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Successive reproductive performance and amino acid profiles in the newly hatched larvae of green mud crab (*Scylla paramamosain*) under captive condition

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Abstract

Three successive reproductive performance and larvae quality from a single matting event of the green mud crab (*Scylla paramamosain*) was assessed by evaluating the reproductive capability, presence of amino acids in the newly hatched larvae and withstand of the larvae against starvation. The results of the present study revealed that, fecundity and egg fertilization rate was significantly higher ($p < 0.05$) in 1st and 2nd spawning event than the 3rd. Production of viable and phototaxis larvae was also higher in 1st spawning event and significantly ($p < 0.05$) decreased among successive spawning. Some of the essential amino acids (aspartic acid, isoleucine, leucine, phenylalanine and lysine) in larvae decreased gradually in successive spawning, indicated as traced. Longer time for commencement of medium death (MD⁵⁰) under starvation for 1st and 2nd spawned larvae indicated better vitality to survive. Findings of this study suggested the safe use of 1st and 2nd spawned larvae for seed production purposes by saving time, space and money for new broods.

Keywords: Mud crab; successive reproduction; performance; larvae; amino acids; starvation

1. Introduction

Global mud crab aquaculture evolved grow out in pens or cages in the mangroves, fattening in the earthen ponds or floating cages and soft shell shedding in floating boxes [56]. All these activities are directly or indirectly dependent on collection of juveniles and adult crabs from the wild sources [56, 57]. Continuous collection of all size groups from the natural sources caused depletion of the stock. Hence decreasing trend in natural seed supply making the mud crab aquaculture unreliable [23, 24, 28] and are the major obstacles against further development of the mud crab sector as a sustainable mode [30]. To minimize the seed crisis, Australia, the Philippines, Vietnam and Indonesia are producing mud crab seeds in commercial scale since 1991 to enhance the mud crab aquaculture [56, 57], but the survival from Z1 to crablet stage seemed very low and inconsistency [39]. The declining trend of the average size of the harvested crabs [23], unavailability of adequate suitable broodstock in due time [52] and unpredictable reproductive performance and larvae quality [10, 46] that varied from brood to brood have been detected as the major reason behind the lower seed production rate.

In these circumstances, the capability of the female mud crab to fertilize 3 successive batches of eggs from the stored sperms from a single mating [45, 56, 57] could be an advantage to mitigate the demand of required broodstock. The hatchery managers and researchers could be benefitted by means of saving time and cost for new broodstock and minimizing the rearing cost and space for the male candidates as because of higher cost involved in broodstock management [47]. While the majority of the hatchery owners and researchers managed to use the larvae that produced from the first spawning only. The larvae of successive 2nd and 3rd spawning remained unused that might be their ignorance regarding successive spawning or from their assumption that successive spawning produce poor quality larvae. Meanwhile, documented information on reproductive performance and larvae quality for the successive spawning is not available yet except the partial observation of Quintio [47].

Reproductive performance and larvae quality differ within species [11], by environmental factors [20] and by maternal nutrition [31]. Of which, maternal nutrition that subsequently transmitted into the eggs [7] greatly affects the quality of eggs and larvae in fish [22, 27] and in crustaceans [10, 27, 43]. The primary component of the egg is lipoprotein (lipid and protein) [11] which are the main source of nutrient for embryonic development in crabs [49] and the level of this two component is considered as the indicator of egg and larvae quality [8].

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Besides the level of protein, the composition of amino acids has also been perceived as an energy source for the eggs that lack of oil globules^[55]. The proper amino acid composition in broodstock has been identified as successfulness for larvae production in crustacean such as *P. monodon*^[38, 39]. The level of some of the essential and nonessential amino acids have been substantially declining during embryogenesis of the European lobster, *Homarus gammarus*^[56] and reportedly noticed the similar observation for the mud crab *S. serrata*^[61] and regarded as traced element. Limited information is available on the role of amino acids in predicting the larvae quality of crustaceans. Whereas, the information regarding the amino acid profile and larvae quality is scarce for the mud crab. Considering the all above, this study was aimed to observe the successive reproductive performance and larvae quality of a typical mud crab species *S. Paramamosain* in considering the amino acid composition and stress test.

2. Methodology

2.1 Source of broodstock

The broodstock used in this study was exclusively grown in captive condition by breeding the base population (P₀) in the hatchery.

