 ± 0.05	0.13 ± 0.09
7	Prophylactic treatment (control, formalin and oxytetracycline)	Clear-Batch	1	4	100	ND	ND
8	Duration of direct ozonation (control: without ozonation and ozonation for 2, 4, 6, 8 and 10 min)	Clear-Batch	1	3	100	ND	ND

*Cylindro-conical fibreglass tanks of 30–100 L and plastic cones of 1 L.

†Values within an experiment in the same column followed by the same superscript letter are not statistically different ($P \geq 0.05$).

For a description of rearing systems, refer to Table 1.

ND, not determined.

rates were used upon daily flushing out of uneaten live food and waste.

Experiment 2

A Clear-Recirc system was compared with two systems where micro-algae were added. Rearing conditions for the Clear-Recirc system were similar to those described in experiment 1. In the Algae-Recirc system, micro-algae were added daily to the recirculating system at a low concentration ranging from 0.1 to 0.2 million cells mL^{-1}. The operation of the rearing tanks was similar to the Clear-Recirc treatment. In the Green-Batch treatment, a classical 'green-water' system, micro-algae concentrations in the culture tanks were kept at a tenfold higher level of 1–2 million cells mL^{-1}. In this system, the culture tanks were initially only filled to 50% of their capacity and gradually increased to 100% by the end of the Z2 stage by adding water and algae daily. Later on, 10–30% of the rearing water was replaced daily by clean seawater and/or algae, depending on the density of micro-algae remaining in the rearing tanks. Upon water exchange, uneaten live food was also flushed out through a mesh screen (mesh sizes as described in experiment 1). The same amount of live food (30–45 rotifers mL^{-1} and 5–10 *Artemia* nauplii mL^{-1}) was fed in all the treatments. In the systems using algae, *Chlorella* was used for Z1–Z3 stages (which is unsuitable as a food source for *Artemia*); from Z4 onwards, *Chlorella* was gradually replaced with *Chaetoceros*.

Experiment 3

In this experiment, a Green-Batch and a Green-Recirc system were set up in order to further evaluate the application of micro-algae on the performance of crab larvae. The first rearing system was a batch system with addition of high concentrations of algae as described in experiment 2. The second system consisted of a combination of the Green-Batch system for early crab stages (Z1–Z2) and a Algae-Recirc system for later stages (Z3 onwards).

Experiments 4–6

In these experiments, the effect of Z1 stocking density (50, 100, 150 and 200 L^{-1}, experiment 4), rotifer feeding densities at 30, 45 and 60 mL^{-1} for Z1–Z2 (experiment 5) and *Artemia* densities at 10, 15 and 20 mL^{-1} for Z3 onwards (experiment 6) was evaluated. These experiments were run in a Green-Batch (experiment 4) or a Clear-Recirc system (experiments 5 and 6) as described above.

Experiment 7

In experiment 7, the effect of prophylactic chemicals on the survival of the larvae was investigated. Three treatments, consisting of a control (no chemicals used), daily addition of formalin at 20 $\mu L\ L^{-1}$ and daily addition of oxytetracycline at 10 $mg\ L^{-1}$, were run in 1 L plastic cones. All cones were placed in a water bath in order to maintain the rearing temperature at 30 °C. Water was replaced almost completely daily. Upon water exchange, the survival was determined.

Experiment 8

To avoid the use of drugs as a prophylaxis, direct ozonation of the culture tanks was tested in aerated 1 L plastic cones. Ozone (O_3) was injected directly via an airstone into every larval rearing cone upon changing water and feed daily. Six treatments with three replicates were arranged consisting of a control (without O_3 application) and O_3 injection for 2, 4, 6, 8 and 10 min (equivalent to a residual O_3 level in the water of 0.06, 0.12, 0.15, 0.17 and 0.19 $mg\ L^{-1}$ as measured by a test kit upon finishing the injection). Other rearing conditions and daily management were similar to those described for experiment 7.

Evaluation criteria

The survival rates in the experiments using large (30–100 L) containers (experiments 1–6) were estimated by volumetric sampling. Depending on the tank volume and the density of the surviving larvae, triplicate 300–1000 mL samples were taken from each tank. Megalopae (M) (DAH 15–18) and first crabs (C1) (DAH 22) were counted individually. In experiments 7 and 8 (using small cones), the average survival rate was calculated by individually counting all surviving larvae in each replicate.

