

Effects of Essential Fatty Acids Containing Natural and Commercial Diets on Larvae Rearing of the Green Mud Crab *Scylla Paramamosain* (Estampador 1949)

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Abstract

Effect of essential fatty acids (EFA) on growth and survival of the green mud crab (*Scylla paramamosain*) larvae was assessed by feeding with natural to commercial diets. The feeding schemes were: larvae reared with *Artemia* (T1); larvae initially fed with rotifers (up to Z2) and ended (Z3 to megalopa) with *Artemia* (T2); and larvae fed with rotifers up to Z2 and ended (Z3 to megalopa) with commercial diet (T3). The commercial diet had significantly ($p < 0.05$) higher levels of docosahexaenoic acid (11.23%), $\sum n-3$'s (15.90%) and $\sum n-6$'s (4.21%); and lacked in eicosapentaenoic acid (2.25%) than rotifer and *Artemia*. The earliest commencement of megalopa stage within 15 days with significantly ($p < 0.05$) higher larval stage index (LSI) of 5.90 ± 0.17 was achieved from the feeding scheme of T2 than other two feeding schemes. This feeding scheme deposited $17.32 \pm 0.19\%$ eicosapentaenoic acid (EPA) and $3.82 \pm 0.11\%$ docosahexaenoic acid (DHA); the $\sum n-3$ to $\sum n-6$ ratio of 0.20 and EPA to DHA ratio of 0.22 in megalopa, that stimulated significantly higher ($p < 0.05$) megalopa survival ($20.00 \pm 6.96\%$) indicating the superiority over rest feeding schemes. Meanwhile, some deformities and mortalities in Z5 and megalopa stages suggested further studies for optimization of specific fatty acid requirements for late larval stages (Z5 and megalopa).

Keywords: Effects; Diets; EFA's; Larvae; Mud crab.

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1. Introduction

Mud crab aquaculture is an emergent sector in south-east Asian countries [1]. Since its starting to date, mud crab aquaculture is solely dependent on natural seeds [2,3]. Natural seeds are not sufficient enough to support the present status of the mud crab industry [4].

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Moreover, recent intervention of soft shell crab shedding has stimulated the scarcity of mud crab seeds [5]. In this circumstances, hatchery based seed production is the best alternate to support the present mud crab aquaculture and further expansion of this sector as well.

In the hatchery conditions, the survival rate of mud crab seeds from the zoea (Z1) to crablet stage is low and inconsistent [6] and ranging from 3.2 % to 10 % [7]. Mass mortality of larvae at the early zoeal stages (Z1 to Z2) is linked with improper nourishment from maternal source [6-8] and mud crab broodstock required high dietary requirement for lipids, fatty acids and protein for maturation and breeding [9]. The second mass mortality happened during metamorphosis in the late zoeal stages (Z5 to megalopa) is reportedly associated with nutritional inadequacy of the supplied diets [10-14]. Among the nutrients, long chained polyunsaturated omega-3 fatty acids (LC-PUFA) of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are evidently responsible for proper functioning of cardiovascular, renal, nervous, immune and reproductive systems as well as the regulatory nutrient for the formation of brain and structure [15,16]. In crustaceans, EPA and DHA play vital role for the cytokine regulation [17] and outer shell formation [18], and the main component of the body shell is chitin. However, supply of improper nutritional diet is resulting in moulting failure of crustaceans, known as Moulting Death Syndrome (MDS). Moulting failure in mud crab larvae is associated with lack of essential fatty acids (EFAs), especially the eicosapentaenoic acid (EPA, 20:5 ω 3) and docosahexaenoic acid (DHA, 22:6 ω 3). Marine crustaceans including mud crab larvae are unable to synthesize these EFAs *in situ* or incapable of bio-converting from the parent omega-3 molecule of ALA linolenic acid (α -18:3 ω 3). Therefore EFAs should be supplied through diet [18,19]. Boosting of EPA and DHA through enrichment of live prey provided better results in crab larvae rearing [20,21]. Some research on dietary EPA and DHA levels for mud crab larvae rearing has been done elsewhere [22]. Meanwhile, mud crab seed production in hatchery condition is a new attempt in Bangladesh and Malaysia. However, the aim of this experiment was to observe the implication of different natural and commercial diets containing various levels of essential fatty acids of EPA, DHA and their rations on growth, survival and metamorphosis of mud crab (*Scylla paramamosain*) larvae.

2. Materials and Methods

2.1. Experimental design and protocol

This experiment was conducted in three treatments based on three different diets (two species of live food and a commercial marine shrimp larval diet) used for different developmental stages of the crab larvae. In treatment-1 (T-1), larvae from Z1 to Z2 were fed with *Artemia* umbrella and from Z3 to megalopa with *Artemia* nauplii; in treatment-2 (T-2), larvae were reared on rotifers from Z1 to Z2 and *Artemia* nauplii from Z3 to megalopa; and in treatment-3 (T-3), larvae were fed rotifers from Z1 to Z2 and formulated

shrimp larvae rearing microbound diet (Artemac, Bio-marine, Hawthorne, California, USA; grain size 20 µm to 80 µm and 60 µm to 120 µm) from Z3 to megalopa. Each of the treatments was run in three replicates. The experiment was conducted in 1000 L round larvae rearing tanks with a water volume of 700 L of each. Larvae were stocked at a density of 80 larvae per liter of water.

The seawater (30 ppt) was pumped from the sea, passed through a gravel bed filter and stored in a fibre glass tank (50 m³ volume). Necessary water, hatchery utensils (glass wares, siphon pipe, bowls, buckets, filter bags, dip scoop net etc.) and hatching/incubation tanks were disinfected with 10–20 ppm of Clorox (5.25 % calcium hypochlorite) followed by dechlorination with sodium thiosulphate [22,23].

Table 1. Detailed experimental design for mud crab larvae rearing.