2.2 Preparation of spawning tanks

Depending on the availability of gravid crabs, two different types of spawning tanks were tried for spawning. For individual spawning, nine plastic buckets with a volume of 50 L each was set up in such a way that each bucket had an independent water circulation system through underwater sand filtration (25 cm sand filling) by aeration with final water supply from a general bio-filter. For that of group spawning, 5m² (4.2 m × 1.2 m) fibre glass tanks were used. The tanks were half-filled with filtered sea water, contained three underwater sand filter tray facilitated with hiding places and setting up of aeration. The tanks were covered with a black plastic net to create darkness.

2.3 Feeding and management of gravid broods

The broodstock were fed at the rate of 5 to 10% of body weight twice a day in the morning (09:30 h) and the evening (16:30 h) with chopped raw/natural feeds. The feeding strategy for a week with different feeds was maintained as: Saturday and Sunday> trash fish; Monday> squid; Tuesday> trash fish; Wednesday> blood cockles; Thursday> trash fish; and Friday> small shrimp. The uneaten feeds and excreta were removed in every morning with the help of scoop net. Approximately 75% of the water was replaced with filtered sea water at weekly intervals. Water salinity of the spawning tanks were maintained 30±1.0 ppt and the temperature was 31±1.0 °C. Gravid and/or mated crabs were reared in the spawning system until they were spawned. When a brood had finished spawning, it was taken out from the spawning tank, weighed and placed in the hatching tank with 30 ppt sea water and aeration.

2.4 Examination of eggs and measurement of size (diameter)

Immediately after spawning, a sub-sample of egg was collected with a sterile forcep, placed onto a glass slide and observed under a compound microscope (Magnus Pro., Plan Achromate, Germany). The diameter of 10 eggs was measured with 40X magnifications by holding an ocular micrometer (0.01 mm accuracy) and the average egg diameter was noted against the respective brood.

2.5 Observation of egg fertilization and calculation of fertilization rate

Observation of egg fertilization was imposed on the second time collected samples for each brood (egg mass deep orange to orange-red) when the cell division started within the eggs. A sub-sample was placed onto a glass slide and monitored under a compound microscope. Fertilized eggs were counted on the basis of progress in embryonic development. Eggs failed to cell division were regarded as unfertilized. After counting of triplicate samples, fertilization rate was estimated.

$$\text{Fertilization rate } f (\%) = \frac{\text{TNES} - \text{NUE}}{\text{TNES}} \times 100$$

Where, TNES= Total number of eggs in sub sample; NUE= the number of unfertilized eggs

2.6 Estimation of the rate of fallen eggs

Brooding eggs from the hatching tank were siphoned out in each morning and collected by a fine meshed nylon (50 µm). Residues onto the net were cleaned, triplicate sub-samples were measured and counted under a compound microscope. The process was continued for an entire housing period until hatching. Fallen eggs were then calculated as:

$$\text{Fallen eggs (DE)} = \frac{\text{No. of dropped eggs in sub sample} \times \text{weight of dropped eggs}}{\text{weight of sub sample}}$$

$$\text{Rate of fallen eggs } (\%) = \frac{\text{DE}}{\text{F}} \times 100$$

Where, DE= total fallen eggs and F= Fecundity

2.7 Collection of larvae, estimation of viable larvae, phototaxis larvae, dead larvae and follicle cells

Immediately after hatching, the spent brood was taken out and weighed. Aeration of the hatching tank was turned off and one corner of the tank lid was opened to allow light. Phototaxis larvae gathered in the lightened corner were gently collected by a glass beaker and placed into a plastic bucket partially filled with sea water (30 ppt) and aeration. Rest of the larvae indiscriminately swam in the water column (nonphototaxis) were also collected in another bucket. Three sub-samples (100 ml each) of larvae suspension from each bucket were taken, counted separately. Both phototaxis and nonphototaxis larvae were then calculated following the formula below:

$$\text{Total phototaxis larvae (PZ) or nonphototaxis larvae (NPZ)} = n \times \frac{\text{VT}}{\text{VF}}$$

Where, n= average number of larvae count in the sub-sample (100 ml) for the respective larvae sample (PZ or NPZ); VT= volume (L) of the respective larvae suspension (PZ or NPZ) in bucket; and VS= volume of sub-sample (100 ml).

Whereas, total produced viable larvae (VZ) were calculated as= PZ + NPZ

Where, PZ= total phototaxis larvae, and NPZ= total nonphototaxis larvae.