Larval development was monitored every 3 days but daily in experiments 7 and 8 by identifying the average zoeal instar stage of a sample of larvae and assigning it a value: Z1 = 1, Z2 = 2, etc. Megalopa stage was assigned a value of 6. To compare the larval development in each treatment, an average larval stage index (LSI) was calculated from the average LSI value of all replicate containers in the same treatment. For large containers (experiments 1–6), five or 10 larvae (in 30 and 100 L tanks respectively) were

Table 3 Experiment 1: survival rates and larval stage index (LSI) values of *Scylla paramamosain* larvae cultured in two different rearing systems

	Days after hatch					
Treatment	**3**	**6**	**9**	**12**	**15**	**18**
Survival rate (%)*						
Clear-Batch	85 ± 6^a	79 ± 9^a	70 ± 6^a	64 ± 7^a	42 ± 6^b	32 ± 5^b
Clear-Recirc	84 ± 4^a	78 ± 8^a	72 ± 5^a	70 ± 5^a	63 ± 9^a	47 ± 6^a
LSI*						
Clear-Batch	1.5 ± 0.2^a	2.7 ± 0.1^a	3.5 ± 0.4^a	4.0 ± 0.0^a	4.6 ± 0.2^a	ND
Clear-Recirc	1.5 ± 0.2^a	2.7 ± 0.1^a	3.6 ± 0.3^a	4.2 ± 0.3^a	4.8 ± 0.1^a	ND

*Survival rates or LSI values in the same column followed by the same superscript letter are not statistically different ($P \geq 0.05$).
For treatment descriptions, refer to Table 1.
ND, not determined.

sampled from each tank to calculate the average LSI. The sampled larvae were staged under a dissecting microscope. In experiment 8, using small containers, all larvae were staged visually upon counting daily survival.

In this research, six larval rearing systems were applied for the experiments. Each rearing system had its own features, i.e. water quality and 'ease of operation'. Therefore, these features in combination made up a treatment as a type of rearing system. They were not considered as variables.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare data. Homogeneity of variance was tested with the Levene statistic (P or α value was set at 0.05). If no significant differences were detected between the variances, the data were submitted to a one-way ANOVA. Tukey's honestly significant difference post hoc analysis was used to detect differences between means and to indicate areas of significant difference. If significant differences were detected between variances, data were transformed using the arcsine-square root (for percentage data, i.e. survival rate) or logarithmic transformations (for LSI value) (Sokal & Rohlf 1995). All analyses were performed using the statistical program STATISTICA 6.0.

Results

Experiment 1

Survival in the Clear-Recirc system at Z4–Z5 stages on DAH 15 and in the megalopa stage on DAH 18 was significantly higher than those in the Clear-Batch system (both at $P < 0.01$) (Table 3). Although slightly higher in the recirculating system, LSI was not significantly different between treatments. The better larval performance in the Clear-Recirc system was accompanied by significantly lower average ammonia levels ($P < 0.01$) and slightly lower nitrite levels (see Table 2).

Experiment 2

On DAH 9, larval survival in the Clear-Recirc system was significantly lower ($P < 0.05$) than in both treatments with micro-algae supplementation (Algae-Recirc and Green-Batch systems) (Table 4). On DAH 12, survival in the Clear-Recirc treatment was lower ($P < 0.05$) than in the Algae-Recirc system, whereas the Green-Batch system had intermediate results. The LSI values on DAH 15 show a similar trend, although not significantly different.

The average levels of ammonia and nitrite in the Clear-Recirc and Algae-Recirc systems were significantly lower ($P < 0.01$) than those in the Green-Batch system (see Table 2). In the Green-Batch system, peaks of ammonia and nitrite concentrations of 3 and 1 mg L^{-1}, respectively, were recorded at the end of the experiment.

Experiment 3

Table 5 presents the larval performance of the crab larvae cultured in two rearing systems. The survival rates and LSI values of both rearing systems were not significantly different. However, the survival rates on later days (from DAH 12–22) in the Green-Recirc system tended to be higher than those in the Green-Batch system. The biofilter had a positive impact on

Table 4 Experiment 2: survival rates and larval stage index (LSI) values of *Scylla paramamosain* larvae cultured in three different rearing systems

	Days after hatch				
Treatment	**3**	**6**	**9**	**12**	**15**
Survival rate (%)*					
Clear-Recirc	74 ± 12^{a}	63 ± 7^{a}	44 ± 6^{b}	26 ± 11^{b}	8 ± 7^{a}
Algae-Recirc	74 ± 12^{a}	63 ± 9^{a}	61 ± 7^{a}	43 ± 7^{a}	15 ± 8^{a}
Green-Batch	74 ± 11^{a}	67 ± 9^{a}	58 ± 9^{a}	35 ± 9^{ab}	13 ± 6^{a}
LSI*					
Clear-Recirc	1.9 ± 0.1^{a}	2.7 ± 0.2^{a}	3.9 ± 0.1^{a}	5.0 ± 0.1^{a}	5.1 ± 0.1^{b}
Algae-Recirc	2.0 ± 0.1^{a}	2.8 ± 0.3^{a}	4.0 ± 0.1^{a}	5.0 ± 0.1^{a}	5.6 ± 0.2^{a}
Green-Batch	2.0 ± 0.1^{a}	2.8 ± 0.2^{a}	4.0 ± 0.1^{a}	5.0 ± 0.1^{a}	5.1 ± 0.1^{ab}

*Survival rates or LSI values in the same column followed by the same superscript letter are not statistically different ($P \geq 0.05$). For treatment descriptions, refer to Table 1.