Treatment	Replication	Larval stages, feeding schemes and feed density					
		Z1	Z2	Z3	Z4	Z5	M
T1	3	Artemia umbrella; 0.5 to 1.5 ind./mL		Artemia nauplii; 1.5 to 10 ind./mL			
T2	3	Rotifer; 40 to 60 ind./mL		Artemia nauplii; 1.5 to 10 ind./mL			
T3	3	Rotifer; 40 to 60 ind./mL		Commercial marine shrimp diet; 0.5 to 1.0 g/ton			

2.2. Live feed and commercial diet

Commercial *Artemia franciscana* cysts (Premium Plus, Golden Dolphin, super *Artemia*, Sdn Bhd, Malaysia) were purchased from the supplier. Hydration, hatching and separation of umbrella type or nauplii of *Artemia* was performed [22,23]. *Artemia* cysts were hydrated and decapsulated in 20 ppt seawater with 10 ppm Clorox and vigorous aeration for 30 min. Decapsulated cysts were rinsed repeatedly with filtered seawater (30 ppt salinity) to remove chlorine, and were then placed in the incubation bucket at the rate of 1.0 g cysts/L of sea water with vigorous aeration for 24 h. After 18-24 h, the umbrella stage and nauplii were obtained, and were transferred into an inverted sharp edged conical jar covered with a plastic lid and left for 30 min. for settling. They were then separated from the shell, and were repeatedly rinsed with clean seawater before feeding them to the mud crab larvae.

Marine phytoplankton (*Nannochloropsis* sp.) was cultured in cylindrical hanging plastic bags filled with disinfected filtered seawater, enriched with 1 mL/L of Conway medium [24] and inoculating the stock solution at the rate of $0.1-0.5 \times 10^7$ cells/mL of culture volume [25,26]. A secondary dose of medium (0.5 mL/L) was added to the culture bags at 7 days of inoculation; the phytoplankton culture reached its peak ($9.4 - 10.5 \times 10^7$ cells/mL) in 15 – 20 days.

The rotifer (*Brachionus plicatilis*) culture was initiated in 300 L rectangular or 1000 L round tanks, filled with seawater (30 ppt salinity). A density of 20 rotifer/mL of culture volume was introduced and fed with baker's yeast at a rate of 0.25 - 0.50 g per million of

rotifers twice a day [27]. The rotifers had grown to a density of 400 ± 50 individual/mL in 5–7 days, and were harvested by siphoning and collected by passing through 50 μm meshed dip nylon net. The harvested rotifers were repeatedly rinsed in filtered seawater and fed with the phytoplankton (*Nannochloropsis* sp.).

Rotifers grown in baker's yeast medium were harvested and enriched before 24–48 h. of feeding with phytoplankton (*Nannochloropsis* sp.) at the density of $\pm 1.5 \times 10^6$ cells/mL per million of rotifer [28]. However, the umbrella type *Artemia* was not enriched, whereas, in the case of late stage feeding (Z3 to Z5) with *Artemia*, nauplii were enriched with commercial fish oil SELCO at the rate of 0.5 g/L of sea water before 4 to 6 hr of feeding time [28].

Commercial microbound diet formulated for rearing marine shrimp larvae (Artemac; Bio-marine, Inc.; Hawthorne, California, USA) was purchased from the supplier and kept in the refrigerator at -20°C for storage. The diet was labelled as follows: grain size 20–120 μm , carbohydrate 12.0 %, protein 57.0 %, lipid 19.0 %, ash 5.0 % and moisture 7.0 %. The feed was also analysed for fatty acid profiles.

2.3. Collection and management of crab broods (Base population, P_0)

Berried broods (P_0) collected from the wild and were purchased from traditional fishermen of Pulau Betong, Penang. The broods were brought to the CEMACS (Centre for Marine and Coastal Studies) hatchery by a covered plastic box filled with seawater (30 ppt salinity) and continuous aeration. Then morphological characteristics of the brood were examined to confirm the species [29]. They were acclimatized to the hatchery condition by placing in aerated seawater (30 ppt and 30°C) in plastic bucket for 1 hr. The broods were then disinfected in a plastic bucket containing 100 ppm formalin (37% formaldehyde) mixed seawater (30 ppt) for a period of 30 min. with aeration followed by repeated washing with filtered seawater (30 ppt) to remove any residual formalin. They were then transferred into the hatching tank for housing/incubation until the larvae were hatched. During incubation time, feeding were ceased, aeration was kept at a moderate flow. The tank bottom was daily cleaned by siphoning and 50–70 % of the water was exchanged everyday with treated seawater (30 ppt). Water temperature, salinity, dissolved oxygen and pH were monitored in regular basis before the water exchange following standard methods [30].

2.4. Preparation of larvae rearing tanks

All the required tanks were prepared by setting up aeration and installing separate light (1,000 Lux; TYH™, 100 W, AC 240 V, 50/60 HZ) for each tank. Tanks were covered with canvas sheet to minimize temperature fluctuation and to prevent external contamination. Tanks were washed cleaned and dried. Before 1 week of initiating larvae culture, tanks were filled up (three fourth) with filtered seawater (30 ppt) and treated with

10 ppm Clorox and left until stocking of larvae. Before stocking, residual chlorine was monitored and dechlorinated by treating with sodium thiosulphate.

2.5. Collection, handling and stocking of larvae

Immediately after full hatching of larvae from the egg mass, the phototaxis strong larvae were gently scooped up by a glass beaker and placed into an aerated plastic bucket filled with sea water (30 ppt). Three sub-samples (100 mL each) of larvae suspension were taken, counted separately and an average was made. Larvae were then stocked in the rearing tank according to the desired density (80 larvae per litre of culture water). Required larvae suspension for desired density was calculated according to the formula in below:

$$\text{Volume of larvae suspension needed to stock (litre)} = \frac{D \times T}{Z} \quad (1)$$

Where, D= desired stocking density/L (80/L); T= volume of each culture tank in litre (1000 L); Z=density of larvae (larvae/L) in the stock bucket.

2.6. Feeding, management and schedules

For the Z1 to Z2 stages, the larvae were fed with rotifer (*Brachionus plicatilis*) at a density of 40 - 60 individual/mL in T2 and T3. The umbrella type *Artemia* was served as feed at a density of 0.5-1.5 ind/mL in T1 for the same larval stage. For the late zoal stages (Z3 to Z5) feeding, *Artemia* nauplii were provided @1.5 to 10 ind/mL of culture water in T1 and T2. Commercial diet was supplied @ 0.5-1.0 g/ton of culture volume for the same stage feeding in T3. Microalgae (*Nannochloropsis* sp) was added in all the larvae culture tanks at the density of 0.5×10^6 cells/mL for the first 3 days of culture. Feeding for each treatment was done thrice in a day at 8 hr intervals (i.e. at 0800 hr, 1600 hr and 2400 hr).