Dead larvae and follicle cells that settled into the bottom was siphoned and collected on to a 50 µm meshed dip nylon net. Larvae were separated from the follicle cells. Dead larvae and follicle cells (FC) were then dried with bolting paper, triplicated sub-samples of each were weighed by a measuring scale (SARTORIUS, BP 110S; D= 0.1 MG, Germany) and

average were made. The average number was then multiplied by the weight of total dead larvae and percent (%) of dead larvae was calculated as:

$$\text{Dead larvae (\%)} = \frac{\text{Total larvae (dead+viable)} - \text{VZ}}{\text{Total larvae}} \times 100$$

Where, VZ= Total viable larvae

2.8 Estimation of fecundity and relative fecundity

Immediately after spawning, sample eggs were collected with a sterile forcep and preserved in 10% formalin. Small sub-sample of preserved eggs were taken, bloated and weighed. The number of eggs in sub-sample were counted by eventually distributed onto a SR (Sedgwick Rafter) by placing under a compound microscope (Magnus Pro, Plan Acromate, Germany) at 40 X magnification and finally average was made for a field. Calculation of the number of eggs in the sub-sample was done as:

Number of eggs (EN) in sub-sample = average number of eggs in a field \times 1000

Then fecundity (F) per brood was calculated using the formula below:

Fecundity (F) = [(WE - WH) - FC] \times EN / average weight of subsample

Where, WE= weight of crab after spawning/extrude; WH = Weight of crab after full hatching; FC = weight of follicle cells; and EN = average number of eggs in sub-sample.

The relative fecundity of crab was expressed as the number of eggs per gram of body weight and was calculated as:

$$\text{Relative fecundity (No/g BW)} = \frac{\text{Fecundity (F) of the brood}}{\text{Total weight (TW) of the brood}}$$

2.8 Disinfection of the spawner and returned into the spawning tank

Immediately after hatching, the broods were disinfected by 100 ppm formalin for 1 hour, repeatedly washed with fresh sea water and returned back into the spawning tank for successive spawning. The whole procedure was repeated for each brood until completion of 3rd spawning from a single mating.

2.9 Proximate composition and amino acid analysis

Sub-samples of each type of feeds and newly hatched larvae for each broods were collected, packed in gipped polybag and stored in the freezer (-20 °C). The samples were then transferred in a low temperature freezer (-180 °C) and kept until analysis. Analysis of proximate composition of crab feeds was done in accordance to the standard protocol [3]. Amino acid was analyzed by using HPLC (High Performance Liquid Chromatography) system.

2.10 Starvation test to the larvae

Starvation test was performed in 1 L plastic jars of each under the temperature ranged between 29 °C to 30 °C. Each of the jars were filled with 500 ml of 30 ppt sea water and 30 viable larvae were placed. Feeding was ceased and the water of each jar was changed daily with the same salinity and similar temperature water to avoid any secondary stress. Mortality of larvae was monitored after every 12 hours. A larva was considered as dead when it stopped swimming, body movement and even moving in the appendages. The stress test was performed for larvae produced from each brood up to 3rd spawning. The stress test was conducted as accordance to the standard methods [9, 51].

2.11 Data analysis

All the data and records were separately computed into MS-Excel. The data were analyzed through SPSS Version 20 and 22. One way ANOVA (Analysis of Variance) and DMRT (Duncan's Multiple Range Test) was done to establish the differences and the ranking. A confidence level of 95% was considered and $p \leq 0.05$ was regarded as significant difference.

3. Results

Results on successive reproductive performance and the larvae quality of captivating broodstock is subsequently presented in below.

3.1 Proximate composition and Amino acid profile in broodstock feeds

All the feeds supplied to the broodstock contained different levels of proximate components. The average levels of moisture, protein, lipid, ash, fibre and nitrogen free extract (NFE) in feed items were 8.40, 63.23, 11.29, 6.86, 5.72 and 4.49 g/100g, respectively (Table 1).