Table 5 Experiment 3: survival rates and larval stage index (LSI) values of *Scylla paramamosain* larvae cultured in two different rearing systems

	Days after hatch					
Treatment	**3**	**6**	**9**	**12**	**15**	**22**
Survival rate (%)*						
Green-Batch	94 ± 6^{a}	88 ± 9^{a}	80 ± 3^{a}	66 ± 15^{a}	44 ± 20^{a}	9 ± 1^{a}
Green-Recirc	94 ± 6^{a}	89 ± 8^{a}	80 ± 5^{a}	68 ± 11^{a}	56 ± 11^{a}	12 ± 3^{a}
LSI*						
Green-Batch	1.4 ± 0.3^{a}	2.7 ± 0.1^{a}	3.8 ± 0.4^{a}	5.0 ± 0.0^{a}	5.2 ± 0.2^{a}	ND
Green-Recirc	1.4 ± 0.2^{a}	2.6 ± 0.2^{a}	3.9 ± 0.3^{a}	5.0 ± 0.0^{a}	5.3 ± 0.1^{a}	ND

*Survival rates or LSI values in the same column followed by the same superscript letter are not statistically different ($P \geq 0.05$). For treatment descriptions, refer to Table 1.
ND, not determined.

water quality in the second part of the experiment, with significantly reduced ammonia ($P < 0.05$) and nitrite ($P < 0.01$) concentrations as a consequence (see Table 2).

Experiment 4

Table 6 shows the survival and development rate of crab larvae stocked at four different Z1 densities (50, 100, 150 and 200 L^{-1}). The survival rates were not significantly different among treatments. Only on DAH 6 was a negative correlation between LSI and larval density observed.

Experiment 5

Table 7 shows the survival rates and the LSI values of crab larvae fed three different rotifer densities in the Z1–Z2 stages. No significant differences were found for any of the parameters. It can, however, be noticed that LSI was always the lowest in treatment 30 rotifers mL^{-1} and the highest in treatment 60 rotifers mL^{-1}.

Experiment 6

Table 8 presents the survival and development rate of crab larvae fed *Artemia* nauplii at three densities (10, 15 and 20 mL^{-1}) from the Z3 stage onwards. No significant differences were observed between the treatments. There seemed, however, to be a weak trend towards higher survival and LSI with increasing *Artemia* density towards the end of the trial.

Experiment 7

Table 9 shows the survival and development rate to the megalopa stage (DAH 22) of larvae receiving different prophylactic treatments. From DAH 6 onwards, the survival rate of larvae in the treatment

Table 6 Experiment 4: survival rates and larval stage index (LSI) values of *Scylla paramamosain* larvae stocked at four different Z1 densities (Z1 L^{-1})

	Days after hatch					
Treatment	**3**	**6**	**9**	**12**	**15**	**22**
Survival rate (%)*						
50	79 ± 9^a	56 ± 19^a	42 ± 16^a	31 ± 17^a	28 ± 12^a	4 ± 6^a
100	80 ± 7^a	74 ± 6^a	71 ± 10^a	56 ± 11^a	45 ± 8^a	5 ± 4^a
150	79 ± 2^a	57 ± 12^a	45 ± 9^a	31 ± 12^a	28 ± 10^a	5 ± 1^a
200	85 ± 5^a	53 ± 17^a	42 ± 8^a	34 ± 3^a	30 ± 5^a	5 ± 1^a
LSI*						
50	1.7 ± 0.2^a	3.0 ± 0.1^a	3.9 ± 0.1^a	5.0 ± 0.1^a	ND	ND
100	1.8 ± 0.1^a	3.0 ± 0.1^{ab}	4.0 ± 0.0^a	5.0 ± 0.1^a	ND	ND
150	1.8 ± 0.2^a	3.0 ± 0.1^{ab}	3.9 ± 0.2^a	5.0 ± 0.1^a	ND	ND
200	1.8 ± 0.2^a	2.7 ± 0.1^b	3.7 ± 0.2^a	4.8 ± 0.1^a	ND	ND

*Survival rates or LSI values in the same column followed by the same superscript letter are not statistically different ($P \geq 0.05$).
ND, not determined.

