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Research Article

Morphological, Histological, and Physiological Responses of Female Orange Mud Crab, *Scylla olivacea* (Herbst, 1796), Fed with Different Dietary Lipid Levels

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The main focus of the study was to assess the effect of lipid levels on the reproduction of the female orange mud crab, *Scylla olivacea*. Four isonitrogenous feed (~420 g/kg) were formulated with different lipid percentages: T1 = 60 g/kg, T2 = 80 g/kg, T3 = 100 g/kg, and T4 = 120 g/kg. A feeding trial was carried out on 120 matured female mud crabs for 90 days with 30 replicates in each treatment. Based on the morphological, histological, and hormonal analysis of each crab, the performances of each experimental diet were tested. The results of the experiment disclosed positive body weight gain (BWG) and specific growth rate (SGR), with the highest value recorded in crabs that were fed T4 (BWG: 14.43 ± 1.14 g; SGR: 0.10 ± 0.01%day^{-1}). The highest gonado-somatic index (GSI) and hepato-somatic index (HSI) were noted in mud crabs that were fed T4, each with a value of 14.51 ± 1.13% and 5.23 ± 0.55%, respectively ($P < 0.05$). The histological assessment revealed that the highest oocyte diameter was recorded in mud crabs fed T4 (192.80 ± 21.93 μm), where the oocyte diameter was correlated with the progesterone (Pg) concentrations in the ovary with $r = 0.73$. Evidence also indicated that Pg and estradiol (E_2) are important in the ovarian development in *S. olivacea* and their concentrations were affected by the lipid levels. An overall analysis revealed that 120 g/kg of lipid in the diet contributes to optimal reproductive performance in *Scylla olivacea*. Yet, a further experiment is expected to be carried out in the future to determine the nutrient-dose response as 120 g/kg lipid may not be the optimum lipid level for reproductive and growth performance in *S. olivacea*. Moreover, the extrapolation of data from this study can be used as guidelines in feed formulation for developments in future research in adult crustaceans.

1. Introduction

Fundamental research on reproduction is important in improving the understanding of the reproductive processes of broodstock in captivity [1]. Critical revisions have concluded a close relationship between the optimal functioning of the ovary and hepatopancreas with reproduction, and both tissues are involved in the steroid hormone

biosynthesis during vitellogenesis [2]. Vitellogenesis consists of sequential changes in the accumulation of yolk that begin from the previtellogenic oocytes up to the accumulation of yolk proteins or vitellin (Vn) in the growing oocytes. During vitellogenesis, vitellogenin (Vg) is synthesized in the ovary and hepatopancreas, where it is sequestered and modified with the addition of polysaccharides and lipids accumulated in oocytes, to the formation of Vn [3, 4]. The presence of Vn during vitellogenesis is closely related to the hormonal changes in crustaceans during their reproductive process [5]. The research carried out on the female swimming crab, *Portunus trituberculatus*, showed that the *in vivo* injections of the exogenous estrogen (0, 0.01, 0.1, and 1 $\mu g\,g^{-1}$ crab weight) in the crabs enhanced Vn in both the ovary and hepatopancreas [6]. In their findings, the highest gonadosomatic index (GSI) was found in crabs with E_2 injections of 1 $\mu g\,g^{-1}$ bodyweight, while no significant differences were found in the survival and hepato-somatic index (HSI) among all treatment methods [6]. The presence of hormones, particularly the vertebrate-type steroids such as progesterone (Pg) and estradiol (E_2) hormones, have been reported in the hepatopancreas, ovary, and hemolymph of Chinese white shrimp *Penaeus chinensis* [7], green mud crab *Scylla serrata* [8], blue pearl crayfish *Cherax albidus* [9], narrow-clawed crayfish *Astacus leptodactylus* [10], and swimming crab *Portunus trituberculatus* [6]. In general, E_2 and Pg play a vital role in crustacean reproduction via enhancing the initiation of related metabolic pathways during vitellogenesis [11, 12], but their concentrations with ovarian maturation stages vary between species. Interestingly, the changes in the vertebrate type of steroids titer during oocyte maturation indicate their importance in governing the vitellogenesis process, which involves the mobilization of lipids from the storage sites [13].