Table 1: Proximate composition of feeds (Mean \pm SD), fed to the mud crab broodstock for ovary maturation

Proximate (g/100g)	Blood cockles	Squid	Small Shrimp	Sardine Fish	Mean \pm SD
Moisture	9.51 \pm 0.12 ^a	8.46 \pm 0.22 ^b	8.06 \pm 0.39 ^{bc}	7.55 \pm 0.25 ^d	8.40 \pm 0.83
Protein	59.53 \pm 0.11 ^d	65.67 \pm 0.32 ^{ab}	61.45 \pm 1.23 ^c	66.26 \pm 0.33 ^a	63.23 \pm 3.27
Lipid	11.39 \pm 0.34 ^c	12.77 \pm 0.34 ^b	6.43 \pm 0.15 ^d	14.58 \pm 0.37 ^a	11.29 \pm 3.49
Ash	3.25 \pm 0.30 ^d	4.15 \pm 0.16 ^b	11.37 \pm 0.48 ^a	4.09 \pm 0.50 ^{bc}	6.87 \pm 1.47
Fibre	6.18 \pm 0.33 ^{bc}	6.25 \pm 0.57 ^b	9.07 \pm 0.04 ^a	5.98 \pm 0.56 ^{bcd}	5.72 \pm 3.79
NFE	10.14 \pm 0.29 ^a	2.67 \pm 0.74 ^{bc}	3.61 \pm 0.60 ^b	1.52 \pm 0.27 ^d	4.49 \pm 3.86

Note: NFE= Nitrogen Free Extract; different superscript in the same row indicates significant difference ($p < 0.05$); shared superscripts in the same row indicate similarity ($p > 0.05$); and $a > b > c > d$

The presence of total essential amino acid (46.96%) and nonessential amino acid (49.74%) was found to provide the proportion of 0.94 in the average samples (Table 2). The cystine was found in a traced level (0.17%).

Table 2: Amino acid composition (% of total amino acids detected) of feeds (Mean±SD) fed to the mud crab broodstock for gonad maturation

Amino acids	Feed items				Mean±SD
	Blood cockles	Squid	Shrimp	Sardine	
Asp	13.15±0.20 ^c	14.17±0.05 ^b	11.80±0.17 ^d	14.90±0.12 ^a	13.51±1.34
Ser	5.67±0.16 ^a	4.57±0.05 ^c	5.20±0.06 ^b	4.03±0.01 ^d	4.87±0.72
Glu	12.47±0.09 ^c	12.89±0.06 ^b	12.33±0.12 ^{cd}	13.54±0.09 ^a	12.81±0.54
Gly	5.60±0.75 ^{abc}	5.66±0.02 ^{ab}	6.50±1.07 ^a	5.06±0.02 ^{bcd}	5.70±0.59
His	2.15±0.05 ^c	2.09±0.05 ^{cd}	2.36±0.07 ^b	4.06±0.06 ^a	2.67±0.94
Arg	11.38±0.10 ^a	8.78±0.03 ^c	11.27±0.12 ^{ab}	6.54±0.01 ^d	9.49±2.31
Threo	4.95±0.11 ^{ab}	4.57±0.04 ^c	4.99±0.07 ^a	4.32±0.01 ^d	4.75±0.32
Ala	4.81±0.06 ^b	4.54±0.02 ^d	4.80±0.05 ^{bc}	5.22±0.02 ^a	4.85±0.28
Prol	3.87±0.08 ^{bc}	3.94±0.03 ^b	4.07±0.07 ^a	3.75±0.05 ^d	3.91±0.13
Cys	0.15±0.06 ^{abc}	0.26±0.01 ^a	0.11±0.02 ^{bcd}	0.16±0.10 ^{ab}	0.17±0.06
Tyro	3.93±0.04 ^c	4.00±0.02 ^b	4.41±0.05 ^a	3.40±0.01 ^d	3.93±0.41
Val	4.53±0.03 ^{bc}	4.24±0.01 ^d	4.55±0.05 ^b	4.90±0.02 ^a	4.56±0.27
Meth	1.44±0.02 ^d	3.66±0.03 ^a	1.70±0.02 ^c	3.44±0.02 ^b	2.56±1.15
Ly	6.34±0.13 ^{cd}	6.68±0.08 ^b	6.35±0.06 ^c	7.58±0.03 ^a	6.74±0.58
Isol	4.34±0.06 ^{cd}	4.70±0.03 ^a	4.41±0.05 ^c	4.58±0.04 ^b	4.51±0.16
Leu	7.47±0.09 ^d	7.85±0.03 ^a	7.74±0.10 ^{ab}	7.72±0.09 ^{abc}	7.69±0.16
Phen	3.74±0.19 ^c	4.11±0.16 ^b	4.81±0.07 ^a	3.56±0.04 ^{cd}	4.06±0.55
∑EAA	46.33±0.37 ^{bcd}	46.65±0.18 ^{bc}	48.19±0.50 ^a	46.70±0.20 ^b	46.96±0.83
∑NEAA	49.65±0.41 ^{abc}	50.03±0.12 ^{ab}	49.22±0.57 ^{cd}	50.07±0.20 ^a	49.74±0.40
EAA: NEAA	0.93±0.02 ^b	0.93±0.01 ^{bcd}	0.98±0.02 ^a	0.93±0.01 ^{bc}	0.94±0.02