Table 7 Experiment 5: survival rates and larval stage index (LSI) values of *Scylla paramamosain* larvae-fed three different rotifer densities (rotifers mL^{-1}) from day 0 to day 6 after hatch

	Days after hatch				
Treatment	**3**	**6**	**9**	**12**	**15**
Survival rate (%)*					
30	89 ± 7^a	53 ± 10^a	30 ± 8^a	13 ± 7^a	10 ± 7^a
45	87 ± 6^a	58 ± 9^a	35 ± 7^a	18 ± 9^a	14 ± 8^a
60	87 ± 5^a	55 ± 7^a	32 ± 6^a	16 ± 11^a	11 ± 10^a
LSI*					
30	1.8 ± 0.2^a	2.7 ± 0.1^a	3.6 ± 0.2^a	3.8 ± 0.2^a	4.0 ± 0.4^a
45	1.8 ± 0.2^a	2.8 ± 0.2^a	3.8 ± 0.1^a	3.9 ± 0.1^a	4.3 ± 0.5^a
60	1.8 ± 0.2^a	2.8 ± 0.2^a	3.8 ± 0.2^a	4.0 ± 0.1^a	4.4 ± 0.5^a

*Survival rates or LSI values in the same column followed by the same superscript letter are not statistically different ($P \geq 0.05$).

using antibiotics was significantly higher than those in the remaining treatments. The survival rates of the control and formalin treatments were similar on most days. From DAH 6 onwards, the LSI values of the formalin treatment were generally higher than for the other treatments (not always significant). On DAH 15 and 18, the antibiotic treatment resulted in lower LSI values ($P < 0.01$).

Experiment 8

Table 10 presents the survival at the Z2, Z3 and Z4 stages of larvae that were daily exposed to different levels of O_3. On DAH 3 (Z2 stage), survival in the control and the treatment with O_3 injection for 2 min (Ozon2) was higher (67–78%) compared with the other treatments (24–45%). On DAH 6 (Z3 stage), the survival of treatment Ozon2 became the highest (52%); however, this was not statistically different from the control (25%). On DAH9, there were no significant differences in survival in all treatments.

Discussion

Rearing system

Recirculation

Water recirculation through a biofilter in the Clear-Recirc system positively affected larval performance compared with manual partial water replacement in the Clear-Batch system (experiment 1). The advantages of recirculating systems in commercial fish and crustacean larval production have been proven before for other species. Research into recirculating

Table 8 Experiment 6: survival rates and larval stage index (LSI) values of *Scylla paramamosain* larvae-fed three different instar-1 *Artemia* densities (*Artemia* mL^{-1}) from 6 days after hatch

	Days after hatch		
Treatment	**9**	**12**	**15**
Survival rate (%)*			
10	26 ± 10^{a}	12 ± 5^{a}	8 ± 3^{a}
15	30 ± 6^{a}	13 ± 7^{a}	10 ± 6^{a}
20	32 ± 8^{a}	19 ± 9^{a}	18 ± 9^{a}
LSI*			
10	3.1 ± 0.2^{a}	3.7 ± 0.4^{a}	4.3 ± 0.5^{a}
15	3.1 ± 0.1^{a}	3.7 ± 0.2^{a}	4.3 ± 0.5^{a}
20	3.2 ± 0.1^{a}	3.8 ± 0.3^{a}	4.6 ± 0.3^{a}

*Survival rates or LSI values in the same column followed by the same superscript letter are not statistically different ($P \geq 0.05$).

Table 9 Experiment 7: survival rates and larval stage index (LSI) values of *Scylla paramamosain* larvae treated daily with prophylactic chemicals

	Days after hatch						
Treatment	**3**	**6**	**9**	**12**	**15**	**18**	**22**
Survival rates (%)*							
Control	85 ± 3^{a}	64 ± 7^{b}	48 ± 8^{b}	34 ± 13^{b}	28 ± 11^{b}	17 ± 7^{b}	9 ± 5^{b}
Formalin	84 ± 7^{a}	66 ± 8^{b}	47 ± 7^{b}	34 ± 12^{b}	26 ± 8^{b}	13 ± 10^{b}	11 ± 8^{ab}
Antibiotics	91 ± 4^{a}	80 ± 2^{a}	74 ± 4^{a}	66 ± 8^{a}	52 ± 6^{a}	34 ± 3^{a}	21 ± 5^{a}
LSI*							
Control	1.8 ± 0.1^{a}	2.6 ± 0.3^{a}	3.4 ± 0.2^{ab}	4.5 ± 0.3^{a}	5.1 ± 0.1^{a}	5.5 ± 0.1^{b}	5.8 ± 0.2^{a}
Formalin	1.8 ± 0.1^{a}	2.8 ± 0.1^{a}	3.7 ± 0.1^{a}	4.3 ± 0.3^{a}	5.1 ± 0.1^{a}	5.8 ± 0.1^{a}	6.0 ± 0.1^{a}
Antibiotics	1.9 ± 0.0^{a}	2.7 ± 0.2^{a}	3.3 ± 0.2^{b}	4.3 ± 0.1^{a}	4.9 ± 0.1^{b}	5.3 ± 0.2^{b}	5.6 ± 0.2^{a}

