Original Research

Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in
Journal of Environmental Biology

JEB™ p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/41/5(SI)/MS_17

Effect of different frozen fresh diets to broodstock growth, reproductive performance and larvae of cleaner shrimp, *Lysmata amboinensis*

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Paper received: 14.01.2020 *Revised received*: 17.03.2020 *Accepted*: 14.08.2020

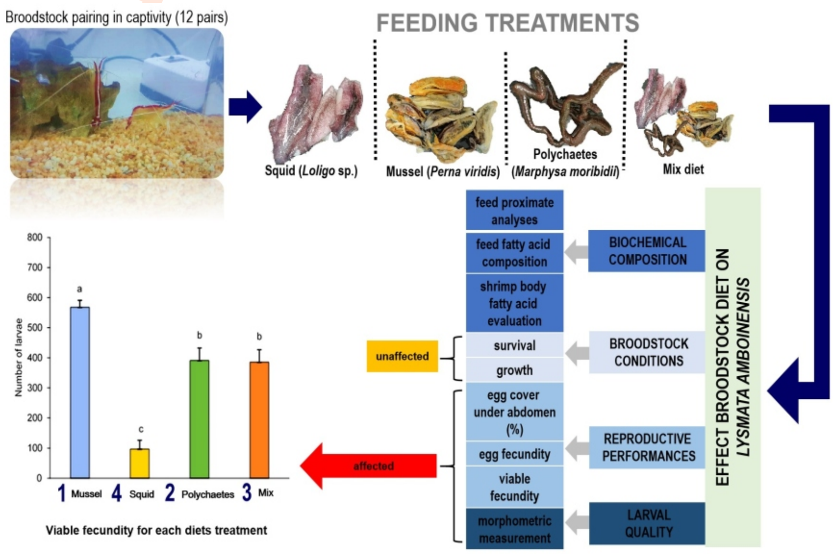
Abstract

Aim: To evaluate the effects of different frozen fresh diets of frozen mussel, squid, polychaetes and the mixture of all feeds) to *L. amboinensis* broodstock.

Methodology : Four diets, comprised of squid (*Loligo* sp.), mussel (*Perna viridis*), polychaetes (*Marphys amoribidii*) separately, as well as the mixture of all four separately were fed to *L. amboinensis* brood stocks. The results were evaluated based on the biochemical compositions (proximate analyses and fatty acid composition of feed), broodstock conditions (survival and growth), reproductive performances (egg cover under abdomen (%), egg fecundity and viable fecundity) and larval quality (morphometric measurement).

Results : Mussel diet produced the highest number of larvae, followed by polychaetes and mix diets. On the contrary, broodstock nourished with squid diet had suffered a heavy loss of eggs throughout the incubation period, with lowered larvae production ($p < 0.05$). The results demonstrated a good relationship between the MUFA content in the diet from the egg produced from this cleaner shrimp whereas the level of 22: 6 n-3 (DHA) in the diet exhibited close relation with egg retention throughout the incubation period.

Interpretation : Broodstock diet influences the egg cover during incubation, egg fecundity, viable fecundity and output in terms of *L. amboinensis* larval morphometric. MUFA has several potential functions in the embryonic development process related to fecundity, while DHA has different functions in early embryogenesis, where it is associated with egg hatchability and larval morphometric of *L. amboinensis.*



Key words : Broodstock, *Lysmata amboinensis,* Reproductive performance, Shrimp

How to cite : Omar, W.N.A., A. Arshad, S.M.N. Amin and A. Christianus: Effect of different frozen fresh diets to broodstock growth, reproductive performance and larvae of *Lysmata amboinensis*. *J. Environ. Biol.*, **41**, 1249-1256 (2020).