At present, extensive studies have been conducted to observe the effects of external factors such as salinity, temperature, and photoperiod on hormonal influence during vitellogenesis and embryonic development [14–16]. However, very few studies have evaluated the effect of diets on the endocrine system, which directly influences reproduction in crabs [17]. Previous investigations have highlighted the importance of the provision of lipid in the diet for broodstock for the ovarian development of pond-reared crabs [18]. It is well established that the nutritional content of crustacean diets directly influences the growth and reproduction of crustaceans. For instance, the optimum growth and reproduction for crustaceans can be achieved with a total lipid level ranging from 75 to 80 g/kg in red claw crayfish (*Cherax quadricarinatus*) Zenteno-Savin et al. [19], 95 g/kg in yellow mud crabs (*Scylla paramamosain*) [20], 100 to 120 g/kg in whiteleg shrimp [21], and 90 g/kg in spiny lobsters (*Jasus edwardsii*) [22]. At the same time, ideal growth is achieved in a crustacean when micronutrients such as n-3 fatty acids are supplied at 8.6 mg/g for whiteleg shrimp [23], 12.7 mg/g for yellow mud crabs [24], and 30 mg/g for giant tiger prawn [25]. Lipids are among the most critical nutrient components required in crabs for energy expenditures, maturation, and growth [26, 27]. The determination of lipid requirements in crabs is generally species-specific and varies according to different developmental stages. Data on lipid requirements for *Scylla* sp. are currently restricted to *Scylla serrata* and *S. paramamosain*. As such, a study on *S. paramamosain* reported that diets containing 95 g/kg of fish oil significantly improved the crabs' survival [20, 28]. Meanwhile, research on *S. serrata* recorded an optimal lipid level of 53–138 g/kg [29], 320–400 g/kg protein with either 60 or 120 g/kg lipid [30], and 100 g/kg [31]. Song et al. [32] tested the effects of various phospholipid levels (9.9 g/kg, 16.5 g/kg, 25.1 g/kg, 36.3 g/kg, 49.5 g/kg, and 61.2 g/kg) on female swimming crab, *Portunus trituberculatus*, and they concluded that 41.5 g/kg showed the highest Pg and E_2 contents in the hepatopancreas, hemolymph, and ovaries.

Hence, the objective of this study is to evaluate the effect of the diet formulation with different lipid levels on the vitellogenesis and reproductive physiology in female orange mud crabs, *S. olivacea*. This study aims to improve the understanding of the mechanisms involved in ovarian development in *S. olivacea* broodstock for the development of feed formulation purposes.

2. Materials And Methods

2.1. Experimental Diets. Four isonitrogenous feed (~420 g/kg) with different lipid levels were introduced during the formulation with T1 = 60 g/kg, T2 = 80 g/kg, T3 = 100 g/kg, and T4 = 120 g/kg. The lipids were sourced from mangrove clam meat, *Polymesoda erosa* (105.1 ± 2.3 g/kg crude lipid) and fish oil (FO). The amounts of some ingredients were altered in accordance with the general lipid requirements of crustaceans following Catacutan [30]. The isoproteic rate of the diets was achieved by adjusting the diet's FO and wheat flour contents. All the dry ingredients were premixed and added to form a homogenous mixture except for probiotics as well as vitamins and minerals for the diet preparation. Then, 50 mL kg^{-1} of water was added to the mixture, which was cooked for a while to activate the agar until everything was well mixed. The mixture was left to cool, and once the temperature of the mixture reached below 50 °C, the probiotics and vitamins and minerals (1 : 1) were added. The resultant pellets were molded into an ice tray cube (3.0 × 1.5 × 1.5 cm) and stored in a sealed bag in a -20 °C freezer until use to maintain the nutrient content. Overall formulation and the determination of proximate analysis are described in Table 1.