Note: different superscript in the same row indicates significant difference ($p < 0.05$); shared superscripts in the same row indicates similarity ($p > 0.05$); and $a > b > c > d$; [Asp= Aspartic acid, Se= Serine, Glu= Glutamic acid, Gly= Glycine, His= Histidine, Arg= Arginine, Threo= Threonine, Ala= Alanine, Prol= Proline, Cys= Cystine, Tyro= Tyrosine, Val= Valine, Meth= Methionine, Ly= Lysine, Isol= Isoleucine, Leu= Leucine, and Phen= Phenylalanine]

3.2 Successive reproductive performance of green mud crab

A total of 9 brood was reared and 100% of the brood

responded until successive third spawning, of which 77.78% of the broodstock was able to fertilize the egg mass for the third spawn (Table 3).

Table 3: Successive reproductive performance (Mean±SD) of the green mud crab *S. paramamosain* developed under captive condition

Reproductive parameters	Spawning event		
	1 st spawning	2 nd spawning	3 rd spawning
No of broods	9	9	9
Brood size (g)	380.39±36.67 ^a (340.60-436.40)	374.32±33.99 ^{ab} (334.60-426.40)	348.44±25.91 ^{abc} (320.60-395.50)
CW (cm)	11.26±0.92 ^a (9.70-12.40)	11.26±0.92 ^a (9.70-12.40)	11.26±0.92 ^a (9.70-12.40)
Mating to spawning interval (days)	43.56±17.06 (19.0-68.0)	N/A	N/A
1 st spawn to 2 nd spawn interval (days)	N/A	47.89±10.88 (35.0-65.0)	N/A
2 nd to 3 rd spawn interval (days)	N/A	N/A	55.86±7.86 (43.0-65.0)
Spawning success (%)	100 ^a	100 ^a	100 ^a
Fertilization success (%)	100 ^a	100 ^a	77.78 ^b
Egg mass color	Orange-reddish	Yellow-orange	Yellowish
Egg fertilization rate (%)	81.33±5.32 ^a (75.0-90.0)	75.56±9.38 ^{ab} (62.0-88.0)	63.43±8.77 ^c (49.00-74.00)
Egg diameter (mm)	0.31±0.01 ^a (0.29-0.32)	0.30±0.01 ^a (0.29-0.32)	0.30±0.01 ^a (0.29-0.32)
Incubation period (days)	9.89±0.60 ^c (9.0-11.0)	10.33±0.50 ^{bc} (10.0-11.0)	10.57±0.53 ^{ab} (10.0-11.0)
Fallen eggs (%)	5.03±0.77 ^c (4.0-6.0)	7.57±1.69 ^b (4.0-10.0)	13.43±2.82 ^a (9.0-18.0)
Fecundity (eggs/crab × 10 ⁶)	2.29±0.57 ^a (1.46-3.28)	2.05±0.25 ^{ab} (1.77-2.51)	1.37±0.16 ^c (1.10-1.57)
Relative fecundity (No. eggs/g BW)	5975±1115 ^a (4055-7519)	5486±390 ^{ab} (4765-5929)	3947±378 ^c (3456-4473)
Fertilization to hatching rate (%)	93.44±1.88 ^a (90.0-96.0)	87.00±3.74 ^b (82.0-92.0)	84.86±3.85 ^{bc} (80.0-90.0)
Production of viable larvae (No/crab × 10 ⁶)	1.73±0.41 ^a (1.24-2.42)	1.35±0.25 ^b (0.97-1.76)	0.74±0.15 ^c (0.47-0.93)
Phototaxis larvae (No/crab × 10 ⁶)	1.67±0.39 ^a (1.20-2.32)	1.17±0.20 ^b (0.89-1.48)	0.42±0.11 ^c (0.25-0.54)
Larvae size (mm)	2.18±0.01 ^a (2.16-2.19)	2.17±0.01 ^a (2.16-2.19)	2.17±0.01 ^a (2.16-2.19)

Note: N/A= not applicable; figures within the parentheses indicate ranges; different superscript in the same row indicates significant differences ($p < 0.05$); shared superscripts in the same row indicate similarity ($p > 0.05$); and $a > b > c$