Introduction

The marine ornamental trade started in 1930s, and currently the rising popularity has made it a global multi-million Euro industry (Rhyne *et al*., 2012; Leal *et al*., 2016b). Presently, most of the ornamental species captured from coral reefs area have been documented to be critically endangered are due to direct or indirect threats associated with human activities (Leal *et al*., 2012; Thornhill, 2012), leading to biodiversity reduction and ecological imbalances (Lin *et al*., 2002; Bunting, 2003). Captive breeding of ornamental species lessen strain on coral reefs besides meeting the increasing market demands (Leal *et al*., 2016a). In recent years, ornamental shrimp has become one of the top traded invertebrates along, with some species of genera *Lysmata* and *Stenopus*, which represents the highest commodity of shrimps traded in the marine aquarium industry (Calado *et al*., 2017). The increasing demand for certain species, which consists of high value white-striped cleaner shrimp, *L. amboinensis*, have threatened the sustainability of this species. *L. amboinensis* known as protandric simultaneous hermaphrodite (PSH) (Bauer, 2000), where it is born as a male and later, it will change into simultaneous, true hermaphroditism, which omits self-fertilization (Fiedler, 1998). Therefore, the energetic requirements of oogenesis and spermatogenesis must be satisfied via brood stock diets (Calado *et al*., 2009). With the exception of certain species, particularly in the genus *Macrobrachium*, the knowledge and information of nutritional requirements for Caridean shrimp broodstock (Harrison, 1990) including *L. amboinensis* are still scarce. In view of the above, this research was conducted with the aim to assess the effects of widely available diets (fresh frozen mussel, squid, and polychaetes, as well as mixture of mentioned feeds) to *L. amboinensis* broodstock that can minimize the production cost, and the effects were evaluated based on broodstock conditions, reproductive performances, larval quality and biochemical compositions via proximate analyses and fatty acid profile.

Materials and Methods

Broodstock maintenance and collection of larvae : This work used following acclimation, the 12 pairs of *L. amboinensis* brood stock shrimp brood stocks with average total length (45.95 ± 0.59 mm), ($p < 0.05$) were paired and transferred to the experimental tanks (0.40 m × 0.21 m × 0.29 m) using under gravel system with constant aeration. Water temperature (25 °C ± 1 °C), salinity (30 ppt) and pH (7.98 ± 0.1) were stable throughout the experimental period. The aquaria were provided with around 2-cm thick layer small pebbles as a substrate of and dead corals for shelter with proper monitoring of ammonia and nitrites .Brood stocks were fed with chopped squid, mussel, and polychaetes ad *libitum* once dailyor on alternate days. Shrimp with eggs in complete embryo stage were separated and individually held in hatching tank with proper aeration. The larvae were gathered using light trap and gently harvested using a sieve (300 µm) and measured.

Experimental procedure : The four dietary treatments with three replicates entailing two shrimps.

Asian green mussel, *Perna viridis*, cut up in pieces sized between 2–3 mm; Squid, *Loligo* sp., cut up in pieces sized between 2–3 mm; Polychaetes, *Marphysa moribidii*, cut up in pieces sized between 2–3 mm and Mussel, squid, as well as polychaetes (mix diet) that are fed in succession of days.

The amount of food was Hettiarachchi and Edirisinghe (2016), equivalent to 5% of total body weight, fed twice daily (morning and evening) and taking account on preliminary observations of consumption and daily feeding response. Before collecting the data, the broodstock were fed with experimental diets for two moult cycles to permit acclimatization with the diet treatments, as well as to reduce the consequences of former maintenance diet.

Parameters recorded : Parameters measured for egg retention under abdomen (%), viable fecundity and fecundity were based on the study of Tziouveli *et al*. (2011). Feeding data collection was done for every replicate in a successive spawn. The experimental parameters recorded were: Broodstock survival (%) expressed as percentage of shrimp survived at the end of study; Broodstock growth (%) expressed as percentage of increase in the initial size for overall length (TL), carapace length (CL), as well as carapace width (CW) of the broodstock at the end of the study and Egg retention under abdomen (%) is denoted as the percentage of eggs available at the carrying area throughout the incubation period. No spawn is indicated by zero percent, while 100% refers to the overall egg cover. The data was recorded every two days and was calculated by the formula:

$$= \frac{\text{The egg mass volume occupied in the abdominal egg carrying area (length × width × depth)(mm}^3\text{)}}{\text{Abdominal length × abdominal width × abdominal depth (mm}^3\text{)}} \times 100$$

Viable fecundity refers to the amount of larvae hatched at a successful spawning event; Egg fecundity was expressed as a number of fertile egg at a successful spawning event. Individual eggs were counted with the help of a dissection microscope (Olympus SZ51 – LGB, Olympus Corporation, Tokyo, Japan) and Larval morphometric data (TL, CL and CW) were measured with a digital microscope Dino-Lite equipped with DinoCapture 2.0 software based on triplicates result for each treatments (n = 90).

