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Efficiency of Algal oil as a Feed Ingredient for the Development of Maturation Diet for the Spent *Penaeus monodon* (Fabricius) Spawners

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study designed to understand the effect of Algal oil as feed ingredients to attain reproductive success in the spent spawners of *P. monodon*. Five diets prepared were D1, D2, and D3 with 2.5%, 5% and 7.5% algal oil respectively, reference diet with fish oil and negative control diet without fish oil. The results showed that the maturation diet increased the moulting frequency and shortened the time of full maturation of the ovary. In D2 fed spawners, the duration of spawnings was minimized and the fecundity was also increased and the Gonado Somatic Index was also high. Compared with control diet, the abortive spawning and gaped ovary formed was also less in algal oil based diet (D2). D2 fed spawners also enhanced the percentage of the formed eggs, fertilized eggs and their hatching and also showed maximum prophenoloxidase activity. Though the reference diet had influenced in all reproductive parameters, the success was inferior to the experimental algal diet. The algal oil (5%) based diet was found to be very efficient in the reproductive success of spent spawners and therefore this diet can be used for the recycling of spent spawners.

Keywords: Shrimp hatchery; maturation diet; algal oil; bacterial clearance; gonado somatic Index.

1. INTRODUCTION

Aquaculture is the fast-growing industry in the world than any other rapidly developing animal feed producing industries. It is assumed that by 2022, aquaculture will provide a 35 % increase in production, compared to the current production [1]. This forecasted value of production can be achieved only through the production of young ones in hatcheries. The berried spawners are the prime requisite for the production of young ones. However if the berried spawners are indiscriminately hunted for hatcheries, the population of fin and shell fishes will be declining in the natural stock of the sea [2]. Generally, the hatchery operation of the tiger shrimp *P. monodon* depends on the wild caught brooders, where the availability of the gravid spawners is highly seasonal. Every hatchery spends more than 50 % running capital for the purchase of gravid spawners [3]. To overcome the above problem regarding the availability of the spawners either in finfish hatchery or in shellfish hatchery, the recycling of the spent spawners are very essential [4].

Reproductive exhaustion through a forced reproduction using eyestalk ablation in shrimp, *P. monodan* spawners will cause sudden changes in its body's biochemical composition mainly lipid and protein [5,6,7]. Consecutive spawns in a short duration significantly affect both the spawner's body cholesterol and triacylglycerides in the hepatopancreas [8] and therefore a maturation diet that supplies the rapid nutritional requirement during this period is very obligatory. Many researchers have turned their study towards the direction of formulating a maturation diet for fishes. Babu et al. in 2008 [9] have investigated the effect of a herbal maturation diet to overcome the forced spawning by eyestalk ablation. Babu and Marian [10] have attempted to overcome the defects of forced reproduction by co-feeding herbal product enriched Artemia to spent gravid spawners and that significantly improved the reproductive performance of larval quality 2.5 times more than the control group.

Algae have also been considered to be the important alternative feed ingredients for aquaculture. Algae have a lot of preferable character for being treated as a good candidate to offer rich protein, lipids and carbohydrates [11]. Patnaik *et al*. [12] found out the use of PUFA rich algal meal in diets for *Litopenaeus vannnamei* enhanced reproductive performance. Fish oil containing Omega-3 fatty acids are an important dietary component for most fishes. Thus, the alternative feed which contains omega-3 rich algal oil [13] in aquaculture is important as it improves the larval nutrition to achieve larval survival rates [14]. Algal cell extract produced from *Chlorella* [15,16], *Dunaliella* [17] and *Spirulina* [18] have already been commercialized. Algal lipids are highly significant at the early stages of fish larvae affecting the spawning and egg quality of many fish species. A deficiency in (n-3) unsaturated fatty acids affects fecundity, fertilization and hatching rates [19].

The indiscriminate fishing of the berried spawners in the sea, leads to the steep decline of the spawners which leads to the hike of the natural spawners price as well as short supply and therefore recycling of the spent spawners is an unavoidable need in shrimp hatcheries. The level of lipid in the hemolymph of the spawners is steeply declined soon after the spawning and advance stages of ovary development and hence an immediate replenishment of lipid is very

essential to make the spawners ready to spawn with quality eggs in a short time. The egg quality and reproductive success mainly depend on the supply of PUFA rich diet [20]. For reproductive success in the spent spawners of *P*. *monodon*, the supply of PUFA rich diet is most obligatory and hence a diet containing PUFA rich algal oil is required for the successful running of shrimp hatchery. The main objective of the present study is to formulate a maturation diet with PUFA rich algal oil for the reproductive success in spent *Penaeus monodon*.

2. MATERIALS AND METHODS

The feed ingredients given in the table were weighed and mixed well in a container by adding a sufficient quantity of distilled water and then the ingredients were made into a dough. The dough was then placed in a container and boiled in a pressure cooker for 20 minutes. After boiling, the dough was taken out of the container and after cooling, vitamins, algal oil (2.5 g (%), 5 g (%) and 7.5 g (%)) and mineral mixture were added separately into the dough and mixed well. Instead of algal oil, fish oil of 5 g weight was incorporated in the reference diet. The negative control contained neither algal oil nor fish oil (Table 1).

2.1 Preparation of Diets

The dough was then allowed to pass through a pelletizer having a perforation diameter of 1.5 mm dye. Then the reference diet (Fish oil), Negative control diet (without algal oil), as well as the experimental diet, were dried in a hot air oven at a temperature of 40° C for 15 hours. The dried pellets were collected and stored in airtight plastic containers.

2.2 Estimation of Protein, Lipid and Carbohydrate

The protein, lipid and carbohydrate composition of the spent *Penaeus monodon* spawners was estimated by the following methods.

2.2.1 Estimation of protein

Protein was estimated by the method of Lowry et. al. [21].