*Survival rates or LSI values in the same column followed by the same superscript letter are not statistically different ($P \geq 0.05$).

Table 10 Experiment 8: survival rates of *Scylla paramamosain* larvae treated daily by ozone for different durations of time (min)

Treatment	**DAH 3 (Z2)**	**DAH 6 (Z3)**	**DAH 9 (Z4)**
Control	78 ± 6^{a}	25 ± 9^{ab}	9 ± 6^{a}
Ozon2	67 ± 6^{ab}	52 ± 14^{a}	11 ± 10^{a}
Ozon4	40 ± 17^{abc}	33 ± 11^{ab}	5 ± 2^{a}
Ozon6	45 ± 25^{abc}	27 ± 15^{ab}	5 ± 5^{a}
Ozon8	31 ± 2^{bc}	19 ± 3^{b}	0 ± 0^{a}
Ozon10	24 ± 15^{c}	12 ± 9^{b}	0 ± 1^{a}

Survival rates in the same column followed by the same superscript letter are not statistically different ($P \geq 0.05$).
DAH, days after hatch; Z, zoea; Control, without ozonation; Ozon 2, 4, 6, 8 and 10, duration of ozone injection from 2 to 10 min, which is equivalent to 0.06, 0.12, 0.15, 0.17 and 0.19 mg L^{-1} of the residual ozone respectively.

systems has also been identified as a priority for shrimp culture (Lawrence & Lee 1997). In these systems, water exchange is minimized through the use of biological, chemical and/or mechanical filtration to maintain good water quality continuously. As they provide less stress and confer constant good water quality to the larvae, these systems are able to maintain a high biological carrying capacity in relatively little space (Quillere, Marie, Roux, Gosse & Morot-gaudry 1993; Twarowska, Westerman & Losordo 1997). For crab larviculture, recirculating systems also appear to warrant further investigation in order to decrease labour requirements and seawater consumption, providing a more stable culture medium

and thus reducing larval stress. If the system design is kept simple, recirculating systems could also be suitable for large-scale production.

Role of supplemented micro-algae

The addition of micro-algae to the recirculation systems resulted in both higher survival and faster development in this study. Micro-algae have been proven to be beneficial by various modes of action. They could help maintain the quality of live food. As in the culture of marine fish larvae, unconsumed rotifers may reside in the tanks for several days and their nutritional value may become severely reduced (Makridis & Olsen 1999). Furthermore, according to these authors, poorly fed rotifers were more sensitive to starvation than well-fed rotifers, as their nitrogen content decreased at a higher rate.

Micro-algae also play an important role in stabilizing water quality via either ammonia uptake or oxygen production (Tseng, Huang & Liao 1991). Because the Clear-Recirc system already provided optimal water quality, it is unlikely that the stabilizing effect on water quality is responsible for the improved performance in the algae-supplemented system. In batch culture systems, this effect would probably be much more pronounced. A direct comparison between a green and clear water batch system was, however, not made in this study.

In a study on the effect of *Chlorella* on the population of luminous bacteria *Vibrio harveyi*, no luminous bacteria were recovered on days 2 and 3 in flasks with *Chlorella*, while those without the micro-algae still harboured luminous bacteria at day 3 (Tendencia & dela Pena 2003). Also, the diatom *Chaetoceros* has been shown to produce natural antibiotics and high concentrations of this marine diatom will eliminate *Vibrio vulnificus* and other pathogenic bacteria, which contribute to the propagation of viruses in the shrimp production environment (Wang 2003).

In conclusion, micro-algae in mud crab larval rearing may play a role in improving and maintaining live food quality and controlling bacteria levels.