Proximate analyses: Gross proximate composition of food items used were evaluated following the methodology of Association of Official Analytical Chemists (AOAC, 1997).

Fatty acid profiles : The overall total fatty acids were drawn out from feeds, tissues and plasma via the technique of Folch *et al*. (1957) with modification by Rajion *et al.* (1985). FAME were analysed with GC column (Agilent 7890N) and a 30 m × 0.25 mm, and 0.25-µm coating thickness Supelco SP-2330 Capillary Column (Supelco, Inc., Bellenfonte, PA, USA).

Statistical analyses : One-way ANOVA was applied to detect possible significant differences of all reproductive parameters involved in the study using the software IBM SPSS (predictive analytics software). The percentage of egg cover under abdomen across the incubation time was compared based on the final percentage value during the incubation period. Duncan's Multiple Range Test was applied of ANOVA indicated a huge dissimilarity ($p < 0.05$).

Results and Discussion

The survival rate of *L. amboinensis* brood stocks were unchanged by the experimental diets because there was an absence of mortalities recorded throughout the study period. The findings demonstrated the suitability of all diets for the general maintenance of broodstocks. Similarly, Tziouveli *et al.* (2011) also reported that the broodstock of this species were able to survive when fed with squid (*Loligo opalescens*), mussel (*Perna canaliculus*), artemia and pellet (commercial Kuruma prawn diet, Ebi Star, Higashimaru, Japan). This showed that this species was easy to maintain due to its hardiness and not fussy in choosing its diet. Apart from its fascinating external morphology and interesting behavior, this may also be a factor that causes marine shrimp hobbyists to rear this shrimp in their ornamental aquarium.

Fig. 1 shows no significant difference in all parameters for broodstocks growth (total length, carapace length and carapace width) in the different diet treatments over the experimental period ($p > 0.05$). The insignificant growth rate recorded for brood stock could be related to almost similar protein content percentage of all diets in the experiment which ranged from 63.93% to 75.10%. Moreover, the reproductive event in brood stocks takes up great amount of energy, thus, there was an expectation on the absence of growth throughout this experiment. The simultaneous hermaphrodites did not exhibit growth as a significant portion of their energy directed to oocyte production, thus, producing slower growth rates. Bolognini *et al.* (2017) mentioned that an extraordinary amount of energy required in the reproductive event as an investment to ensure offsprings' survival.

Fig. 2 shows mussel, polychaetes and mix diets displayed high percent of egg cover at the end of incubation