2.7. Water management and monitoring of water quality variables

For the first 2 days, the water in the culture tanks was not changed except topping up (100 L and 150 L for 1st and 2nd day of culture, respectively) to the maximum mark of culture volume (1000 L). Later on, the water was changed from 30 % to 50 % daily with 30 ppt saline water up to the Z5 stage. Water salinity was reduced to 27 ppt from Z5 to megalopa stage by gradual decreasing of 0.5 ppt in each water change. Water quality variables, like temperature (°C), salinity (ppt), pH, dissolved oxygen (mg/L), ammonia (mg/L) and nitrate (mg/L) were monitored in each day before water change following standard methods [30].

2.8. Determination of survival and growth

Survival of larvae was monitored each day by counting larvae in triplicate sub-samples of 250 mL collected from different locations in each tank. The survival rate of larvae was calculated as in the following equation:

$$\text{Survival rate (\%)} = \frac{NL_0 - NL_n}{NL_0} \times 100 \quad (2)$$

Where, NL_0 = Number of surviving larvae on day “0” and NL_n = Number of surviving larvae on the day “n”.

Growth of the larvae expressed as larval stage index (LSI) was estimated by picking 10 larvae from each tank and observing under a compound microscope to monitor the current stage of development [31]. A larva was considered for a specific stage (Z1 to Z5) depending upon morphological development (Z1 to Z5 or M). Discrete numbers of 1, 2, 3, 4, 5 and 6 were assigned for the developmental stages of zoea-1 (Z1), zoea-2 (Z2), zoea-3 (Z3), zoea-4 (Z4), zoea-5 (Z5) and megalopa (M), respectively. Larvae in a tank were considered as a specific larval stage when more than 80% of the larvae reached to that stage [32]. Finally, larval stage index (LSI) was calculated by using the formula in below:

$$\text{Larval Stage Index (LSI)} = \frac{(L1 \times N1) + (L2 \times N2)}{TN} \quad (3)$$

Where, L1= counted number of zoea at late stage; N1= assigned number for zoea at late stage; L2= counted number of zoea at early stage; N2= Assigned number for zoea at early stage; and TN= total number of larvae observed in sample.

2.9. Biochemical analysis

For proximate and fatty acid analysis, each type of feed (rotifer, nauplii of *Artemia* and microbound diet) was collected immediately before feeding. The initial larvae sample was collected immediately after hatching and also those of produced megalopa for each feeding treatment were also collected. All types of the collected samples were packed separately in air tight zipped polythene bags, labeled accordingly and stored in the freezer (-20°C). The collected samples were transferred into the low temperature freezer (-180°C) and stored until proximate composition and fatty acid analysis were to be performed.

Moisture content was determined by drying the samples onto the infrared moisture determination scale (Precisa HA 60). Crude protein was analyzed according to Kjeldahl methods following digestion with concentrated sulphuric acid (H_2SO_4), distillation with 40 % sodium hydroxide (NaOH) and titration with 0.1 N hydrochloric acid (HCl) by using an automated protein quantification apparatus (VAPODEST 50S, Gerhardt, Wiesbaden, Germany). A constant factor of 6.25 was used to convert nitrogen into protein.

Crude lipid was analyzed through lipid extraction method using chloroform and methanol mixture 2:1 (v/v) [33]. Crude fibre was estimated on the residues left onto the filter paper by igniting and digestion with 5 % H_2SO_4 and 40 % NaOH. The ash content

was estimated by burnt the samples in a muffle furnace at 550 °C for 5 to 6 hr. Quantitative analyses of fatty acids of the samples were performed as accordingly on collected fatty acid methyl esters (FAME) [34]. An internal standard, 1 µL caproic acid methyl ester (6: 0) diluted in chloroform (1: 499, v/v) was added to 3 µL dried FAME. The sample was vortexed and 1 µL was injected to gas chromatography (GC) for analysis.

2.10. Data analysis

The collected data was computed in Microsoft Excel and was analyzed through SPSS version 20 and 22. ANOVA was done to observe the differences between treatments. A confidence level of 95 % was considered and $p \leq 0.05$ was regarded as a significant difference between the treatments.

3. Results

3.1. Water quality variables

Water temperature ranged from 29.2°C to 31.8°C, salinity from 27.0 ppt to 30.0 ppt, pH from 7.7 to 8.4, Dissolved oxygen from 5.2 mg/L to 6.8 mg/L and NO₂ between 0.00 mg/L and 0.28 mg/L, respectively in all the treatments of larvae rearing Table 2.

Table 2. Range of water quality variable during larvae rearing with different feeding schemes.

Variables	T-1	T-2	T-3
Water Temperature (°C)	29.4-30.0	29.3-29.8	29.2-29.8
Salinity (ppt)	27.0-30.0	27.0-30.0	27.0-30.0
pH	7.7-8.2	7.7-8.4	7.8-8.4
Dissolved Oxygen (mg/L)	5.3-6.4	5.3-6.8	5.2-6.5
NO ₂ (mg/L)	0.00-0.25	0.00-0.25	0.00-0.28

Concentration in NH₄ gradually increased up to 1.50 mg/L in T3 with increasing the larvae rearing duration and a slimy layer on the bottom and tank wall was noticed. The levels of NH₄ in T3 showed a significant difference ($p < 0.05$) from day 6 of culture onward than T1 and T2 (Fig. 1).

3.2. Proximate and fatty acid composition of larvae feeds

Among the tested feeds, commercial diet contained significantly higher ($p < 0.05$) levels of protein (56.43 %) followed by *Artemia* (54.51 %) and rotifer (47.81 %). As to the lipid level, *Artemia* ranked at the top with 14.41 % lipid and had no significant difference ($p > 0.05$) to the lipid level of commercial diet (13.49 %). However, rotifer contained a significantly lower ($p < 0.05$) lipid level (12.18 %) among all the diets (Table 3).