Choice of system

In experiment 3, the Green-Recirc system (which is a combination of a Green-Batch system during the rotifer feeding stage and a Algae-Recirc system thereafter) seemed to be better than the Green-Batch system. The Green-Batch system seems to be more appropriate for early stages of crab larvae (Z1–Z2) as it is less stressful for the early zoeae and easier to gradually fill up the tanks with fresh seawater, algae and rotifers than flushing out old rotifers in the recirculation system. In the recirculating system, the young larvae may be prone to physical damage and may spend considerable energy trying to swim up against the current. Early crab larvae are delicate due to their small size and the three long spines on the carapace that are easily damaged when they are entrapped on the mesh screen during flushing out of uneaten feed in the recirculation system (Davis 2003). The nutritional effect of micro-algae is probably also more pronounced during the rotifer feeding stage than during the *Artemia* feeding stage. Furthermore, it is not necessary to recirculate water during these first days, as the concentrations of ammonia and nitrite are still low. Using the Algae-Recirc system in later stages is more favourable for reducing the increasing ammonia and nitrite concentrations as more waste material is produced by the crab larvae. Moreover, as the larvae develop into more efficient predators, feed is consumed faster, and maintenance of optimal feed quality is less of an issue. Many studies successfully applied a similar combined rearing technique due to its benefit for the larvae and convenience for management, particularly for large rearing containers. Under green-water culture conditions, water is not exchanged for the first 3 days. Thereafter, water exchange is slowly increased from 10–20% day^{-1} for Z2–Z3 to between 40% and 50% day^{-1} at the end of the rearing cycle (Z4–M) (Mann, Asakawa & Pizzuto 1999; Quinitio, Parado-Estepa, Millamena, Rodriguez & Borlongan 2001). In Japan, a mesocosm system is used for culturing larvae in larger tanks (> 10 m^3). The tanks are partially filled with green water at Z1 (20–25% volume), tanks are then filled up with clean seawater during the course of the Z2–Z3 stages and during the Z4 and M stages water is exchanged on a flow-to-waste basis (Hamasaki, Suprayudi & Takeuchi 2002).

Other rearing techniques

Z1 stocking density

No significant effect of larval density was observed from 50 to 200 Z1 L^{-1}. This would suggest that the larvae can be grown at 200 Z1 L^{-1}. Variation in the final survival between replicate tanks also seemed to decrease at higher densities. For *S. paramamosain*, Djunaidah, Mardjono, Wille, Kontara and Sorgeloos (2001) found a tendency of increased survival to Z5 as a function of the Z1 stocking density (i.e. survival

rates of 27%, 39% and 63% being obtained at densities of 50, 75 and 100 Z1 L^{-1} respectively). Baylon and Failaman (1999) also reported higher survival and metamorphosis of *Scylla serrata* at 50 Z1 L^{-1} compared with lower densities of 10 and 25 Z1 L^{-1}. Increased survival at higher larval densities somehow seems contradictory. However, indirectly, food ration might be responsible. Excess food in treatments with low larval densities may pollute the water and may thus cause mortality. In our study, we noted higher concentrations of ammonia and nitrite in the treatment having 50 Z1 L^{-1} (see Table 2). For the highest stocking densities tested in our study (200 Z1 L^{-1}), the larval development rate seemed slightly impaired. This high stocking density may have caused competition for feed, resulting in slower development. Therefore, Z1 stocking densities in the range of 100–150 Z1 L^{-1} might be optimal.

Rotifer density for feeding early larval stages (Z1–Z2 stages)

Although there was a trend towards increased survival and growth with increasing rotifer density, no significant differences in larval survival or growth were found between the different rotifer densities tested. Although not significant, the highest survival was generally observed at 45 rotifers mL^{-1}; while a density of 60 rotifers mL^{-1} resulted in the fastest larval development. The differences were, however, not very marked, and moreover such high feeding rates might be economically unrealistic. We can therefore conclude that feeding 30 rotifers mL^{-1} is enough for optimal larval performance. In practice, however, the intermediate density of 45 rotifers mL^{-1} was frequently used for feeding early larval stages. Other studies indicated that high rotifer densities (30–80 mL^{-1}) are required for optimal growth and survival of *S. paramamosain* (Djunaidah, Mardjono, Lavens & Wille 1998; Zeng & Li 1999) and *S. serrata* (Suprayudi, Takeuchi, Hamasaki & Hirokawa 2002). For *S. paramamosain* larvae, feeding 30 and 60 rotifers mL^{-1} resulted in a significantly higher survival compared with feeding only 15 rotifers mL^{-1} (Djunaidah *et al.* 2001). These authors found that the individual dry weight of Z5-fed 15 rotifers mL^{-1} was significantly lower than those of Z5 fed with higher rotifer densities. Practically, feeding 30 rotifers mL^{-1} at Z1 and increasing gradually to 45 mL^{-1} at Z2 proved to be sufficient for a stocking density of 100 Z1 L^{-1} in our trials in larger rearing tanks (500–1000 L). Increasing the ration by larval stages in this way compensates for the increased ingestion of crab larvae as they grow (Baylon, Bravo & Manigo 2004). For early larvae, however, food amount cannot be reduced to their maximum ingestion potential as they are quite inefficient predators and therefore might require a minimal density to maximize encounter.