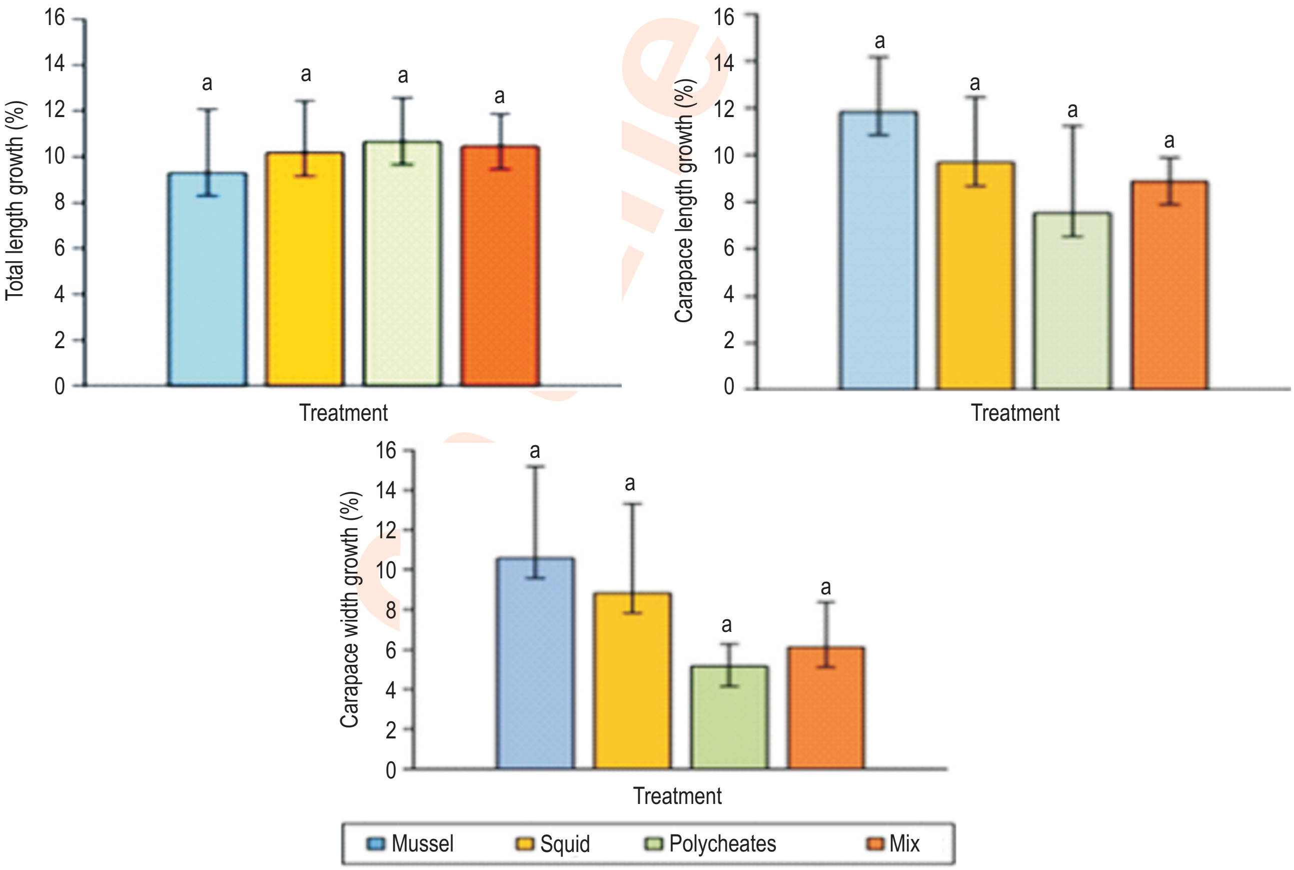


Fig. 1 : Growth rate of *Lysmata amboinensis* broodstock (percentage increment in total length, carapace length and carapace width) over the span of study under different diets (means ± S.E, n = 3 replicates per treatment) .

period (90.33%, 83.67% and 85.33%) without a great difference ($p > 0.05$). Besides, these three diets also demonstrated similar egg loss over the course of 17 days with reduction of approximately 10%–16%. Squid diet had the lowest initial egg cover (85.0%) and the highest egg loss during incubation (58.67%) ($p < 0.05$). Squid contained the highest level of protein (75.10%) and polychaetes had the highest amount of lipid (12.05%) (Table 1). Lipid component offers suitable energy sources for broodstock maturation and continuous spawning event as it related to the production of reproductive tissue (Naessens *et al.*, 1997).

Feeds showed significant effect on egg fecundity ($p < 0.05$) (Fig. 3). The broodstock fed with mussel diet produced the highest fecundity (1985), followed by mix diet (1905), polycheates (1667) and squid (959). As the percent of egg cover under abdomen reduced throughout the incubation period, it totally affected the viable fecundity at the end of the study. Proximate analyses showed that squid had the lowest lipid content resulting in poor spawning performance as the broodstock fed with squid showed reduced egg fecundity and poorer egg retention. Although polychaetes contained the highest amount of lipid, too high of lipid content may negatively affect the ingestion rate because the shrimp broodstock tend to be satisfied when reached their energy requirements (Aranyakananda and Lawrence, 1994), hence causing nutrient deficiencies (D'Abramo, 1997). The mussel diet produced the highest number of larvae (568), followed by polychaetes and mix diets (391 and 386, respectively) (Fig. 4). Shrimp fed with squid showed significantly low ($p < 0.05$) reproductive output (97). Examining the experimental diets in the aspect of fatty acid profile and broodstock body can deliver some possible clarifications.