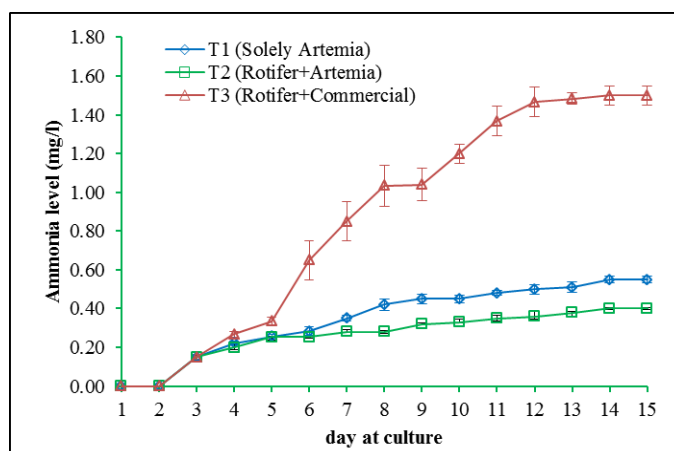


Fig. 1. Trend of ammonia (NH_4) under different feeding regimens of the green mud crab larvae rearing.

Table 3. Proximate composition of feeds (mean \pm SD) fed to the mud crab larvae under different feeding treatments.

Proximate (g/100g)	<i>Artemia</i>	Rotifers	Commercial diet
Moisture	13.39 \pm 1.02 ^a	11.12 \pm 0.59 ^b	8.40 \pm 0.18 ^c
Protein	54.51 \pm 0.40 ^b	47.81 \pm 0.48 ^c	56.43 \pm 0.06 ^a
Lipid	14.41 \pm 0.54 ^a	12.18 \pm 0.51 ^c	13.49 \pm 0.32 ^b ^a
Ash	7.66 \pm 0.81 ^c	11.26 \pm 0.26 ^b	12.52 \pm 0.28 ^a

Note: different superscripts in same row indicates significant differences ($p < 0.05$), shared superscripts in the same row indicate an insignificant difference ($p > 0.05$), and $a > b > c$.

In case of fatty acid composition of the diets (Table 4), SFA (Saturated fatty acid) was dominated (36.76 %) in rotifers followed by commercial diet (29.82 %) and *Artemia* (23.03 %). The SFA level in rotifers was very close to that of the initial larvae sample (39.87 %). The MUFA (Monounsaturated fatty acid) level was dominated in *Artemia* (35.62 %) followed by rotifers (33.65 %) and commercial diet (30.26 %). The level of PUFA (Polyunsaturated fatty acid) was significantly higher ($p < 0.05$) in *Artemia* (41.35 %) than in commercial diet (39.92 %) and rotifers (29.59 %).

In the commercial diet, ARA (Arachidonic acid) and DHA (Docosahexaenoic acid) levels were higher than in other feeds. On the other hand, EPA (eicosapentaenoic acid) was higher in the rotifer, followed by *Artemia* and commercial diet. The commercial diet contained significantly higher ($p < 0.05$) levels of n-3 and n-6 fatty acids than the other two types of tested feeds. The ratio of DHA: EPA was significantly higher ($p < 0.05$) in commercial diet (5.03 \pm 0.48:1), followed by rotifer (0.29 \pm 0.05:1) and *Artemia* (0.16 \pm 0.02:1) (Table 4).

Table 4. Fatty acid composition (% of total fatty acid detected) of initial larvae sample and tested feeds (mean \pm SD) fed to the mud crab larvae.