The broodstock had completed spawning within 43.56 ± 17.06 days with the shortest spawning at 19 days after mating. The interval between first and second spawning and second to third spawning were 47.89 ± 10.88 days and 55.86 ± 7.86 days, respectively. Egg fertilization rate of $81.33 \pm 5.32\%$ in the first spawn were similar ($p > 0.05$) to the second spawn ($75.56 \pm 9.38\%$), but differed significantly ($p < 0.05$) with the third spawning event ($63.43 \pm 8.77\%$). The amount (%) of fallen eggs showed the significant increment ($p < 0.05$) among successive spawning with the lowest (5.03%) in the first spawning and highest (13.43%) for the third spawning. The fecundity showed a decreasing trend among the successive spawning with the highest (2.29×10^6) in the first spawning and lowest (1.37×10^6) in the third spawning. The fecundity showed statistically insignificant between first and second spawning but it was significantly different ($p < 0.05$) with the third spawning. The production of phototaxis larvae was $1.67 \pm 0.39 \times 10^6$, $1.17 \pm 0.20 \times 10^6$ and $0.42 \pm 0.11 \times 10^6$ for the

first, second and third spawning event, respectively, that showed significant difference ($p < 0.05$) between the successive spawning. The egg diameter and newly hatched larvae did not show any significant difference ($p > 0.05$) among successive spawning (Table 3).

3.3 Amino acid profiles in successive larvae sample of domesticated broodstock

Total essential amino acid (\sum EAA) in larvae sample showed gradual decreasing with significantly different ($p < 0.05$) between successive spawning (Table 4). The major amino acids that decreased in third spawned larvae included aspartic acid, isoleucine, leucine, phenylalanine and lysine. On the other hand, the total unessential amino acid (\sum NEAA) showed a significant increase ($p < 0.05$) in third spawned larvae in comparison to the first and second spawned larvae with an increase of glycine, alanine and proline (Table 4).

Table 4: Amino acid composition (% of total amino acids detected) of larvae samples (Mean \pm SD) under successive spawning of captive broodstock

Amino acids	Larvae type		
	1 st spawned larvae	2 nd spawned larvae	3 rd spawned larvae
Aspartic acid	14.84 \pm 0.26 ^a	12.63 \pm 0.16 ^b	11.54 \pm 0.53 ^c
Serine	3.63 \pm 0.43 ^{bc}	3.88 \pm 0.14 ^{ab}	4.31 \pm 0.31 ^a
Glutamic acid	16.52 \pm 0.27 ^a	14.95 \pm 0.22 ^{bc}	15.53 \pm 0.36 ^{ab}
Glycine	5.76 \pm 0.18 ^{bc}	5.93 \pm 0.04 ^b	7.53 \pm 0.33 ^a
*Histidine	2.05 \pm 0.09 ^a	1.93 \pm 0.19 ^{ab}	1.78 \pm 0.12 ^{abc}
*Arginine	3.29 \pm 0.27 ^b	2.84 \pm 0.09 ^{bc}	3.75 \pm 0.28 ^a
*Threonine	1.56 \pm 0.06 ^b	1.53 \pm 0.10 ^{bc}	2.36 \pm 0.08 ^a
Alanine	3.67 \pm 0.16 ^b	3.65 \pm 0.11 ^{bc}	4.67 \pm 0.13 ^a
Proline	9.17 \pm 0.15 ^c	11.02 \pm 0.14 ^b	17.85 \pm 0.15 ^a
Cystine	0.84 \pm 0.14 ^{ab}	0.91 \pm 0.08 ^a	0.69 \pm 0.10 ^{abc}
Tyrosine	0.93 \pm 0.04 ^c	1.13 \pm 0.12 ^b	1.35 \pm 0.06 ^a
*Valine	5.86 \pm 0.19 ^{bc}	5.90 \pm 0.43 ^b	7.09 \pm 0.39 ^a
*Methionine	1.88 \pm 0.09 ^b	1.65 \pm 0.45 ^{bc}	2.53 \pm 0.31 ^a
*Lysine	8.46 \pm 0.36 ^a	7.14 \pm 0.06 ^b	2.11 \pm 0.14 ^c
*Isoleucine	4.57 \pm 0.16 ^{ab}	4.63 \pm 0.04 ^a	0.68 \pm 0.06 ^c
*Leucine	7.22 \pm 0.22 ^{ab}	7.24 \pm 0.03 ^a	1.13 \pm 0.02 ^c
*Phenylalanine	3.55 \pm 0.17 ^a	3.31 \pm 0.02 ^{ab}	2.24 \pm 0.54 ^c
\sum EAA	38.44 \pm 0.75 ^a	36.17 \pm 0.30 ^b	23.66 \pm 0.22 ^c
\sum NEAA	55.35 \pm 0.32 ^b	54.10 \pm 0.53 ^c	63.48 \pm 0.83 ^a
\sum EAA: \sum NEAA	0.69 \pm 0.02 ^a	0.67 \pm 0.01 ^{ab}	0.37 \pm 0.01 ^c