The fatty acid content in the experimental diets, especially 14:0, 16:1, OA, DHA, MUFA and PUFA n-6 showed critical difference between the treatments ($p < 0.05$) (Table 2). All diets showed high contents of total PUFA and SFA without critical difference among the experimental diets ($p > 0.05$). The diet mussel showed a paramount level in 14:0 (Myristic) and 16:1 (Palmitoleic), while polychaetes displayed the highest contents of 18:1n-9, total MUFA and total PUFA n-6. The squid diet showed high level of DHA and overall PUFA n-3, but very low in total MUFA compared to other diets, ($p < 0.05$). Tziouveli (2011) reported that shrimp fed with mussel-squid displayed the lowest production of *L. amboinensis* larvae (22). However, in this study, feeding broodstock with mussel alone produced the highest viable fecundity (568). DHA level seemed to play critical role in egg retention, fecundity and early larvae production. This hypothesis is also supported by the result of previous study done by Wen *et al.* (2002) that DHA has a function in early embryogenesis that affects the hatchability of eggs. High DHA level has been reported in broodstock diet improved embryogenesis and early larval development (Xu *et al.* 1994; Cahu *et al.* 1995). Cahu *et al.* (1995)

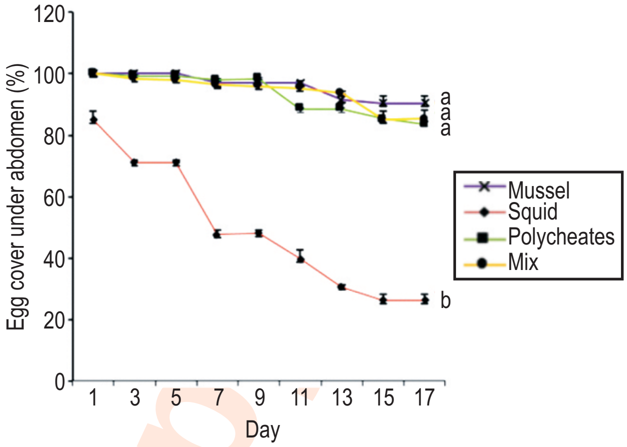


Fig. 2 : Egg cover expressed as a percentage of total egg incubate area during 17 days incubation period for different diets (means ± S.E, n = 3 replicates per treatment). Different letters show significant difference in egg cover percentage, ($p < 0.05$).

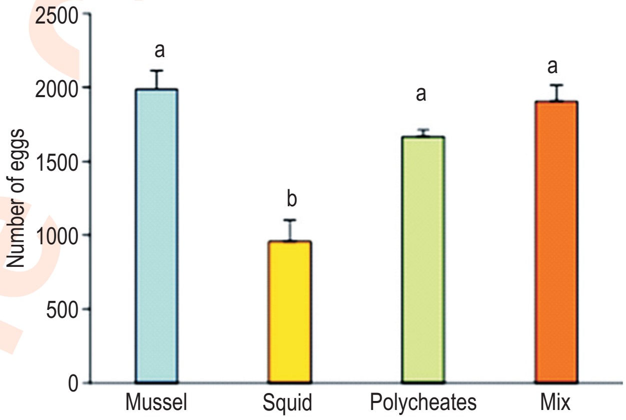


Fig. 3 : Fecundity for each diets treatment. Different letters show significant difference between diets treatments for fecundity ($p < 0.05$) (means ± S.E, n = 3 replicates per treatment).

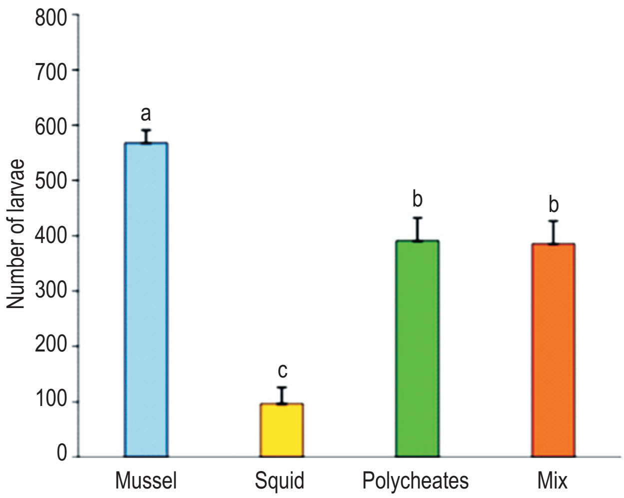


Fig. 4 : Viable fecundity for each diets treatment. Different letters show significant difference between diets treatments for viable fecundity ($p < 0.05$) (means ± SE, n = 3 replicates per treatment).