Fatty acids	Initial larvae (Z1)	<i>Artemia</i>	Rotifers	Commercial diet
C14:0	0.74 \pm 0.07 ^d	1.89 \pm 0.02 ^c	3.40 \pm 0.01 ^b	3.86 \pm 0.14 ^a
C16:0	20.64 \pm 0.41 ^c	15.22 \pm 0.13 ^d	23.45 \pm 0.13 ^a	21.61 \pm 0.50 ^b
C18:0	15.29 \pm 0.25 ^a	5.06 \pm 0.04 ^c	8.24 \pm 0.02 ^b	4.08 \pm 0.02 ^d
C20:0	1.11 \pm 0.10 ^a	0.43 \pm 0.08 ^c	0.73 \pm 0.02 ^b	0.13 \pm 0.02 ^d
C22:0	0.97 \pm 0.08 ^a	0.21 \pm 0.00 ^c	0.60 \pm 0.01 ^b	ND
C24:0	1.11 \pm 0.08 ^a	0.22 \pm 0.03 ^c	0.35 \pm 0.06 ^b	0.15 \pm 0.01 ^{dc}
Σ SFA	39.87\pm0.41^a	23.03\pm0.11^d	36.76\pm0.11^b	29.82\pm0.62^c
C14:1	ND	0.24 \pm 0.00 ^b	1.94 \pm 0.00 ^a	0.14 \pm 0.00 ^c
C16:1	4.02 \pm 0.07 ^d	11.58 \pm 0.03 ^a	9.01 \pm 0.06 ^b	6.60 \pm 0.10 ^c
C18:1n9t	ND	0.19 \pm 0.03 ^c	2.09 \pm 0.00 ^a	0.34 \pm 0.03 ^b
C18:1n9c	11.52 \pm 0.76 ^d	23.10 \pm 0.14 ^a	16.49 \pm 0.20 ^{cb}	17.00 \pm 0.17 ^b
C20:1n9	1.06 \pm 0.13 ^c	0.29 \pm 0.07 ^d	2.40 \pm 0.04 ^b	4.72 \pm 0.30 ^a
C22:1n9	ND	ND	0.77 \pm 0.10 ^a	0.61 \pm 0.07 ^b
C24:1n9	0.27 \pm 0.06 ^c	0.22 \pm 0.01 ^{dc}	0.95 \pm 0.07 ^a	0.85 \pm 0.02 ^b
Σ MUFA	16.86\pm0.69^d	35.62\pm0.10^a	33.65\pm0.09^b	30.26\pm0.45^c
C18:2n6t	0.32 \pm 0.04 ^b	ND	0.40 \pm 0.02 ^a	0.31 \pm 0.02 ^{cb}
C18:2n6c	1.00 \pm 0.09 ^d	11.45 \pm 0.06 ^c	12.65 \pm 0.05 ^a	11.77 \pm 0.09 ^b
C20:2	0.99 \pm 0.08 ^a	0.22 \pm 0.01 ^{dc}	0.58 \pm 0.02 ^b	0.22 \pm 0.01 ^c
C22:2	ND	11.69 \pm 0.12 ^a	ND	7.51 \pm 0.39 ^b
C18:3n3 (ALA)	0.22 \pm 0.02 ^d	8.43 \pm 0.09 ^a	3.01 \pm 0.04 ^b	2.30 \pm 0.08 ^c
C20:3n3	0.22 \pm 0.02 ^{cb}	0.24 \pm 0.01 ^b	0.59 \pm 0.01 ^a	0.13 \pm 0.01 ^d
C20:5n3 (EPA)	15.70 \pm 0.92 ^a	5.12 \pm 0.03 ^c	7.70 \pm 0.04 ^b	2.25 \pm 0.21 ^d
C22:6n3 (DHA)	13.39 \pm 0.35 ^a	0.84 \pm 0.10 ^d	2.22 \pm 0.06 ^c	11.23 \pm 0.07 ^b
C18:3n6	ND	0.50 \pm 0.00 ^a	0.29 \pm 0.01 ^b	ND
C20:3n6	ND	2.63 \pm 0.18 ^a	1.26 \pm 0.02 ^b	0.64 \pm 0.02 ^c
C20:4n6 (ARA)	11.43 \pm 0.81 ^a	0.24 \pm 0.02 ^{dc}	0.89 \pm 0.00 ^c	3.56 \pm 0.13 ^b
Σ PUFA	43.27\pm0.62^a	41.35\pm0.21^b	29.59\pm0.15^d	39.92\pm0.17^c
Σ UFA	60.13 \pm 0.41 ^d	76.97 \pm 0.11 ^a	63.24 \pm 0.11 ^c	70.18 \pm 0.62 ^b
Σ (n-3)	29.53 \pm 0.61 ^a	14.63 \pm 0.19 ^c	13.52 \pm 0.14 ^d	15.90 \pm 0.24 ^b
Σ (n-6)	11.43 \pm 0.81 ^a	3.37 \pm 0.16 ^c	2.44 \pm 0.02 ^d	4.21 \pm 0.12 ^b
UFA: SFA	1.51 \pm 0.03:1 ^d	3.34 \pm 0.02:1 ^a	1.72 \pm 0.01:1 ^c	2.35 \pm 0.07:1 ^b
(n-6): (n-3)	0.39 \pm 0.03:1 ^a	0.23 \pm 0.01:1 ^{cb}	0.18 \pm 0.00:1 ^d	0.26 \pm 0.01:1 ^b
DHA: EPA	0.86 \pm 0.07:1 ^b	0.16 \pm 0.02:1 ^d	0.29 \pm 0.05:1 ^c	5.03 \pm 0.48:1 ^a

Notes: ND= not detected; different superscripts in same row indicate significant differences ($p < 0.05$), shared superscripts in the same row indicate an insignificant difference ($p > 0.05$), and $a > b > c > d$

3.3. Survival rate of larvae

The survival was higher in T2 and T3 for the initial three days (Fig. 2) when larvae were fed with rotifers. On the other hand, an initial mortality was noticed in T1 within 72 h, when the larvae were fed with *Artemia* umbrella.

On the fifth day of rearing, a sudden fall in survival was marked in all the treatments, while it was drastically so in T1 and T3, when the feeding was changed from *Artemia* umbrella to nauplii and rotifer to commercial diet, respectively. In T2, the mortality was

steady and the survival rate was found to be significantly higher ($p < 0.05$) from the fourth to the fifteenth day of rearing (Fig. 2).

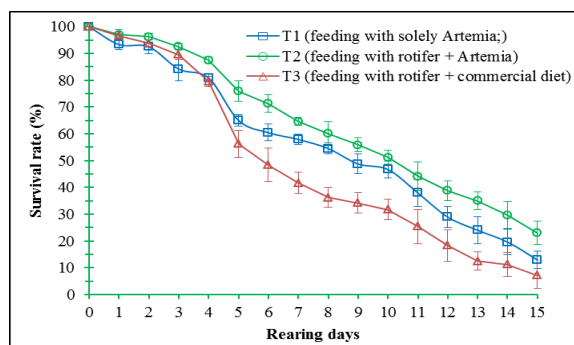


Fig. 2. Trends in daily survival (%) of the green mud crab larvae fed with different feeding schemes.

Table 5. Survival rate (%) of the green mud crab larvae (Mean \pm SD) at different larval stages reared with different feeds.

Larvae stages	T1	T2	T3
	(<i>Artemia</i>)	(Rotifer + <i>Artemia</i>)	(Rotifer + Commercial diet)
Z2	84.17 \pm 4.39 ^{cb}	92.50 \pm 1.25 ^a	89.58 \pm 1.91 ^{ba}
Z3	60.00 \pm 3.75 ^b	72.50 \pm 2.50 ^a	41.67 \pm 4.02 ^c
Z4	48.75 \pm 3.75 ^b	56.67 \pm 1.91 ^a	32.50 \pm 4.33 ^c
Z5	25.67 \pm 6.00 ^b	40.42 \pm 1.91 ^a	11.25 \pm 4.51 ^c
M	10.67 \pm 3.41 ^b	20.00 \pm 6.96 ^a	3.00 \pm 2.65 ^c

Notes: Z2.....Z5= zoea stage 2 to 5 and M= megalopa; different superscripts in same row indicate significant differences ($p < 0.05$), shared superscripts in the same row indicate a similarity ($p > 0.05$), and a>b>c.