2.2.2 Estimation of lipid

Lipid was estimated by the method of Folch et al. [22]

2.2.3 Carbohydrate estimation

Carbohydrate was estimated by the method of Roe [23]

2.3 Experimental Design

In these ways, five separate diets were prepared. They were the reference diet (5 g (%) fish oil) and negative control (without any oil), and three experiment diets (D1- 2.5 g (%), algal oil, D2- 5 g (%), algal oil and D3- 7.5 g (%) algal oil. The spent spawners (egg laid spawners) of three different size groups 150-200, 201-250 and 251-300 g were selected for this study (Table 2). Once the spent spawners were found acclimated to the hatchery environment, the animals were uni-eye stalk ablated. The experimental feeding started from the day of the stocking into the maturation tank. The animals were fed on the basis of 15 -20% of its body weight. The control spawners were also uni-eye stalk ablated but are fed only with the negative control diet (without algal oil incorporated pellet diet). The reference diet spawners were fed with the diet having fish oil instead of algal oil. The experiment was repeated four times for each treatment. Five replicates were maintained for all experimental groups. The maturation tank was circular in shape that holds 7 ton water quality in capacity (r =1.5 m and h = 0.9 m).

2.4 Feeding

The spent spawners of three size groups (150-200, 201-250, 251-300) were selected for this experiment. Each group of the experiment consisted of 10 males and 10 females. The total body weights of the twenty animals were taken initially to allocate the feeding schedule. The animals were given the maturation diet based on 15- 20% body weight. The feeding was given three times daily; morning after water exchange (7 am), evening (4 pm) and night (10 pm). The feed uptake was calculated on a dry weight basis. Before feeding, the unfed were collected separately. The animals were fed till more than 90% of the animal spawned.

2.5 Moulting Frequency

The number of moulting in thirty days after the ablation was counted by tagging the animals in both experimental and control diet-fed groups. Four sets of experiment with twenty animals were studied and the experiment was repeated three times.

2.6 Algaloil Maturation Diet on Ovary Development

Reproductive success of feeding the algal oil maturation diet was assessed by feeding the maturation diet to three groups of the experimental spent spawners. The time of ovary development was determined by the times (days) required to observe the first stage ovary after ablation. The time for maturation was assessed by the times (days) required to become 4th stage ovary from the 1st stage ovary. The reference of the diet spent spawners were also subjected to all observation but fed only with a negative control diet (without algal oil).

2.7 Percentage of Clumping Eggs, Spawning and Gaped Ovary

The percentage of clumping (abortive spawning) and the percentage of spawning were studied by the method described by Babu *et al*., [24]. The percentage of clumping was calculated by weighing the clumped and individual eggs separately by an electronic balance. From the total of both clumped and individual eggs, the percentage of clumping was calculated. To study the percentage of spawning, the spawned eggs were collected separately and weighed. The remaining unspawned eggs were taken out along with the ovary by a longitudinal incision on the dorsal side of the animals. All eggs were carefully separated from the ovary and weighed. From the total weight of the spawned and unspawned (collected from an ovary) egg, the percentage of spawning was calculated. The number of gaps in an ovary was counted by holding the spawners in hands and showing them towards the light. This helps to make the ovary visible to count the number of gaps in the ovary.

2.8 Fecundity of Spent Spawners After Ablation

The spent spawners after ablation were fed with the experimental maturation diets. The eggs collected from the four subsequent spawnings on different days after ablation and fed with experimental diets were collected and counted by keeping the eggs in 100-litre plastic buckets under heavy aeration. This aeration keeps all the eggs in suspension and with a 10 ml beaker, five samples were collected from a different depth of the bucket. By counting the eggs obtained from the five samplings, the average of five counts is derived and thereby the total eggs were calculated for the 100-litre volume of water. In the same manner, the eggs obtained from II, III, IV spawning were counted and expressed in millions.

2.9 Study of Egg Quality

In this experiment, the eggs obtained in the 1st, 2nd and 3rd spawning after feeding with a maturation diet were studied for the percentage of the formed embryo, percentage of the fertilized eggs and percentage of the deformed embryo. The percentages of the formed eggs were calculated by observing the development of larvae in the egg after ten hours of spawning. This will be confirmed after referring to the normal developments of the fertilized eggs.

2.10 Gonadosomatic Index (GSI)

For gonadosomatic index (GSI) estimation, females were weighed individually after wiping it dry. The gonad was dissected out carefully and weighed by using an electronic balance. The GSI are calculated by using a formula. It is the calculation of gonadal weight to the proportion of body weight and it is calculated by the formula [25].

$$\text{Gonadosomatic index (GSI)} = \frac{Weight\ of\ Gonad}{Weight\ of\ Animal} \times 100$$

2.11 Algal oil Maturation Diet on the Survival of Nauplii

As the nauplii quality depends upon the quality of eggs, this experiment was conducted. By observing the larvae under a microscope every six hours interval, the percentage of stage conversion was assessed. The number of larvae that undergo metamorphosis into the next larval stage was considered as 100%. The substages among the nauplii stage were identified by the standard procedure obtained from the manual written by Treece and Yates [26]. The total number of nauplii entered into each substage was assessed and from this, the percentage survival in each sub stages was calculated.

2.12 Immunological effect of Maturation Diet

2.12.1 Preparation of bacterial solution

The bacteria such as *Vibrio harveyi*, *Vibrio parahemolyticus* and *Aeromonas* sp. were

cultured overnight in marine broth at 37° C and to prepare the solution for injection, cultures were pelleted at 6000 rpm for 10 minutes, washed three times in filtered (0.2 µm) and sterilized saline (3%), and resuspended in sterile saline to obtain a concentration of 5×10^6 cells/ml. Bacterial counts were determined using a bright-line hemocytometer at 100X.

2.12.2 Hemolymph sampling

Hemolymph was collected from the ventral part of the haemocoel of the second abdominal segment using 25 gauche needles and 1ml syringe filled with 0.2 ml cold modified Alsever's solution (AS;19.3 mM Na citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA (ethylene diamine tetraacetic acid): pH 7.2) as an anticoagulant. This prevents melanisation and keeps the hemocytes in a quiescent state [27]. The puncture procedure prevented the extraction of the tissue particle during the hemolymph sampling. Directly after sampling, the hemolymph was stored in 5 ml Eppendorf cups and kept on ice until analyzing within 1 hour after sampling. The hemolymph of the experimental, reference and control diet were collected.