Table 1 : Proximate composition of experimental diets used to feed *L. amboinensis* broodstocks (expressed as % dry matter basis)

	Mussel	Squid	Polychaetes
Crude protein	69.74±0.49^{ab}	75.10±0.26^{a}	63.93±0.33^{b}
Crude lipid	7.62±0.23^{b}	1.79±0.01^{c}	12.05±0.64^{a}
Crude carbohydrates	12.71±0.16^{b}	18.51±0.32^{a}	13.42±0.07^{b}
Moisture	72.09±0.27^{b}	82.25±0.15^{a}	80.25±0.83^{a}
Ash	9.93±0.20^{a}	4.60±0.47^{b}	10.60±0.66^{a}

Values are means of triplicate samples (± S.E). Different letters showed significant difference between diets treatments ($p < 0.05$)

Table 2 : Fatty acid content (% of total fatty acids) of experimental diets used to feed *L. amboinensis* broodstock

Fatty acid profiles	Mussel	Squid	Polychaetes
14:0	9.13±0.38^{a}	5.69±0.43^{b}	4.61±1.55^{b}
16:0	19.29±0.49^{a}	24.55±3.74^{a}	23.68±3.76^{a}
16:1	11.52±0.43^{a}	5.14±1.25^{b}	4.74±0.68^{b}
18:0 (SA)	10.98±1.23^{a}	12.91±2.32^{a}	14.24±0.54^{a}
18:1n-9 (OA)	4.82±0.87^{b}	6.11±0.94^{b}	11.95±2.21^{a}
18:2n-6 (LOA)	2.35±0.39^{a}	6.13±1.25^{a}	9.85±3.65^{a}
18:3n-3 (ALA)	4.63±0.58^{a}	4.40±0.70^{a}	5.37±0.48^{a}
20:4n-6 (AA)	8.37±0.73^{a}	6.09±0.67^{a}	10.58±1.98^{a}
20:5n-3 (EPA)	9.77±0.31^{a}	7.48±0.45^{b}	4.37±0.78^{b}
22:5n-3 (DPA)	9.17±2.15^{a}	9.72±1.35^{a}	8.19±0.61^{a}
22:6n-3 (DHA)	9.98±1.55^{b}	14.16±1.68^{a}	5.76±1.92^{b}
ΣSFA	39.40±1.48^{a}	41.59±5.06^{a}	42.53±2.61^{a}
ΣMUFA	16.34±1.04^{a}	10.99±0.76^{b}	16.69±1.58^{a}
ΣPUFA	44.27±2.50^{a}	47.97±3.79^{a}	44.12±0.71^{a}
ΣPUFA n-3	33.55±2.58^{ab}	35.50±3.90^{a}	23.68±2.72^{b}
ΣPUFA n-6	10.72±1.09^{b}	11.92±0.84^{b}	18.09±2.16^{a}

Values are means of triplicate samples ± S.E. Different superscript letters in the same column represent significant differences (p < 0.05); Σ Saturated fatty acid (SFA): 14:0, 16:0, 18:0 ΣMonounsaturated fatty acid (MUFA): 16:1, 18:1n-9 ΣPolyunsaturated fatty acid (PUFA): 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3

showed that at high DHA level in the diet (17.5% to 19.8% of total fatty acid), eggs of *Penaeus indicus* are able to incorporate this level of DHA which is sufficient for adequate hatching.