Similar to our study, most studies investigating the effect of rotifer density added the live food in one single ration. Under these circumstances, theoretic densities are only attained upon feeding and gradually decrease as larvae consume the prey. Optimal live food quantities cannot, however, be separated from feeding frequency. Because zoea larvae can consume their optimal ration within 1 h, Genodepa, Southgate and Zeng (2004) suggested that they can be fed once a day. Because of the severe reduction in the nutritional value of rotifers with longer retention times in rearing containers (Makridis & Olsen 1999) and the fact there is a minimum prey density needed for the passive feeding behaviour of zoea larvae (Heasman & Fielder 1983; Zeng & Li 1999), the interaction between the optimal ration and feeding frequency should be further investigated.

Artemia for feeding later larval stages (from Z3 onwards)

We found no difference between feeding Z3 a daily feed ration of 10, 15 or 20 *Artemia* nauplii mL^{-1}. Especially in later larval stages (Z4–Z5), there was, however, a tendency towards higher survival with increasing ration. In this respect, it might be beneficial to increase the *Artemia* density by crab stage from 10 to 15 mL^{-1}. High live feed densities would increase the chance for early larvae to encounter and capture feed organisms (Zeng & Li 1999) and therefore would improve the larval performance (Brick 1974; Heasman & Fielder 1983; Quinitio *et al.* 2001). On the other hand, older larval have a higher ingestion capacity. Optimal rations should therefore be determined for each larval stage separately. In this respect, studies on individual larvae are very useful to determine prey consumption. According to our previous experiments (Nghia 2004), each Z3, Z4, Z5 and megalopa larva was capable of consuming on average 15, 25, 37 and 114 newly hatched *Artemia* day^{-1} respectively. Therefore, at a stocking of 100 larvae L^{-1}, the daily *Artemia* feeding densities theoretically should be at least 1.5, 2.5, 3.7 and 11.4 mL^{-1} for Z3, Z4, Z5 and megalopa stages respectively. For Z1, Z2 and Z3 stages of *S. serrata*, the

number of *Artemia* nauplii ingested by the larvae at a lower food density of 2.5 mL^{-1} was comparable to that at 5 mL^{-1}, and for Z4–Z5, at 5 mL^{-1}, it was comparable to 10 mL^{-1} (Baylon *et al.* 2004). In that study, *Artemia* was, however, co-fed with rotifers at a density of 15–20 mL^{-1}. If *Artemia* was the only food, the optimal *Artemia* ration would therefore probably be higher than 2.5–5 mL^{-1}. In another study on *S. serrata*, a daily optimum food concentration of 10 *Artemia* nauplii mL^{-1} was established for zoea survival (Brick 1974). In the mass seed production of *S. serrata*, newly hatched *Artemia* are given starting late Z2 at 0.5–3 mL^{-1} and 5–7-day-old *Artemia* are routine fed from late Z5 to early megalopa (Quinitio & Parado-Estapa 2003). Older *Artemia* provided a larger-sized prey for zoeae to megalopae and hence, the density was reduced.

For megalopae of *S. paramamosain*, we found a threefold higher number of ingested newly hatched *Artemia* nauplii compared with Z5 (114 and 37 *Artemia* respectively) for a similar prey density (Nghia 2004). This means that megalopae are voracious predators, capable of chasing their prey actively and consume large amounts of feed in a short time. From this, it could be beneficial if megalopae are fed frequently smaller rations in order to optimize feed quality and reduce cannibalism. Genodepa *et al* (2004) similarly indicated that in contrast to earlier larval stages, which can be fed once per day, *S. serrata* megalopae may need to be fed more often to maximize ingestion. These authors found no significant differences in the ingestion rate of megalopae fed microbound diets at rations ranging from 12.5% to 100% of the standard ration (equivalent to 5 *Artemia* nauplii mL^{-1} in 1 h). Baylon *et al* (2004) also found a high increase in *Artemia* ingestion in the first few days of the planktonic phase of the megalopa stage. Later on, megalopae become more benthic as they prepare for the second metamorphosis to first crab. Swimming *Artemia* are no longer accessible, and minced shrimp or mussel meat are a more suitable feed.