2.12.3 Phenol oxidase activity

Pro-phenol oxidase activity in hemolymph samples was determined using L-dihydroxyphenylanine (L-DOPA) as a substrate [28]. 200 µl TBS was added to the experimental cuvette containing 30 µl of hemolymph sample. Then 60 µl of L-DOPA solution (1.6 mg/ml in TBS) was added followed by immediate mixing. An addition of 200 µl of TBS as a diluent and the enzyme activity was determined by measuring the absorbance of dopachrome at 490 nm against a blank containing 260 µl of TBS and 60 µl of L-DOPA. The absorbance was measured at 1 and 3 minutes after the addition of 200 µl of TBS. Prophenol oxidase enzyme activity was expressed as units, defined as the amount of enzyme giving an increase in absorbance at 490 nm of 0.001 per min/mg/protein.

2.13 Statistical Analysis

Statistical analysis was performed for all the experiments however, statistical analysis of peak values are represented in the Tables. The statistical analysis for each experiment was carried out using one way ANOVA followed by Tukey's multiple comparison tests with statistical significance set at $p<0.05$. All the analysis was performed with SPSS 11.5. Origin (version 6) software was used to represent data in the form of Tables.

3. RESULTS

3.1 Proximate Analysis

The feed ingredients and formulation is given in Table1.The experimental setup and feeding is given Table 2. The total protein, lipid and carbohydrate content was estimated for experimental (D1, D2, D3) and reference diet (Table 3). Based on the result, protein content was maximum in D3 with a 5% level of significance followed by other diets. Similarly, lipid content was also high in D2 (12±0.23) and D3 (13±0.42) at a significant level of $p<0.05$. Total carbohydrate content was also estimated for diets used, among which D1 and D2 showed maximum carbohydrate content with 42±0.34 and 41±0.02 respectively with a 5% level of significance.

Table 1. Feed formulation and Composition of Feed Ingredients (g/100g)

Feeding Ingredients	Types of feeds/ Amount of feed ingredients (g/100g)				
	D1	D2	D3	Reference diet	Negative control
Fish meal	40	40	40	40	40
Shrimp Head Meal	6	6	6	6	8.5
Soya bean meal	15	15	15	15	15
Groundnut oil cake	15	15	15	15	15
Wheat flour	12.5	10	7.5	10	12.5
Tapioca powder	5	5	5	5	5
Algal oil	2.5	5	7.5	-	-
Fish oil	-	-	-	5	-
Gelatin	2.5	2.5	2.5	2.5	2.5
Multivitamins	1	1	1	1	1
Minerals	0.5	0.5	0.5	0.5	0.5

Table 2. Feed type and Experimental diet

Name of Diet	Male and Female spent used for individual test	Triplicates	Total animal used in each diet
D1	20 (10 male and 10 female)	3	60
D2	20 (10 male and 10 female)	3	60
D3	20 (10 male and 10 female)	3	60
Reference Diet	20 (10 male and 10 female)	3	60
Negative Control	20 (10 male and 10 female)	3	60
		Total	300

Table 3. Efficiency of different diets on the Protein, Lipid and Carbohydrate content in Experimental, Reference and control spawners

	D1	D2	D3	Reference Diet	Negative control
Protein	47±0.98**	47±0.43**	48±0.20*	47±1.07**	47±0.73
Lipid	11±0.89**	12±0.23*	13±0.42*	11±0.12**	8±0.92
Carbohydrate	42±0.34*	41±0.02*	39±0.71**	42±0.49*	45±1.27

** Denotes significance at 5% level; ** denotes significance at 1%; ± indicates the standard deviation of three replicates*

D1 – 2.5 g (%) algal oil, D2 – 5 g (%) algal oil, D3 – 7.5 g (%) algal oil, Reference diet- 5 g (%) fish oil, Negative control

3.2 Feed Intake by the Spent Spawners

The trend of feed intake invariably differs as per the size of the spawners. The maximum feed consumption of all size range spawners (150-200, 201-250 and 251-300 g) consumed the maximum of 25.50, 37.68 and 44.72 g feed/day respectively. All size groups of experimental animals and positive control animals consumed more quantity of maturation diets (D1, D2 and D3) than the control. The reference diet-fed animal (positive control) consumed 20.66, 30.22 and 40.35 g feed/day respectively and all the parameter were statistically analyzed significant level was denoted in Table 4.

3.3 Moulting Frequency

In thirty days after the natural spawning and ablation, the ablated spawners under the size group 150- 200 g are moulted 2.16 times but the other size groups like 201- 250 and 251-300 g are moulted 1.07 and 1.05 times respectively. About diet, the ablated spawners under the size group 150-200 g are moulted 1.76 times and other size groups such as 201-250 g and 251-300 g moulted 0.94 and 0.88 times respectively. At the same time, the ablated control spawners of size group 150-200 g are moulted 1.67 times within 30 days after ablation, but the other size group fed with the control diet did not moult. All the values were statistically analysed (Fig 1).

3.4 Effect of Algal oil Maturation Diet on Reproductive Success

Three size groups of the experimental uni-stalk ablated spent spawners 150- 200, 201-250 and 251-300 g were selected for this study. The size of the spawners and algal oil maturation diet (D1, D2 and D3) had their combined effect on the quick development of the ovary after ablation. In the experimental spawners, the time taken for the start of ovary development in 150-200, 201-250 and 251-300 g size spawners after ablation and feeding with algal oil maturation diets were 6.35, 6.80 and 8.85 days respectively. But the negative control diet-fed spawners of size group 150-200, 201-250 and 251-300 g took 12.17, 17.65 and 15.10 days to attain the start of the development of the ovary. The reference diet-fed spawners of size group 150-200, 201-250 and 251-300 g took 10.26, 11.04 and 12.65 days to attain the start of the development of the ovary. The time taken to reach the 4^{th} stage from the 1^{st} stage also varied with the size of the animals and diet of the animals. The experimental spawners of size group 150-200, 201-250 and 251-300 g took 3.26, 3.80 and 3.17 days to reach the 4^{th} stage from the 1^{st} stage. All the experimental values were analysed using a statistical tool, the reference diet and negative control diet-fed spawners of the same size group took 7.44, 7.36, 6.28 and 11.72, 9.63, 8.36 days respectively to attain the 4^{th} stage from 1^{st} stage (Table 5).