2.2. Feeding Trial and Experimental Conditions. A total of 150 healthy mature female orange mud crabs, *Scylla olivacea*, with a complete set of functional feeding appendages were sampled from Setiu Wetland, Terengganu coastal waters, Malaysia. The mean body weight (BW) and carapace width (CW) of the samples were in a range of 186.42 ± 37.90 g and 10.24 ± 0.66 cm, respectively. Each treatment group consisted of 30 individuals of mud crabs, including the control group. Only crabs with Stage 4 of ovarian maturation were taken into the study as the ovary at this stage undergoes ripening, thus indicating its readiness to spawn [11]. As the ovaries are not externally visible due to the hard and thick carapace, hence, we have inspected the maturity of mud crab ovary by carefully depressing and pushing forward

Table 1: Feed formulation and proximate composition for each feed.

Ingredients	Inclusion (dry matter, g/kg) with lipid (L) g/kg			
	T1	T2	T3	T4
Mangrove clams flesh mix	526.8	526.8	526.8	526.8
Wheat flour	257.9	237.9	217.9	197.9
Fish oil	5.3	25.3	45.3	65.3
Soy lecithin	20	20	20	20
[1]Vitamin and mineral mix (1 : 1)	30	30	30	30
Molasses	50	50	50	50
[2]Probiotics	50	50	50	50
Agar	60	60	60	60
	1000	1000	1000	1000
Proximate analysis (%)				
Dry matter	22.46 ± 0.77	21.02 ± 1.45	23.35 ± 3.17	23.92 ± 0.65
Crude lipid	6.67 ± 0.15	8.29 ± 0.47	10.49 ± 0.91	12.73 ± 0.31
Crude protein	42.89 ± 1.14	42.31 ± 1.47	42.39 ± 1.53	42.04 ± 1.06
Crude fibre	0.02 ± 0.01	0.04 ± 0.02	0.05 ± 0.02	0.08 ± 0.00
Crude ash	8.33 ± 0.46	8.23 ± 2.99	8.83 ± 1.14	8.09 ± 0.15

[1]Vitamin and mineral mixtures: vitamin C (L-ascorbyl-2-monophosphate): 40, 000 mg/kg; vitamin B1: 4, 600 mg/kg; vitamin B6: 4, 100 mg/kg; nicotinic acid: 14, 800 mg/kg; choline: 34, 800 mg/kg; lysine: 80, 340 mg/kg; selenium: 18 mg/kg; BHT: 150 mg/kg; BHA: 100 mg/kg; propyl gallate: 1 mg/kg; citric acid: 50 mg/kg (Sano Tops: Inve Aquaculture). [2]Probiotics: strains of *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus pumilus* (Sano-life Pro-2 Powder: Inve Aquaculture).

the first abdominal segment following method adapted from Quinitio et al. [33]. Mud crabs with mature ovaries will have ovaries that occupy the whole cephalothoracic cavities of the carapace. Mud crabs from control group ($n = 30$) were promptly dissected for the ovary and hepatopancreas, and the hemolymph was collected for analysis of (i) morphology, (ii) histology, and (iii) physiology (hormonal).

The broodstock was transported to the hatchery of the Institute of Tropical Aquaculture and Fisheries AKUATROP, Universiti Malaysia Terengganu (UMT), for acclimatization for a 7-day period. Prior to acclimatization, the crabs were disinfected with 25 ppm of formalin for 30 min to kill parasites [34]. After disinfection, the crabs were stabilized in a 1-tonne liter tank, under a simple recirculated water system, equipped with moderate aeration, water pump, and UV light. Water salinity and water temperature in all tanks were maintained at 25 g/L and 26–29 °C, respectively. Prior to feeding experiment, the crabs were fed with chopped yellow scadfish, *Selaroides leptolepis*. After a successful acclimation, the crabs were starved for 24 hours, before feeding experiment can be proceeded.