Note: different superscript in the same row indicates significant difference ($p < 0.05$); shared superscripts in the same row indicate similarity ($p > 0.05$); and a>b>c>d; *EAA

3.4 Withstand of larvae against starvation

The larvae of the third spawning event showed rapid declining than the second and first spawning (Fig. 1). The third spawned larvae survived for 72 hours, whereas, the

second and first spawned larvae survived up to 108 hours and 120 hours, respectively. Fifty percent of the larvae was died within 24 hours for third spawning, but it was about 60 hours for both the second and first spawning (Fig. 1).

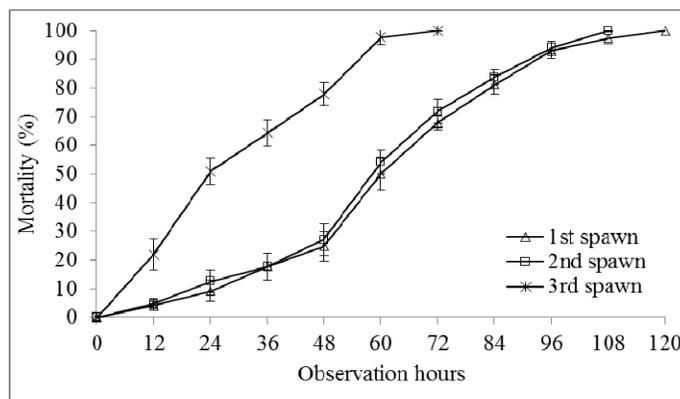


Fig 1: Mortality pattern of the green mud crab under starvation stress test

4. Discussion

4.1 Successive reproductive performance of green mud crab

Both quantitative and qualitative larvae production is the main concern for sustainable aquaculture for the mud crab species to ensure the profitability. The physiological condition and performance of the larvae in the decapod crustaceans has been revealed as the larval quality, which has been exclusively monitored from the general characteristics, viz., morphology at hatch, behavior of the larvae, stress resistance, biochemical composition and production (survival) [50]. Female mud crab is blessed with the ability to storage the sperms and capable to fertilize 2 to 3 successive batches of egg mass from a single event of mating [12, 35]. In this study, the same domesticated broods (Table 3) were treated with similar feeding schemes having alike nutrient contents (Table 1 and 2) up to the successive 3rd spawning from a single mating event. Despite the same age group, the broods had spawned at different intervals after mating and unlike interims in successive spawning. Such type of spawning differences as the general phenomenon in crustacean might associate with individual degree of maturation, the level of sex pheromones and secretion of spawning hormone from the Y-organs [6, 15, 17] and also due to the environmental factors like, temperature, light, salinity as well as nutrition [21, 40, 58].

It has been reported that the fecundity for the brachyurans usually declines with each successive spawning under a single mating event [16] and similar reports has also been demonstrated for mud crab of *Scylla serrata* [42, 48]. From this study, an apparent decrease in fecundity and relative fecundity has been noticed between first and second spawning, but not significantly different ($P>0.05$), whereas, differed significantly ($p<0.05$) with the third spawning (Table 3). The reasons behind this might due to the first life time spawning of the mother crab and some of the broods were found to extrude small quantity of eggs in the first spawning, but extruded large amounts for the second spawning. In this study, 100% percent of the broods responded in the successive third spawning, but fertilization success was 100% for the first and second spawning, whilst, 77.78% for the third spawning (Table 3). This lower fertilization success in the third spawning might associate with the inefficient utilization of the sperm by the respective spawner or might be associated with the limitation of sperm allocation during mating [53]. Virtually, in this study virgin males and females were employed for mating and were inefficient in reproductive mechanism. Some of the males repeatedly mated with different females within short intervals from previous mating. Such type of repeated mating without adequate interval for regeneration of spermatids might cause sperm limitations for fertilization of the successively spawned egg mass [53], as because 2 to 8 healthy spermatids has been reportedly required for fertilization of a single egg of the blue crabs [41]. In this study, egg mass color has changed from orange-reddish to yellowish under successive spawning and the production of viable or phototaxis larvae decreased significantly ($p<0.05$) under successive spawning (Table 3). Such type of color change in the egg mass might linked to the limitations in astaxanthin, which is regarded as the main component for the egg mass coloration [19, 29, 44]. A similar color changes in the egg mass under successive spawning was previously reported [42] and color of the egg mass was regarded as an indicator for egg quality [14, 42]. Whereas, some researcher mentioned no association of the egg color with the