Table 4. Feed in take by the spent Spawners

Animal size (g)	Feed intake (gram/day)				
	D1**	D2*	D3*	Reference diet**	Negative control
150-200	20.02±1.72	25.50±4.80[a]	24.83±2.01[a]	20.66±1.88	17.68±4.65
201-250	32.84±1.28	37.68±3.52[b]	36.33±2.09[b]	30.22±2.07	30.17±4.80
251-300	40.00±2.44	44.72±4.25[c]	43.83±1.92[c]	40.35±1.71	38.65±3.84

** Denotes significance at 5% level; ** denotes significance at 1% level different alphabet between experiment denotes significance at 5% level using Tukey's multiple comparison test; ± indicates the standard deviation of three replicates*

D1 – 2.5 g (%) algal oil, D2 – 5 g (%) algal oil, D3 – 7.5 g (%) algal oil, Reference diet- 5 g (%) fish oil, Negative control

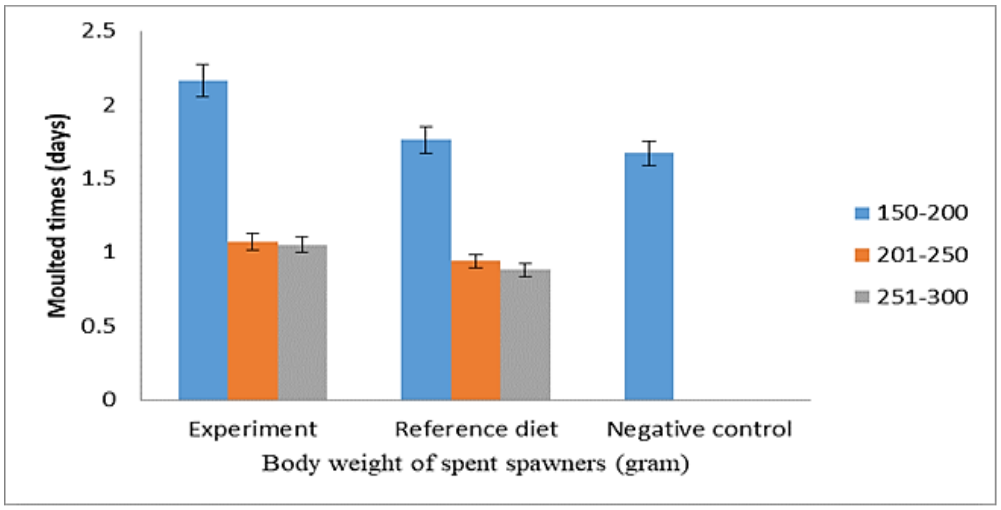

Fig. 1. The effect of different diets on Moulting frequency after ablation

Table 5. Effect of algal oil maturation diet on reproductive success in *P. monodon* spent spawner after uni-stalk ablation

Animal size (gm)	Time for start of ovary development after ablation (days)				
	D1*	D2**	D3**	Reference diet*	Negative control
150-200	9.35±1.09[a]	6.35±1.50	6.42±1.13	10.26±1.71[a]	12.17±2.30
201-250	9.14±0.95[a]	6.80±1.35	6.85±0.91	11.04±1.58[b]	17.65±2.13
251-300	10.82±1.46[b]	8.85±1.26	8.95±1.53	12.65±1.66[bc]	15.10±2.48
Animal size (gm)	**Time for maturation of ovary 1st – 4th stage (days)**				
	D1*	D2**	D3**	Reference diet*	Negative control
150-200	6.62±1.60[a]	3.26±1.36	3.50±1.41	7.44±1.26b	11.72±2.16
201-250	6.81±1.63[a]	3.80±0.97	3.95±1.64	7.36±1.47b	9.63±1.56
251-300	6.09±0.78[a]	3.17±0.83	3.22±0.84	6.28±1.10a	8.36±1.53

** Denotes significance at 5% level; ** denotes significance at 1% level; different alphabet between experiment denotes significance at 5% level using Tukey's multiple comparison test; ± indicates the standard deviation of three replicates*

D1 – 2.5 g (%) algal oil, D2 – 5 g (%) algal oil, D3 – 7.5 g (%) algal oil, Reference diet- 5 g (%) fish oil, Negative control

3.5 Clumping of Eggs or Abortive Spawning

The percentage of clumping of eggs (or) abortive spawning was studied in the experimental diet (D1, D2 and D3), reference diet-fed and control spent spawners for three subsequent spawnings after the first natural spawning and all the values were statistically analysed. In experimental spawners, the percentage of clumping and percentage of spawning in the 1st, 2nd and 3rd spawning were 5.61, 7.81, 10.54 and 95.4,

88.52, 85.18 respectively and there was no gap found in the ovary of experimental spawners. About diet-fed spawners, the percentage of clumping and percentage of spawning were 7.89, 10.23, 18.92 and 84.87, 76.88, 72.56 respectively in the 1st, 2nd, and 3rd spawning. The number of gaps in the ovary was 0.98, 1.32, and 1.42 in the 1st, 2nd, and 3rd spawning respectively. In negative control spawners, the percentages of clumping and spawning gaped ovary was 20.72, 25.18, 30.54 and 70.68, 60.85, 53.14 respectively in the 1st, 2nd, and 3rd spawning. The number of gaps in the ovary was 1.14, 2.17 and 2.83 respectively in the 1st, 2nd, and 3rd spawning (Fig. 2).