Feeding trial was conducted for each experimental feed for 90 days. The crabs were randomly divided into four groups (T1 = 60 g/kg, T2 = 80 g/kg, T3 = 100 g/kg, and T4 = 120 g/kg) with 30 replicates for each treatment. The crabs were separated individually in a cage (33.0 × 45.0 × 11.0 cm). During the feeding trial, the mud crabs were fed with 5% of BW once a day, which was during dusk (2000 hours) as mud crabs are active at night [35]. A 100% water exchange was done every week. At the end of each feeding trial, all crabs ($n = 30$) from each tank were subjected to dissection of the hepatopancreas and ovaries for analysis. The performances of each experimental diet were tested based on the morphological, histological, and hormonal analyses of the crabs. All experimental protocols for this research followed the Research Ethics Guidelines for the Ethics of Research on Animals 2014 (UMT; https://studylib.net/doc/8738550/research-ethics-guidelines—pusat-pengurusan-penyelidikan).

2.3. Morphological Analysis. The body weight gain (BWG) was calculated for morphological analysis by subtracting the final body weight from the initial weight to the nearest 0.1 g using a digital electronic balance following the measurement used by Ahamad-Ali et al. [36]. The specific growth rate (SGR) was assessed based on methods adapted from Romano and Zeng [37]. The gonado-somatic index (GSI) and hepato-somatic index (HSI) were recorded for morphological analysis according to the methods of Hidir et al. [38] by weighing the tissues of the ovary and hepatopancreas, respectively, over the total body weight of each crab. The calculations for each parameter are as follows:

$$\text{(i) Body weight gain, BWG (g)} = \text{final weight} - \text{initial weight},$$

$$\text{(ii) Specific growth rate, SGR (\%day}^{-1}) = \frac{(\ln \mathrm{Wf} - \ln \mathrm{W0})}{\mathrm{t}} \times 100, \quad (1)$$

where W_f = final weight (wet weight), W_0 = initial weight (wet weight), and t = day of culture.

(iii) Gonado − somatic index (GSI)/hepato − somatic index (HSI)

$$\text{GSI or HSI (\%)} = \frac{\text{ovary/hepatopancreas weight}}{\text{body weight}} \times 100\% \quad (2)$$

2.4. Histological Analysis. The histological study was done based on the standard histological procedure by Mumford [39]. Five intermolt mud crabs from each treatment were dissected, and the parts of the ovaries and hepatopancreas tissues (0.1–0.2 g) were fixed in Davidson's fixative solution between 24 and 48 h before immersing them into 70% ethanol. The samples were placed into the histological cassette and forwarded into a tissue processor for 19 h before embedding them with paraffin wax. Routine thin section paraffin histology was performed at 5 μm thickness on a microtome and attached to a slide using Mayer's albumin. Hepatopancreatic tubules and ovarian oocytes were transversally sectioned. Each of the sections was then dewaxed, dehydrated, and stained with hematoxylin-eosin. The slides containing these sections were then mounted with Dibutylphthalate Polystyrene Xylene (DPX) before they were observed under an Advanced Research Microscope (Nikon Eclipse 80i, Japan) together with NIS-Elements D (Version 4.11) Imaging Software.

The diameter (in μm) of perfectly sectioned tissues of hepatopancreatic tubules ($n = 80$) and ovarian oocytes ($n = 80$) was measured and calculated for each treatment. The tubule and oocyte diameters were measured parallel to the long axis of each mounted slide using the Advanced Research Microscope by selecting the tubules/oocytes with the widest diameter. The sizes of oocytes and tubules were recorded to the nearest hundredths. The measurement of each cell was taken as close to the equatorial plane as possible plane to ensure that only sections of whole cells were used, with the cells that showed the largest possible two-dimensional surface area and complete sections of nucleus within each cell [40]. Each measurement was made on a digital image at 100×.

The histological assessment of hepatopancreas indicated the presence of four different cell types—the E-cells (embryonic), F-cells (fibrillar), B-cells (blister-like), and R-cells (resorptive)—which lie on the pseudostratified epithelium that is arranged along the hepatopancreatic tubule. The tubule lumen of each mud crab displayed a star shape, and B- cells were found adjacent to the tubule lumen. The identifications of the cells were made by referring to Franceschini-Vicentini et al. [41]. The hepatopancreatic cells, particularly the B- and R-cells, were counted manually by observing the histology images. Generally, R-cells can be recognized by having cylindrical with subapical vacuoles with the nucleus located at the basal region of the cell, whereas B-cells were identified by large vacuoles which often projects into the lumen of the tubule. The data of both B- and R-cells were presented as cell counts per tubule.