The survival rate of larvae according to the different developmental stages of all feeding treatments is shown in Table 5. The survival rate for the Z2 stage was similar between T2 (92.90 %) and T3 (89.58 %) also between T3 and T1 (84.17 %), but it was significantly different ($p < 0.05$) between T2 and T1. From the Z3 to Z5 stages, the survival rates were significantly ($p < 0.05$) different among the treatments with highest values in T2 followed by T1 and T3. At the megalopa stage, survival rate was 10.67 %, 20.00 % and 3.00 % in T1, T2 and T3, respectively and differed statistically ($p < 0.05$). Megalopa produced in T3 seemed very weak and were smaller in size (Table 7) thus unable to reach to the crablet stage (crab instars). Feeding treatments with enriched rotifers as a first feeding (Z1 to Z2) followed by enriched *Artemia* nauplii as later stages (Z3 to megalopa) augmented higher survival rates in Z5 and megalopa stages.

3.4. Larval stage index (LSI)

Trends in the larval stage index (LSI) under different feeding treatments are presented in Fig. 3. It could be seen that, the LSI of larvae was similar up to day four. Later on the LSI

began to vary between different feeding treatments and continued up to the fifteenth day. From day four onwards, T2 showed the highest LSI values followed by T1 and T3 (Fig. 3).

Larval stage index (LSI) at every three days is presented in Table 6. On day three, the LSI values were insignificant ($p>0.05$) among the treatments, indicating synchronized growth in all treatments. On the sixth day of rearing, the highest LSI value (3.00) was achieved in T2 with SD value of 0.00, which indicated synchronized growth in all the replicates and it was significantly ($p<0.05$) higher than T3. However, it was statistically insignificant ($p>0.05$) between T1 and T2. The earliest commencement of highest LSI value of 5.0 (Z5 stage) was obtained in T2 on the twelfth day of culture and it was significantly higher ($p<0.05$) than T1 (4.67) followed by T3 (4.37). On the fifteenth day of culture, the LSI values were 5.51, 5.90 and 5.33 in T1, T2 and T3, respectively. The LSI value on the fifteenth day was statistically significant ($p<0.05$) between T2 and T1 followed by T3, but it was insignificant ($p>0.05$) between T1 and T3. The level of LSI value on the fifteenth day of culture indicated that more than 80 % of larvae in T2 metamorphosed to the megalopa stage, whilst in T1 and T3 achieved ≤ 50 % of megalopa on the fifteenth day of rearing (Table 6).

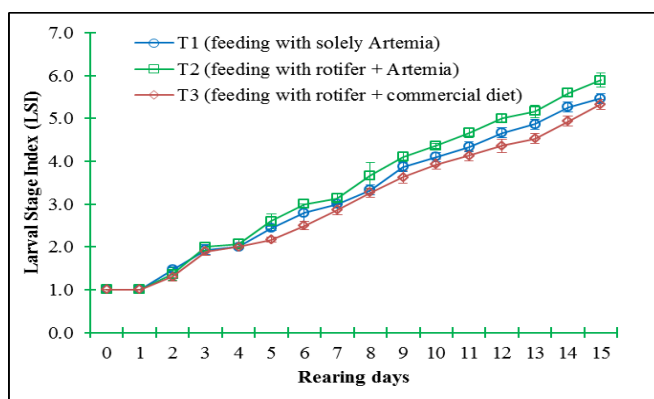


Fig. 3. Daily pattern of larval stage index (LSI) under different feeding schemes during larvae rearing period of the green mud crab.

Table 6. Larval stage index (LSI) (mean \pm SD) on each 3 day interval of the green mud crab larvae reared with different feeding schemes.

Rearing days	T1 (<i>Artemia</i>)	T2 (Rotifer + <i>Artemia</i>)	T3 (Rotifer + Inert)
Day 3	1.93 \pm 0.12 ^{ba}	2.00 \pm 0.00 ^a	1.90 \pm 0.10 ^{cba}
Day 6	2.80 \pm 0.20 ^{ba}	3.00 \pm 0.00 ^a	2.50 \pm 0.10 ^c
Day 9	3.87 \pm 0.12 ^{ba}	4.10 \pm .10 ^a	3.63 \pm 0.15 ^{cb}
Day 12	4.67 \pm 0.12 ^b	5.0 \pm 0.10 ^a	4.37 \pm 0.15 ^c
Day 15	5.47 \pm 0.12 ^b	5.9 \pm 0.17 ^a	5.33 \pm 0.12 ^{cb}

Notes: ND= not determined, different superscripts in the same row indicate significant differences ($p<0.05$), shared superscripts in the same row indicate an insignificant difference ($p>0.05$), and $a>b>c$.

3.5. Larvae sizes at different stages

Size of larvae at different stages under different feeding treatments is as shown in Table 7. Up to the Z2 stage, larvae size was similar in all treatments. From Z3 to the megalopa stages, larvae size was significantly smaller ($p < 0.05$) in T3, but it was similar in T1 and T2 (Table 7).

Table 7. Size (mm) of the mud crab larvae (mean \pm SD) at various larval stages under different feeding schemes.

Larvae stages	T1 (<i>Artemia</i>)	T2 (Rotifer + <i>Artemia</i>)	T3 (Rotifer + Inert)
Z1 (initial)	2.17 \pm 0.01 ^a	2.17 \pm 0.01 ^a	2.17 \pm 0.01 ^a
Z2	2.20 \pm 0.01 ^a	2.20 \pm 0.01 ^a	2.20 \pm 0.01 ^a
Z3	2.88 \pm 0.01 ^a	2.88 \pm 0.01 ^a	2.82 \pm 0.01 ^b
Z4	2.92 \pm 0.02 ^{ba}	2.93 \pm 0.02 ^a	2.87 \pm 0.03 ^c
Z5	3.69 \pm 0.02 ^{ba}	3.71 \pm 0.01 ^a	3.64 \pm 0.04 ^c
M	3.31 \pm 0.02 ^{ba}	3.32 \pm 0.01 ^a	3.20 \pm 0.02 ^c

Notes: ND= not determined, Z1: zoea-1..... Z5: Zoea-5 and M: megalopa; different superscripts in same row indicate significant differences ($p < 0.05$), shared superscripts in the same row indicate an insignificant difference ($p > 0.05$), and $a > b > c$

3.6. Fatty acid composition of megalopa under different feeding treatments

As shown in Table 8, the saturated fatty acid (SFA) content was significantly different ($p < 0.05$) in megalopa between all feeding treatments with the highest level of 46.60 % in T3 where larvae were fed with rotifer and commercial diet. Monounsaturated fatty acid (MUFA) was similar to the megalopa sample produced from all treatments. Polyunsaturated fatty acid (PUFA) seemed statistically different ($p < 0.05$) among all treatments with the highest level (42.68 %) in megalopa derived from T1 where larvae were reared with *Artemia* umbrella followed by *Artemia* nauplii enriched with SELCO (commercial marine fish oil).