3.6 Fecundity of Spent Spawners after Ablation

The fecundity of the spent experimental (D1, D2 and D3), reference diet and control animals was assessed continuously for four subsequent spawnings. The duration between the two adjacent spawnings was also assessed in both experimental, reference diet and control spawners. The fecundity of the experimental spawners fed with D2 in I, II, III and IV spawning were 0.93, 0.76, 0.64 and 0.57 million eggs respectively. About diet, the fecundity of spawners in I, II and III spawning were 0.72, 0.43, 0.31 and 0.17. In negative control diet-fed spawners, the fecundity in I, II and III spawning were 0.61, 0.32 and 0.17 million respectively and no IV spawning in negative control groups. The total fecundity after ablation in experimental animals was 2.9 million eggs. The total fecundity in the reference diet and negative control animals was 1.63 and 1.10 million eggs. The time duration between 0&I, I&II, II& III, III &IV were 7.47, 5.12, 4.48, 3.69 days respectively in experimental spawning and for reference diet and negative control, the durations were 12.19, 7.55, 8.67 and 15.72, 9.64, 11.72 respectively in I, II and III spawning and significant level among the experiment was mentioned in Table 6.

3.7 Algal Oil Maturation Diet on Egg Quality

In the experimental diet (D2), the percentage of formed eggs obtained in the I, II and III spawning was 94.17, 87.40, and 85.18 respectively at a 5% level of significance. About the diet, the percentage of formed eggs were 72, 67.33 and 58.33 in the I, II and III spawning respectively. In negative control spawners, the percentage of formed eggs were 65.68, 42.14 and 33.10 % in I, II and III spawning respectively. The percentage of fertilized eggs in the experimental diet (D2) fed spawners were 92.60, 80.05, 76.54 respectively in I, II and III spawning. But about diet and negative control, the percentage of formed eggs were 75.67 and 63.66, 44.67 and 65.84, 56.13 and 41.65 respectively in I, II and III spawning with the significant value of $p<0.05$. The percentage of hatching in experimental diet (D2) fed spawners was maximum (92.36) in the first spawning followed by 85.60 and 82. 48 in the II and III spawnings. About diet, the maximum percentage of hatching (75.83) was observed in the I spawning followed by 68.67, 60.83 respectively in the II and III spawnings. In the negative control, the maximum percentage of hatching (62.89) was observed in the I spawning followed by 40.45 % in the II spawning and 30.84 % in the III spawning (Table 7).

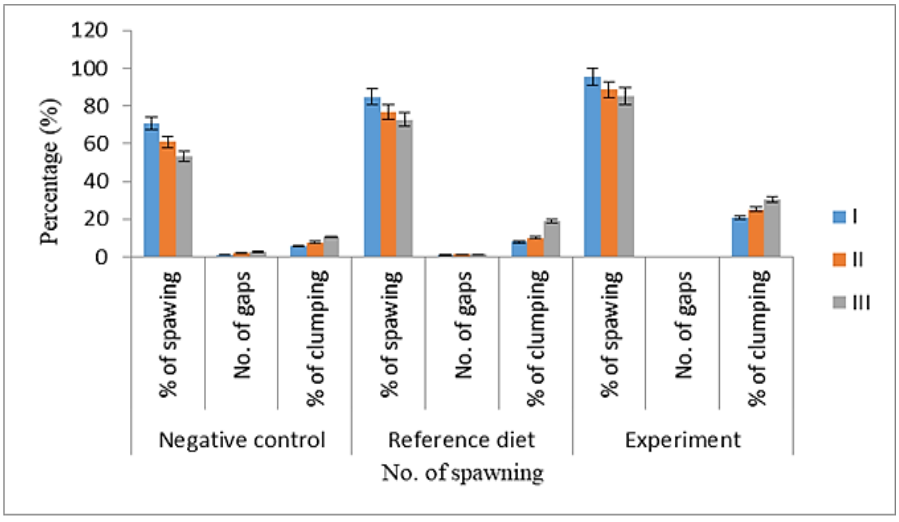

Fig. 2. The effect of maturation diet on spawning quality

Table 6. The efficiency of different diets on fecundity and duration of subsequent spawning of spent spawners after ablation

Time of Spawning	Fecundity of Spawner (Million)				
	D1**	D2*	D3*	Reference diet**	Negative control
I	0.48±0.02	0.93±0.14^{bc}	0.85±0.07^{bc}	0.72±0.09	0.61±0.18
II	0.20±0.01	0.76±0.12^{b}	0.60±0.01^{ab}	0.43±0.01	0.32±0.16
III	0.43±0.00	0.64±0.09^{ab}	0.56±0.01^{a}	0.31±0.00	0.17±0.04
IV	0.22±0.00	0.57±0.17^{a}	0.46±0.01^{a}	0.17±0.12	0
Total eggs in four Spawning	1.36±0.08	2.9	2.47±0.09	1.63±0.02	1.10
Time of Spawning	Duration between two spawning after ablation (days)				
	D1*	D2**	D3**	Reference diet**	Negative control
I	9.31±1.25 ^{bc}	7.47±2.61	7.67±1.33	12.19±1.78	15.72±2.77
II	8.69±0.20 ^{b}	5.12±1.68	5.81±0.02	7.55±0.55	9.64±2.05
III	7.85±0.02^{ab}	4.48±1.07	4.64±0.32	8.67±1.26	11.72±1.86
IV	5.19±0.18^{a}	3.69±1.80	3.78±0.69	0	0

** Denotes significance at 5% level; ** denotes significance at 1% level; different alphabet between experiment denotes significance at 5% level using Tukey's multiple comparison test; ± indicates the standard deviation of three replicates*

D1 – 2.5 g (%) algal oil, D2 – 5 g (%) algal oil, D3 – 7.5 g (%) algal oil, Reference diet- 5 g (%) fish oil, Negative control