However, the results of this study contradict to the well documented function of DHA in the reproduction response, particularly in embryogenesis and early larval development of penaeid shrimp. Higher DHA content in squid diet (14.16% of total fatty acid) affected the egg retention and gave the lowest egg fecundity. Previously, in penaeids shrimp, Bray *et al*. (1990) also reported that extremely high content of DHA may result in low hatchability because it becomes negatively inhibitory, which affects the level of other important fatty acids. Tziouveli *et al*. (2011) suggested that the high DHA level in squid may partly clarify the effect of lesser egg cover prior to hatch conceding egg production in *L. amboinensis*. Tziouveli *et al*. (2012) later showed that DHA content of 11% administered to broodstock of *L. amboinensis* contributed the optimal outcome for embryo hatchability, as well as succeeding larval production. Figueiredo *et al*. (2008) reported that low consumption of DHA during embryogenesis based on fatty acid profiles of *A. cinereum* eggs showed that there may not be a requirement for DHA-rich diet for this specie. This also proved the amount of DHA in the diet to fulfil the broodstock requirement for better reproduction performance in terms of embryogenesis, egg retention, fecundity which finally lead to larval production, were species dependent.

Diet treatments have significant effect on larval morphometric (total length, carapace length and carapace width) (Table 3). In this study, larvae produced from broodstock fed with squid displayed higher value of body total length and produced bigger head in terms of their carapace width measurement. The findings suggest that significant larval morphometric may be related to high DHA contents in the squid diet. Narciso and Morais (2001) reported an extremely high amount of DHA in the brain and retina that play a specific role in neural and visual membrane systems (Sargent, 1995). Martin *et al*. (1994) demonstrated that the presence of DHA in high concentrations in phospholipids from the brain, retina and testes is unusual, and it plays an essential role in the development of central nervous system. Mourente and

Table 3 : Larval morphometric measurements of *L. amboinensis* under different diet treatments

Treatment	Total length	Carapace length	Carapace width
Mussel	2.62±0.02[b]	0.91±0.01[a]	0.48±0.00[b]
Squid	2.69±0.01[a]	0.69±0.00[c]	0.52±0.00[a]
Polychaetes	2.43±0.01[c]	0.90±0.01[a]	0.47±0.00[b]
Mix	2.41±0.01[c]	0.87±0.00[b]	0.48±0.00[b]

Different letters showed significant difference between diets treatments ($p < 0.05$) (means ± S.E, n = 90 replicates per treatment)

Table 4 : Fatty acid composition (% of total fatty acids) of *L. amboinensis* broostock fed with different diets

Fatty acid profiles	Mussel	Squid	Polychaetes	Mix
14:0	5.01±0.27[a]	3.00±0.36[a]	5.67±2.14[a]	4.47±1.11[a]
16:0	18.09±0.72[a]	22.37±3.72[a]	20.33±0.01[a]	19.40±0.46[a]
16:1	7.47±0.42[ab]	5.63±1.57[b]	11.55±1.67[a]	9.86±1.41[ab]
18:0 (SA)	16.46±0.33[a]	13.06±2.2[ab]	12.50±0.16[ab]	11.97±0.95[b]
18:1n-9 (OA)	16.78±0.65[a]	17.47±11.16[a]	19.89±1.13[a]	17.78±1.59[a]
18:2n-6 (LA)	2.20±0.79[b]	4.01±0.27[a]	3.24±0.14[ab]	2.37±0.35[b]
18:3n-3 (ALA)	2.72±0.13[a]	2.37±0.03[ab]	2.17±0.73[ab]	1.36±0.02[b]
20:4n-6 (AA)	8.62±0.67[ab]	7.48±0.53[b]	11.12±0.14[ab]	13.97±3.06[a]
20:5n-3 (EPA)	11.99±0.81[a]	6.08±0.38[b]	3.44±0.62[c]	6.35±0.40[b]
22:5n-3 (DPA)	4.41±0.92[a]	9.93±3.22[a]	7.98±2.17[a]	5.87±1.60[a]
22:6n-3 (DHA)	6.23±0.00[a]	8.58±1.64[a]	2.11±0.78[b]	7.61±0.66[a]
Σ SFA	39.57±0.65[a]	38.43±5.58[a]	38.50±2.31[a]	34.85±1.23[a]
Σ MUFA	24.25±1.07[b]	23.09±0.10[c]	31.44±0.53[a]	27.64±0.18[ab]
Σ PUFA	36.17±1.72[a]	38.46±5.48[a]	30.05±2.83[b]	37.52±1.40[a]
Σ PUFA n-3	25.35±1.60[ab]	26.97±5.22[a]	15.70±2.84[b]	21.18±1.32[ab]
Σ PUFA n-6	10.83±0.12[d]	11.50±0.26[c]	14.36±0.00[b]	19.05±0.00[a]