2.5. Hormone Collection. The hemolymph of crabs was collected prior to the commencement of the feeding trial (Day 1), and it was recorded as initial reading. At the end of the feeding trial, the crabs were sacrificed for ovaries, while the hemolymph was collected again and recorded as final readings. The crabs were anesthetized on ice for 5 min before the withdrawal of hemolymph. 1 mL of hemolymph was taken from the 3rd walking leg using a 1 mL syringe provided with a 25G needle. To prevent coagulation, the hemolymph withdrawal was performed with a syringe containing 0.2 mL anticoagulant [15]. 1.0 L anticoagulant was prepared following Söderhäll and Smith's [42] method by mixing the citrate/EDTA buffer (26.3 g of 0.45 M NaCI; 18.0 g of 0.1 M glucose; 8.8 g of 30 mM trisodium citrate; 5.5 g of 26 mM citric acid and 3.7 g of 10 mM EDTA) (SIGMA) at pH 4.6, pre-cooled to 8 °C. The hemolymph samples were then centrifuged at 1,000 rpm for 5 min, and the plasma was separated from hemocytes to prevent hemocytes lysate from interfering with the proteins in hemolymph when measured in ELISA. The samples were then stored in a 1.5 mL microcentrifuge tube and frozen at -20 °C until analysis. Meanwhile, the ovary tissues from dissected *S. olivacea* were cut into small fragments (0.1 to 0.2 g) and frozen at -20 °C until analysis by ELISA [43].

2.5.1. Hormone Assay. The concentrations of Pg and E_2 in the hemolymph and ovary of *S. olivacea* were analyzed using a commercial ELISA kit (Cayman Chemical, Ellsworth Rd, Ann Arbor, MI, USA) following the manufacturer's protocol. Upon analysis, the samples were thawed at room temperature, homogenized, and centrifuged (12,000 rpm at 4 °C for 10 min), and the resultant supernatant (serum) was collected. Each treatment was assayed in six replicates. A Microplate Reader (Multiskan FC, Version 1.00.94) was used to read the plate at a wavelength of 404–420 nm, and the plate was read when the absorbance of the maximum binding wells (B_0) was between 0.3 and 1.5 arbitrary unit per milliliter (AU/mL). Data of Pg and E_2 concentrations in the hemolymph and ovary in each sample were expressed as nanogram per milliliter (ng/ml) and nanogram per milligrams, respectively.

2.6. Statistical Analysis. All data (BWG, SGR, GSI, HSI, oocyte diameter, tubule diameter, B- and R-cells, as well as Pg and E_2) were tested for normality based on the measurement of Shapiro-Wilk and Skewness and Kurtosis before proceeding with the ANOVA tests. Data from morphological and histological tissues sectioning as well as steroid hormonal analysis were expressed as mean ± SD. ANOVA tests were performed using SPSS (IBM SPSS Statistics 20) at $P < 0.05$ (95%) where a statistically significant difference between feeding trials was determined by Tukey's HSD multiple comparisons. Pearson's correlation (r) and linear regression (R^2) were carried out using the Microsoft Excel 2013 to determine the relationship between (i) the morphological examination of both hepatopancreas and ovaries during the feeding trial, (ii) the histological sectioning of both oocyte and hepatopancreatic tubule, and (iii) the steroid hormone concentrations.

3. Results

3.1. Morphological Analysis of S. olivacea During Feeding Trial. Data for BWG, SGR, GSI, and HSI for *S. olivacea* during the feeding trial are presented in Figure 1. The data was