In conclusion, a ration of 10 *Artemia* nauplii mL^{-1} appears to be sufficient for the optimal performance of Z3 larvae. An increase in prey density in the Z4–Z5 stage may, however, be beneficial. These food amounts appear to be higher than what most other studies recommend (2.5–10 *Artemia* nauplii mL^{-1}; Brick 1974; Baylon *et al.* 2004). Perhaps the recirculating system used in this study resulted in a greater loss of prey organisms (e.g. more *Artemia* were entrapped on the overflow screen) than in the small batch culture systems used in other experiments. The small nauplii size of the *Artemia* strain (Vinh Chau strain) used in our study could be another reason that led to increased ingestion. In practice, (larger sized) highly unsaturated fatty acid-enriched *Artemia* were normally used in order to reduce the prey amount to 5–10 mL^{-1}. Megalopa probably should be fed more frequently and, towards the end of that developmental stage, a non-moving food may be better.

Prophylactic chemicals

Laboratory cultures of crab larvae often suffer severe mortality from disease, particularly from epibiotic bacteria and larval mycosis (Armstrong, Buchanan & Caldwell 1976; Hamasaki & Hatai 1993a, b). A study on *S. serrata* indicated a significantly higher survival up to DAH 7 (over 90%) when using oxytetracycline, whereas almost complete mortality occurred in the control treatment (Mann 2001). The author considered that potentially up to 80% of the larval mortality could be attributed to bacteriological causes. The results of our study also indicated that bacteria are, more than any other factor tested, a main cause of larval mortality. Antibiotics more than doubled survival up to the crab stage.

However, antibiotics have not always been used in a responsible manner in aquaculture. A major consequence of using antibiotics has been the proliferation of resistant bacteria and the transmission of resistance to other bacterial species (Benson 1998). The development of antibiotic resistance by pathogenic bacteria is considered to be one of the most serious risks to human health at the global level (FAO 2002). Formalin is more acceptable than antibiotics as it shows no accumulation in animal tissues (Jung, Kim, Jeon & Lee 2001). Recently, however, Japan has strictly banned the use of formalin in aquaculture as it may cause cancer in humans, reduces oxygen levels in the water and causes algae to die off (VASEP 2003). Moreover, in our experiments, formalin did not significantly improve larval survival compared with the negative control. Pathogenic bacteria are considered to be one of the most serious causes for the high mortality of early crab larvae. It can be safely assumed that all inputs (seawater, broodstock, live feed and daily management in hatcheries) into the culture tank are potential sources of infection (Blackshaw 2001). Strict hygiene at all steps is always advised for hatchery activities. However, this advice is not always followed, especially in backyard hatcheries. Therefore, other techniques should be

investigated as alternatives for the use of chemicals. Ozonation and probiotics could be interesting in this respect (Davis 2003; Nghia 2004). Ozone is a powerful oxidant and is becoming more and more popular in various aquaculture systems for disinfection and improving water quality by oxidation of inorganic and/or organic compounds (Tango & Gagnon 2003). In our study, direct application did not significantly improve survival compared with a negative control. However, there was a tendency of a residual O_3 concentration of 0.06 mg L^{-1} to improve larval performance. Variability within this treatment was, however, very high, which could be indicative of the fact that dosing was not careful enough. Longer O_3 exposure times (4–10-min exposure, equivalent to 0.12–0.19 mg L^{-1} residual O_3) all decreased overall survival. These high O_3 concentrations probably caused physical damage to the crab larvae. In *Penaeus monodon* juveniles, 0.34–0.5 mg L^{-1} residual O_3 caused loss of balance, immobility and destruction of the gill lamellar epithelium (Meunpol, Lopinyosiri & Menasveta 2003). Ozone treatment should therefore be investigated further, with determination of proper doses for each larval stage. Ultimately, the microbial flora will need to be controlled and there is evidence that this can be achieved using recirculating systems in which O_3 treatment is combined with the inoculation of the biological filter with selected nitrifying and probiotic bacteria (Gatesoupe 1991; Rombaut, Suantika, Boon, Maertens, Dhert, Top, Sorgeloos & Verstraete 2001).

Conclusions and suggestions

The combination of a green-water batch system for early stages and a recirculating system with microalgae supplementation for later stages, a stocking density of 100–150 Zl L^{-1}, feeding density of 30–45 rotifers mL^{-1} for early stages and 10–15 *Artemia* nauplii mL^{-1} for later stages are recommended for larval rearing of *S. paramamosain*.

The optimal ration for crab larvae should, however, be adjusted depending on various factors, e.g. species, larval stages, larval status, prey size, rearing system and rearing techniques. A feeding regime with frequent addition of small quantities of feed is worth investigating.

Antibiotics improved larval survival, proving again that bacterial interference is one of the major causes of mortality. Formalin could not significantly improve survival compared with the control. Both products, moreover, are not encouraged for commercial mud crab larviculture as they are unsafe. Direct ozonation as an alternative to prophylactic chemicals is worth investigating.

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