Values are means of triplicate samples ±S.E. Different superscript letters in the same column represent significant differences ($p < 0.05$). ΣSaturated fatty acid (SFA): 14:0, 16:0, 18:0 ΣMonounsaturated fatty acid (MUFA): 16:1, 18:1n-9 ΣPolyunsaturated fatty acid (PUFA): 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3

Tocher (1992) claimed how DHA influences the brain weight and composition of fatty acid of turbot, *Scophthalmus maximus L.* as DHA was found specifically accumulated in brain lipid. Moreover, Suprayudi *et al*. (2004) reported that the larvae of mud crab require high amount of DHA and EPA at appropriate levels for maintaining high survival and produce good quality larvae in terms of carapace size. In another research, Takeuchi *et al*. (1999) described that DHA plays an important role in increasing carapace width of swimming crab larvae. Therefore, we summarized that higher DHA in squid might have relationship that give effect to the larval morphometric measurement, in terms of producing the longest total length and widest carapace size of *L. amboinensis* larvae.

The results obtained in the present study suggested that high concentration of monounsaturated fatty acid (MUFA) with focusing in significant higher content of 16:1 (Palmitoleic) in mussel, may assist metabolic functioning and support energy requirements during the embryonic development which finally could be associated for better egg retention and high egg production compared to other diets. As mentioned by Balina *et al*. (2018), reproductive event in broodstocks uses great amount of energy. It is broadly acknowledged that MUFA is utilized to provide energetic function (Cavalli *et al*., 2001; Rosa *et al*., 2003). This hypothesis was supported by shrimp body fatty acid evaluation as it showed that shrimp fed with squid have the lowest amount of MUFA (Table 4), which reflected insufficient energy in the body to serve and maintain the reproductive event and finally produce low larvae output.

Simoes *et al*. (1998) and Lin and Zhang (2001) elucidated that different broodstock diets of *L. amboinensis* and *L. wurdemanni* seemed to have no effect on their reproductive performance. In contrast to this study, the diet treatments led to marked variances in the reproductive output of *L. amboinensis*. Broodstock diet is well known to give major impact in shrimp's reproduction (Lin and Shi, 2002). According to Lin *et al*. (2002), diet nutritional equitableness is species dependent. It affects various elements of reproduction, thus influencing the nutritional profile and broodstock nutritional necessities. The diet appears to have an impact on the egg cover during incubation, egg fecundity, viable fecundity and output in terms of larval morphometric of *L. amboinensis*.

However, it should be noted that reproductive performance may be related not only on lipid and fatty acid contents, but also sufficient amount of vitamins, minerals and carotenoids (Perez-Velazquez *et al*., 2003; Williams, 2007). Mussel, *Perna viridis* also has content of high vitamin A, minerals like calcium, potassium, sodium, iodine and amino acid, methionine (Saritha *et al*., 2015) and is used regularly as broodstock diet for penaeid shrimp (Hertrampf and Piedad-Pascual, 2000; FAO, 2007).

Broodstock diet influence the egg cover during incubation, egg fecundity, viable fecundity and the output in terms of *L. amboinensis* larval morphometry. Through fatty acid profile analyses, it was possible to evaluate effect of different diet on reproductive performance of *L. amboinensis*. The results showed that the content of MUFA in the diet correlates well with the egg production of this cleaner shrimp, whereas the level of 22: 6 n-3 (DHA) in the diet exhibited close relation with the egg retention throughout the incubation period. These relationships suggest that MUFA may play some potential role in the embryonic development process related to fecundity whereas DHA may play some other role in early embryogenesis related to egg hatchability and larval morphometric of *L. amboinensis*. This information provides a better understanding on this species nutrition demand which is important to increase the chances of mass production to meet the industry demand in future.

Acknowledgments

This study was funded by an internal grant from Universiti Putra Malaysia (UPM); project no GP-IPB/2014/9440403. We thank the staff at the International Institute of Aquaculture and Aquatic Sciences (I-AQUAS), Universiti Putra Malaysia for their assistance during the conduct of this experiment.

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