Table 8. Fatty acid composition of megalopa (mean \pm SD) fed with different feeds at larvae rearing.

Fatty acids	<i>Artemia</i> (T-1)	Rotifer + <i>Artemia</i> (T-2)	Rotifer + Inert (T-3)
C14:0	0.55 \pm 0.01 ^c	0.66 \pm 0.01 ^b	1.64 \pm 0.02 ^a
C16:0	17.26 \pm 0.04 ^b	16.92 \pm 0.11 ^c	21.44 \pm 0.12 ^a
C18:0	14.82 \pm 0.02 ^b	13.08 \pm 0.08 ^c	15.87 \pm 0.20 ^a
C20:0	0.45 \pm 0.02 ^b	0.18 \pm 0.00 ^c	6.55 \pm 0.17 ^a
C22:0	0.64 \pm 0.02 ^c	1.27 \pm 0.01 ^a	1.10 \pm 0.04 ^b
C24:0	1.20 \pm 0.04 ^a	0.28 \pm 0.02 ^b	ND
ΣSFA	34.92\pm0.03^b	32.38\pm0.19^c	46.60\pm0.07^a
C14:1	0.27 \pm 0.01 ^c	0.40 \pm 0.01 ^b	1.12 \pm 0.02 ^a
C16:1	3.84 \pm 0.02 ^c	5.25 \pm 0.03 ^b	7.84 \pm 0.10 ^a
C18:1n9t	0.40 \pm 0.03 ^b	0.19 \pm 0.01 ^c	0.67 \pm 0.02 ^a
C18:1n9c	16.65 \pm 0.20 ^b	18.98 \pm 0.09 ^a	11.28 \pm 0.04 ^c
C20:1n9	1.24 \pm 0.08 ^a	1.13 \pm 0.06 ^{ab}	0.76 \pm 0.06 ^c
ΣMUFA	22.40\pm0.16^a	25.96\pm0.09^a	21.67\pm0.12^a

Fatty acids	<i>Artemia</i> (T-1)	Rotifer + <i>Artemia</i> (T-2)	Rotifer + Inert (T-3)
C18:2n6t	0.22±0.01 ^b	ND	0.60±0.08 ^a
C18:2n6c	5.92±0.02 ^c	7.39±0.03 ^b	14.25±0.19 ^a
C20:2	1.13±0.02 ^b	1.35±0.01 ^a	0.49±0.02 ^c
C18:3n3 (ALA)	4.42±0.04 ^b	5.64±0.01 ^a	2.69±0.06 ^c
C20:3n3	0.25±0.01 ^{bc}	0.26±0.00 ^b	0.92±0.02 ^a
C20:5n3 (EPA)	23.20±0.09 ^a	17.32±0.19 ^b	1.28±0.11 ^c
C22:6n3 (DHA)	0.90±0.01 ^c	3.82±0.11 ^b	10.30±0.43 ^a
C18:3n6	ND	0.20±0.00 ^b	0.75±0.02 ^a
C20:3n6	6.44±0.03 ^a	4.95±0.03 ^b	ND
C20:4n6 (ARA)	0.19±0.03 ^c	0.26±0.03 ^b	0.45±0.02 ^a
∑PUFA	42.68±0.19^a	41.65±0.26^b	31.73±0.19^c
∑UFA	65.08±0.03 ^b	67.62±0.19 ^a	53.40±0.07 ^c
∑(n-3)	28.77±0.11 ^a	27.03±0.30 ^b	15.19±0.42 ^c
∑(n-6)	6.63±0.06 ^a	5.41±0.04 ^b	1.20±0.04 ^c
UFA: SFA	1.86±0.00:1 ^b	2.09±0.02:1 ^a	1.15±0.01:1 ^c
(n-6): (n-3)	0.23±0.00:1 ^a	0.20±0.00:1 ^b	0.08±0.00:1 ^c
DHA: EPA	0.04±0.00:1 ^{bc}	0.22±0.00:1 ^b	8.09±0.97:1 ^a

Notes: ND= not detected; different superscript in same row indicate significant differences ($p < 0.05$), shared superscripts indicate similarity, and $a > b > c > d$.

The lowest level (31.73 %) of PUFA was in T3, in which larvae was cultured with rotifer followed by commercial diet. Both n-3 and n-6 fatty acids were statistically different ($p < 0.05$) among the treatments with the highest level in megalopa sample obtained from T1, followed by T2 and T3. Both EPA and DHA also differed statistically ($p < 0.05$) among megalopa of all treatments. The EPA was highest (23.20 %) in T1, followed by T2 (17.32 %) and T3 (1.28 %), while the DHA was highest (10.30 %) in T3 followed by T2 (3.82 %) and T1 (0.90 %).