quality of eggs and larvae [10]. In this study, the proportion of fallen egg was 5.03% for the first spawning and increased gradually to 7.57% for second and 13.43% for the third spawning (Table 3). The subsequent increase in fallen eggs under successive spawning might be related to low fertilization rate and long-time rearing of the broodstock under captive condition. In addition, egg dropping might happen due to the larger volume of eggs in comparison to the abdominal space to attach [26], poor attachment in the setae [33] and infestation of ciliates, protozoans, fungus and parasites [48]. In a study, a proportion of 5% to 22% of dropped eggs were mentioned during incubation of mud crab [10, 18].

From this study, the fecundity, relative fecundity, the quantity of viable larvae and the amount of phototaxis larvae of the domesticated mud crab for the first spawning were 2.29 ± 0.57 (eggs/crab $\times 10^6$), 5975 ± 1115 (No eggs/g BW), 1.73 (No/crab $\times 10^6$) and 1.67 (No/crab $\times 10^6$) (Table 3), showed consistent with the observation of the previous authors [12, 18, 32, 36, 48]. This study observed the decrease in reproductive performance of *Scylla paramamosain* under the successive spawning. Such type of lowered fecundity and viable larvae production under successive spawning were previously reported for giant mud crab *Scylla serrata* [42, 48] and for white shrimp *Litopenaeus vannamei* [4].

4.2 Amino acid profile in successively spawned larvae of green mud crab

The presence or absence of some amino acids could affect the egg and larvae quality in fishes [22, 25], in crustacean [2], in lobster [55] and in crabs [34, 60]. Although, the effect of specific amino acid is independent or interactive is not well understood. The main component of the egg yolk is vitellus. Furthermore, vitellogenin is composed with high amount of essential amino acids [25], which is the main precursor of yolk protein [5]. Amino acid profiles in the newly hatched larvae are reflected from the level of amino acids reserved in the eggs that subsequently transmitted to the larvae. About 70% of the free amino acid pool is being used as an energy source during oogenesis, whereas, the rest portion is used for further protein synthesis [54].

In this study, a gradual decrease in essential amino acids, especially, isoleucine, leucine, phenylalanine and lysine was noticed under successive spawning (Table 4). On the other hand, a degradation of the egg mass color, fecundity and larvae viability (Table 3) as well as reducing the larval capability to withstand against starvation for the third spawned larvae has been observed (Fig. 1). Lacked in methionine, arginine, isoleucine and cysteine in the unhatched egg was observed and treated these amino acids related to egg viability [60]. A decrease in essential amino acids of lysine, valine and histidine in newly hatched larvae of *Maja brachydactyla* in the successively spawned year end sample under captive condition was observed and mentioned as nutritional deficiency [2]. Likewise, the declining of arginine, histidine, lysine, methionine, tyrosine and threonine was noticed during embryogenesis of *Scylla serrata* [60]. On the other hand, the level of protein plays a crucial role as the source of energy to withstand against starvation and protein degradation is an important metabolic energy source during long starvation in crabs [13, 60]. However, findings of this study on amino acid profiles in successively spawned larvae and withstand of larvae against starvation is consistent with the observation of the previous researchers [2, 60].

5. Recommendation and conclusion

Development of exclusively captivated broodstock and evaluation of reproductive performance and larvae quality under successive spawning from a single event of mating is the first observation by this study. In spite of gradual declining of fecundity under successive spawning, the quality of larvae, its biochemical composition and capabilities to withstand for a longer period against starvation was reasonably. Though the amount of phototaxis larvae reduced with successive spawning, but up to the second spawning the amount of phototaxis larvae still sufficient enough to operate one larvae cycle with moderate stocking densities (50 to 100 larvae/l) [46, 59]. However, considering the amount of larvae, its physical characteristics and bio-chemical properties, this study suggested that the larvae up to the second spawning could be safely used for seed production purposes.

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