Table 7. Effect of maturation diet on egg quality

Time of spawning	Percentage of formed egg				
	D1**	D2*	D3*	Reference diet**	Negative control
I	69.67±2.32	94.17±2.67^{b}	93±3.74 ^{b}	72±2.94	65.68±4.50
II	57.83±3.56	87.40±3.58^{ab}	85.33±3.29 ^{ab}	67.33±4.10	42.14±3.65
III	43±1.63	85.18±3.43^{a}	83±1.63 ^{a}	58.33±3.68	33.10±5.86
	Percentage of fertilized egg				
	D1**	D2*	D3*	Reference diet**	Negative control
I	74.5±2.48	92.60±3.48^{c}	90.3±2.05 ^{c}	75.67±3.68	65.84±4.65
II	62±2.94	80.05±3.89^{b}	78.67±3.29 ^{b}	63.66±3.09	56.13±4.70
III	51.16±1.43	76.54±3.90^{a}	74.33±1.24 ^{a}	44.67±2.49	41.65±3.86
	Percentage of Hatching				
	D1**	D2*	D3*	Reference diet**	Negative control
I	78.33±1.24	92.36±3.18^{c}	91.33±2.62 ^{c}	75.83±1.02	62.89±5.50
II	70.83±2.71	85.60±4.12^{b}	84.67±3.09 ^{b}	68.67±1.69	40.45±3.69
III	60.83±2.09	82.48±4.03^{a}	85.83±2.39 ^{a}	60.83±1.84	30.84±5.72

** denotes significance at 5% level; ** denotes significance at 1% level; different alphabet between experiment denotes significance at 5% level using Tukey's multiple comparison test; ± indicates the standard deviation of three replicates*

D1 – 2.5 g (%) algal oil, D2 – 5 g (%) algal oil, D3 – 7.5 g (%) algal oil, Reference diet- 5 g (%) fish oil, Negative control

3.8 Gonadosomatic Index (%)

The gonadosomatic indices in the experimental spawners were 6.84, 5.76 and 5.27% in I, II and III spawning respectively. Though the same trend of decrease was observed in the subsequent spawning, the indices were found to be less than the experimental spawners. The GSI in the reference diet and negative control were 6.32, 5.08, 4.86 and 5.26, 3.48, 2.54 respectively in the I, II and III spawning (Fig. 3).

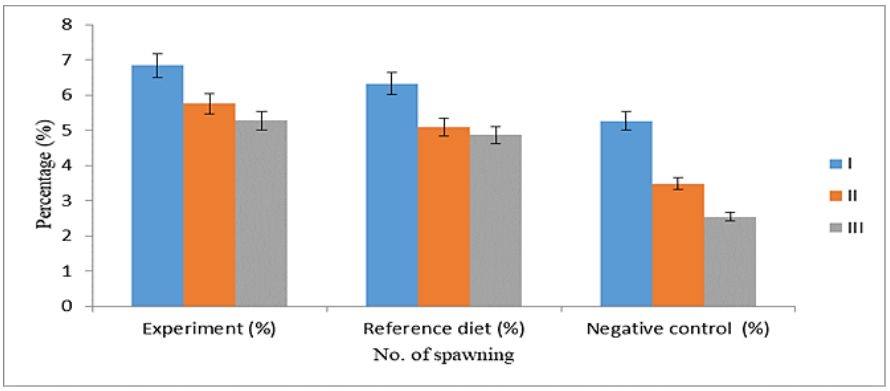

Fig. 3. The effect of different diets on Gonadosomatic index (%)

3.9 Effect of algal oil maturation diet on the survival of Nauplii

To study the efficiency of the algal oil maturation diet on egg quality, the percentage of metamorphosis from N6-Z1 is developed through feeding with an algal oil maturation diet. The study was carried out in terms of the percentage of larval metamorphosis from N1-Z1. In the experimental animals, the percentage of conversion from N6-Z1 was 80.34, but for diet and negative control, it was only 68.83 and 52.68 respectively. The percentage of larval tolerances in sudden hyposaline shock in the larvae obtained from the experimental diet (D2) fed spawners were 80.12, 77.25, 74.55, 73.40, 70.19 and 72.04 respectively in N1, N2, N3, N4, N5 and N6. But about diet and negative control, the stress tolerances of larvae were found to be inferior to the larvae obtained from the experimental diet-fed spawners and they were statistically proved (Table 8).

3.10 Lipid Estimation

The body lipid level in the experimental, reference diet and control animals (251-300 g animal size) during different developmental stages of the ovary was studied. The lipid level of 22.23, 20.10 and 15.62% were recorded in the experimental diet (D2) at a 5% level of significance during the early, ripening and ripened stages respectively of ovary development. The reference diet and control diet-fed animals showed less body lipid when compared with the experimental diet-fed spawners (Table 9).

Table 8. Influence of algal oil maturation diet on Nauplii survival

Larval stage	Percentage of stage conversion				
	D1**	**D2***	**D3***	**Reference diet****	**Negative control**
N1- N2	76.68±2.84	92.67±4.80^{d}	83.83±1.64 ab	72±2.94	65.43±5.77
N2- N3	80.16±2.01	90.50±3.86^{cd}	89.5±1.08^{c}	64.66±2.86	77.50±5.05
N3- N4	70.16±1.84	86.43±4.05^{bc}	85.83±2.46 ^{bc}	68.33±3.68	68.10±4.93
N4- N5	78.66±1.31	85.14±3.54^{b}	83.16±1.31 ^{ab}	72±2.94	56.15±4.80
N5- N6	65.83±1.43	83.26±3.48ab	82.16±2.24 ^{ab}	62.5±1.77	58.45±4.13
N6- Z1	70.66±1.31	80.34±3.45^{a}	79.33±1.24 ^{a}	68.83±3.27	52.68±5.17
Larval stage	**Percentage of Larval Tolerance (Hypo salinity (35-10ppt)**				
	D1**	**D2***	**D3***	**Reference diet****	**Negative control**
N 1	68.83±1.64	80.12±4.80^{d}	79.5±2.54 ^{c}	72.33±2.62	42.50±5.73
N2	60.16±1.84	77.25±3.95^{cd}	76.83±1.92 ^{bc}	63.5±3.18	40.68±4.80
N3	60.83±2.09	74.55±4.08^{bc}	73.83±3.88 ^{ab}	62.16±1.43	43.49±4.20
N4	68.5±1.87	73.40±4.05^{bc}	72.66±2.49 ^{ab}	65.33±2.49	47.70±4.26
N5	62.67±2.05	70.19±4.55^{a}	69.33±3.68 ^{a}	60.5±3.08	45.30±5.64
N6	60.5±1.77	72. 04±3.13^{b}	71.67±2.62 ^{a}	61.83±3.56	40.40±4.83