4. Discussion

Better survival rate and earliest metamorphosis to the subsequent larval stages are the utmost expectation in crustacean larvae rearing. Newly hatched mud crab larvae which lacks of yolk sac are hungry and need immediate feeding [35-37]. They are free swimmer and natural predator to the live prey, but the swimming activities of Z1 larvae are not so swift as to capture prey of the first movable objects, that indicates inefficient predator behavior and are passive feeders depending on options of encounter with food [38]. Thus, first feeding is found to be vital to minimize the initial mortality and to enhance the vitality for successful metamorphosis into subsequent larval stages. Availability of feeds, their size, digestibility and nutritional quality should be considered. In this larvae rearing experiment, the survival rate at the Z2 stage was similar in T2 and T3, where larvae were allowed to feed with enriched rotifer. On the other hand, larvae in the same stage in T1 that were fed with *Artemia* umbrella provided lower survival rate (Fig. 2 and Table 5). This indicated the suitability of rotifers as initial feeding of mud crab larvae rearing by virtue of their smaller size compatible to the mouth gap of the larvae [39-41]. In this

experiment, rotifer enriched with algae (*Nannochloropsis* sp.) might have enhanced the digestibility of the newly hatched larvae, which ultimately benefitted the larvae by developing their digestive system [42,43]. On the other hand, feeding with umbrella stage *Artemia* (T1) was ultimately a mixture of umbrella and nauplii, while some remained in cyst forms, as because of asynchronized hatching of the *Artemia*. Mud crab larvae at the Z1 stage were unable to penetrate the shell of *Artemia* cyst [44]. Moreover, the *Artemia* nauplii were bigger in size than the mouth gap of the larvae. These physical features of the *Artemia*, might have lowered the survival rate of Z2 in T1 in this experiment.

In terms of nutritional content in this experiment, rotifers contained a high amount of EPA and DHA in compared to *Artemia* (Table 4). Zooplankton fed with phytoplankton (*Nannochloropsis* sp.) stored a vast amount of wax esters rich in HUFA's (highly unsaturated fatty acids), which are able to support the metabolic activity and cell membrane development. Though mud crab larvae consumed *Artemia* umbrella, they were not suitable to meet the nutritional demands for sustainable survival and successful metamorphosis to the later stages. A significant improvement in survival was noticed when only the *Artemia* was enriched [44]. Similar observations were made in the larvae rearing of the Chinese mitten crab *Eriocheir sinensis* [45]. Meanwhile, successful results in mud crab larvae rearing was reported as fed solely with umbrella type *Artemia* or combined rotifer and *Artemia* umbrella as initial feed [40], thus supporting the results of T1 in this experiment.

In this experiment, larvae fed with enriched rotifer up to Z2 and later on feeding on enriched *Artemia* nauplii, showed better survival rates, development and metamorphosis to megalopa at 15 DAH (day at hatching) and successfully reached to the crablet stage (Figs. 2-3 and Tables 5-6). Similar outcomes were recorded for *S. serrata* larvae rearing in Australia and in Japan [46]. However, significant differences in the survival of mud crab larvae were not observed when only *Artemia* was enriched. But significantly faster development at 15 DAH (day at hatching) was recorded by feeding with enriched rotifer for the first 6 days after hatching in Vietnam [47].

The larvae of T3 in this study, reared with enriched rotifer up to Z2 and followed by commercial diet, showed lower survival rate in the later stages of rearing (Fig. 2), lingered the intermoult period (Fig. 3 and Table 6) and were unable to moult in the crablet stage. All these might have happened due to physical properties of the diet. In visual observations, the commercial diet was found to float over water for some times, attached to the tank walls and after absorbing water the feed slowly sank to the bottom and formed a slimy layer. It was assumed that major portion of the nutrient, thus leaked into the water and deteriorated the culture tank by elevating NH_4 (Fig. 1). Mass mortality of larvae in this treatment might be associated with lingering of the intermoult period (Table 6). Excess deposition of DHA in megalopa (Table 8) might another possible causative factor in sudden mortality of larvae in T3, as also observed previously [43], and in coupled with environmental stress due to elevated ammonia level (Fig. 1). In this experiment, larvae in T3 (fed with commercial diet) failed to reach the crablet stage. Meanwhile, formulated

feed developed for marine shrimp larvae had some success in mud crab larvae rearing [48].

In this experiment, a portion of larvae (10 to 25 %) was found to contain additional growth or deformities in chelipod, improper exuvia shedding in comparison to the normal larvae of Z5 stage; and faced moulting difficulties. In addition, megalopa which took a longer time for metamorphosis also encountered moulting problem with the deformity in chelipods and eventually died during, immediately after or within 2 days after metamorphosis into the megalopa stage.

Such types of moulting failure and mortality during moulting from Z5 stage to megalopa and at the megalopa stage was earlier mentioned in *S. serrata* and *S. paramamosain* larvae rearing as well as in swimming crab larvae rearing [49,50] and it was associated with improper nourishment of essential fatty acids [51]. Actually, when live feeds (rotifer or *Artemia*) were enriched, all the live feed did not receive equal amounts of enrichment. Meanwhile, nutrients in the live feed gradually decreased as the time passed after enrichment. In addition, larvae rearing in larger vessels (1000 L volume) did not allow uniform distribution of prey. Moreover, all the larvae did not eat at the same time and the same amount of feed. Thus the fast feeder larvae might receive a large amount of nutrients while the slow or late feeder might receive a low amount. Both excess and/or shortage in the essential n-3 fatty acids (HUFA's) like the EPA (20:5n-3) and DHA (22:6n-3) were reported as being responsible for such types of abnormal growth in Z5 as well as deformities at the megalopa stage [11,13] and mass mortality occurred due to excessive levels of DHA [51].

Despite the gradual mortalities in some larval stages, larvae of T1 and T2 in this experiment successfully reached to the crablet stage, but not the larvae in T3. The reasons behind this might be the different ratios in EPA and DHA in the megalopa samples. Better survival of *Scylla tranquebarica* was obtained when DHA: EPA ration was lower (0.07) [20]. Similarly, superior survival and earliest metamorphosis of *Scylla serrata* larvae was reported with a DHA: EPA of 0.3 [13]. Whilst, in Vietnam, for *Scylla paramamosain*, first growth and metamorphosis were triggered by DHA: EPA ratio of 0.6 to 4 [47]. This indicated variations in fatty acid requirements for different species and locations. Therefore, with respect to the DHA/ EPA ratio (Table 4 and 8), the results of this experiment is in agreement with the findings of previous researcher [13, 20, 47, 51].

5. Conclusion

The results of this experiment revealed that, enriched rotifer as a first feeding followed by enriched *Artemia* nauplii for later stage feeding provided better performance in terms of growth, metamorphosis and survival. Commercial diet could be used at a minimum level as supplemental in combination with natural feeds. The level of enrichment with specific fatty acids (EPA and DHA) and their specific ratios need to be emphasized in future studies for the enhancement of the larvae rearing protocol.

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