** denotes significance at 5% level; ** denotes significance at 1% level; different alphabet between experiment denotes significance at 5% level using Tukey's multiple comparison test; ± indicates the standard deviation of three replicates*

D1 – 2.5 g (%) algal oil, D2 – 5 g (%) algal oil, D3 – 7.5 g (%) algal oil, Reference diet- 5 g (%) fish oil, Negative control

Table 9. The efficiency of Algal oil maturation diet on body lipid level during different stages of Ovary development in *P. monodon*

Maturation stage	D1**	D2*	D3*	Reference diet**	Negative control
Early developmental stages of ovary	17.16±1.02	22.23±0.82^{bc}	21.03±1.61^{bc}	15.5±1.47	12.25±0.50
Ripening stage of ovary	15.7±0.83	20.10±0.43^{b}	19.83±0.62^{b}	16.83±1.31	10.58±0.61
Ripened stage of ovary	12.43±1.02	15.62±0.56^{a}	14.1±0.82^{a}	12.33±0.47	8.12±0.48

** denotes significance at 5% level; ** denotes significance at 1% level; different alphabet between experiment denotes significance at 5% level using Tukey's multiple comparison test; ± indicates the standard deviation of three replicates*

D1 – 2.5 g (%) algal oil, D2 – 5 g (%) algal oil, D3 – 7.5 g (%) algal oil, Reference diet- 5 g (%) fish oil, Negative control

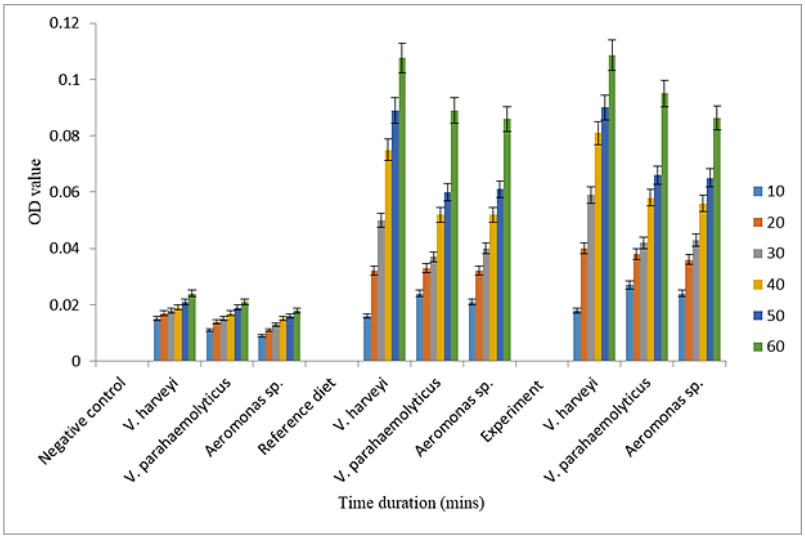

Fig. 4. The prophenol oxidase activity of haemolymph of *P. monodan* after challenging with bacterial pathogens

3.11 Prophenol Oxidase Activity Assay

The *Aeromonas* sp., *Vibrio harveyi* and *Vibrio parahaemolyticus* were injected into the hemolymph of spent spawners of size group 251-300 g. The prophenol enzyme assay was conducted in the hemolymph at different time intervals from 10 minutes to 60 minutes. The spectroscopic absorbance of phenoloxidase in the hemolymph was taken every 10 seconds. The results showed that the enzyme activity increased gradually for all the pathogens when the time duration of the injection was increased. After 1 hour of injection, particularly the data taken at 50 seconds, *Vibrio harveyi* showed the highest activity of prophenoloxidase enzyme with 0.1085±0.046 at a 5% levels of significance, followed by *Vibrio parahaemolyticus* (0.0952±0.068) and *Aeromonas* sp. (0.0864±0.078). In reference diet-fed animals, the highest prophenol oxidase enzyme activity for *Vibrio harveyi was* 0.1076±0.058 and followed by *Vibrio parahaemolyticus* (0.0891±0.075) and *Aeromonas* sp. (0.0859±0.078), but the negative control showed very low prophenol oxidase enzyme activity for *Vibrio harveyi* was 0.0242±0.036 and followed by *Vibrio parahaemolyticus* (0.0212 ± 0.021) and *Aeromonas* sp. (0.0181 ± 0.028) respectively (Fig. 4).

4. DISCUSSION

Food intake is one of the criteria to assess the efficiency of any feed and the diet which is preferred by any animal, should increase the level of feed intake and hence the assessment of feed intake has been accounted for in the present experiment. The result showed that the diet intake was maximum in the experimental diet than the reference and control diets and this proved the efficiency of the experimental diet.

The moulting frequency mainly depends upon the size of the spawners as well as the maturation diet. The moulting frequencies in the

small-sized animals are more than the larger animals, but irrespective of the size of the animal, the maturation diet had influenced the moulting frequency. In the early stages of the crustaceans, the number of moulting is more than the older ones. The present result corroborates the study of Botsford [29]. In his study, it is stated that the duration of the intermolt depends upon the size and the age of the animals. But the maturation diet influenced the animal to increase the moulting frequency irrespective of the size. The hormone responsible for the moulting may be activated by the influence of factors in the algal oil [30,31].

The reproductive success in terms of time for ovary development and time for maturation of ovary were studied to understand the efficiency of maturation diet on the experimental, reference and negative control spawners. In the experimental spawners, the time needed for the initiation of the development of the ovary (1st stage) was minimum than that of the reference and negative control spawners. But, the size of spawners also plays an important role in the initial development of the ovary. The time required for the initiation of the ovary development decreases with an increase in the size of spawners. The time for full maturation of the ovary (4th stage) also depends upon the effect of maturation diet as well as the size of animals. The maturation diet evoked different responses in spawners having different body size. The spawners of higher size reached the final stage of the ovary development in a short time than the spawners of less body weight. But they invariably revealed a positive response to the maturation diet when compared with the reference diet and negative control.

During spawning, the eggs are released in two types. One is by normal spawning, in which the eggs are spawned individually and the other type called abortive or defective spawning where the eggs are released in a clump (or) cluster (Babu, 2001). The later type of spawning happens due to malnutrition and infection. This is most prevalent in captive induced spawning where the spawners are induced to spawn in the captive environment [32]. The algal oil incorporated maturation diet reduced the percentage of clumping when compared with the reference diet and control. The clumping percentage decreased to a considerable level in maturation diet-fed spawners, but due to the subsequent spawning, the lack of required food reserve in their body leads to 10.5 % clumping in the 3rd spawning. But in the reference diet and negative control fed spawners, the percentage of clumping in the 3rd spawning was 18.92 and 30.54 % respectively, which showed the efficiency of the maturation diets.

The algal oil maturation diet enhanced the fecundity in all four spawnings after ablation. The total eggs in one experimental diet fed spawner were 2.9 million, but for the reference diet and negative control, the total eggs were only 1.63 and 1.10 million respectively. Four spawnings were obtained in the experimental animals after ablation, but only three spawnings were obtained in control after ablation. The time gap between the two adjacent spawnings was less in the experimental animals than the reference diet and negative control animals. The reason for the decrease in the fecundity in the reference and negative control may be due to the forced reproduction by eyestalk ablation, that exhaust the biochemical reserves in the body of the ablated animals during spawning and this has been reported by several researchers in *P. monodon* [33,34,35] But in the experimental animals, the algal oil refills the required reserve in the subsequent spawning. This result is supported by a study reported by Babu *et al*. [36]. In this study, the forced reproduction was overcome by co-feeding the spawners with a herbal maturation diet.

The quality of eggs was assessed by the development of formed and deformed embryo in the eggs, percentage of egg fertilization and percentage of hatching. These three qualities determining the factors were found in favour of the maturation diet-fed spawners than the reference diet and control. Though the deformed larvae in the experimental spawners were reported in the 2nd and 3rd spawning due to the effect of multiple spawning, the percentage of deformities was minimum in the experimental animals than the reference diet and control. The same trends were noticed in the fertilization percentage and percentage of hatching.

The gonado somatic index in the female spawners was high in the maturation diet-fed animals. Despite the index reduced during the subsequent spawning (2nd and 3rd), the index was higher than the reference diet and control. In this experiment, the percentage of stage conversion in the larvae obtained from the experimental diet-fed spawners were found to be

superior to the larvae obtained from the reference diet and negative control fed spawners. An earlier report of [37] stated that the *L. vannamei* juveniles and post larvae showed a good growth performance when DHA is present in their diet but a recent report stated that the post-larvae of *L*. *vannamei* showed five times higher level of growth performance when DHA is present in the diet [38].

Another way of defining the reproductive success of any maturation diet is by studying the quality of nauplii obtained from the spawners, fed with a maturation diet and by the percentage of stage conversion (N1-Z1). This has been evidenced in the larvae obtained from the experimental spawners than the reference diet and negative control and when the nauplii of each stage were subjected to a sudden hyposaline shock, the reduction in the survival was common in both the experimental, reference diet and control larvae, but the percentage of survival was more in the experimental larvae when compared to the reference diet and control. Alvarez et al. [39] stated that the survival to salinity stress has been found positively correlated to the size of post-larvae of the shrimp. Correlating to this statement, Castile *et al*. [40] reported that the increased growth during post-larval stages has been related to the continued growth during the juvenile stages.

The percentage of spawning (or) the percentage of egg release from the ovary depends upon the physical condition, the stress of spawners, the nature of spawning environment as well as the lipid level in the body [24]. This experimental diet increased the percentage of spawning than the reference and negative control diet. The gaped ovary is another important type of problem observed in spawners. Though this has been sparsely reported by the researchers, it plays a major role in reproduction such as shrimp spawning [24]. The number of gaps in the matured ovary was found to be reduced by the experimental diet.

The body lipid level in the experimental, reference diet and negative control animals during different developmental stages of the ovary was studied. All three size groups of the reference diet and negative control animals showed less body lipid when compared with the experimental diet-fed spawners. Santigosa *et al*. [41] stated that the EPA and DHA are given by this microalgal oil are well digestible and deposited in the filet thereby promoting the growth of the fish.

The experimental diet fee spawners showed a good prophenoloxidase activity than that of the reference diet and negative control. The earlier report of Nonwachai *et al*. (2010) reported that the feeding of shrimp with microalgae will improve disease tolerance. The algal oil containing a high amount of LC-PUFAs (docosahexaenoic acid and arachidonic acid) will significantly improve the immune parameters such as the total haemocyte count, phenoloxidase activity, superoxide dismutase activity, and bactericidal activity in the post-larval stage of the *Litopenaeus vannamei*, thus resulting in the improved survival rates against *V. harveyi* infection.

5. CONCLUSION

This study concludes that the algal oil incorporated maturation diet improved the spawner health and reproductive success and therefore reduces the number of spawner requirement in shrimp hatcheries because the hatchery people spend more than 50 % of their running cost for the purchase of natural spawners and the recycling of spawners with maturation diet in turn reduces the hunt of berried spawners from the sea. This study also proves that the exhaust of energy due to the forced reproduction can be met by feeding the spawner with the algal oil incorporated maturation diet and hence the algal oil can be used in shrimp hatchery for the successful spawner development.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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