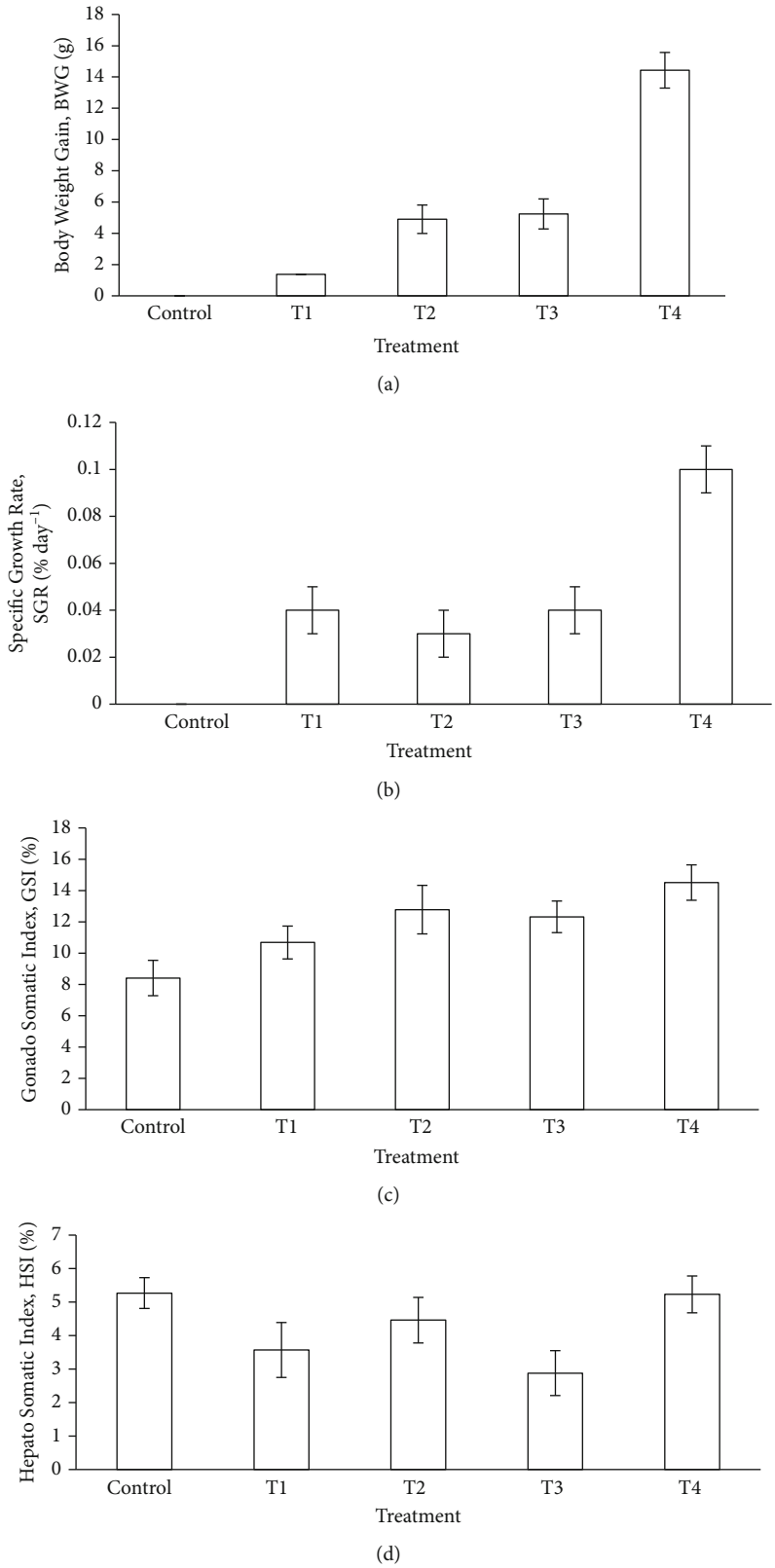

Figure 1: Comparison of (a) body weight gain (BWG); (b) specific growth rate (SGR); (c) Gonado-Somatic index (GSI); and (d) hepato-somatic index (HSI) of wild (control) female orange mud crabs with crabs fed formulated feed with different lipid levels (T1 = 60 g/kg, T 2 = 80 g/kg, T3 = 100 g/kg, and T4 = 120 g/kg lipid) during 90 days of feeding trial. [a,b,c]Means with different superscripts indicate significant differences ($P < 0.05$). The comparisons are between columns in the same period of time. No data were computed for BWG and SGR in control group as the samples were collected and dissected *in situ*.

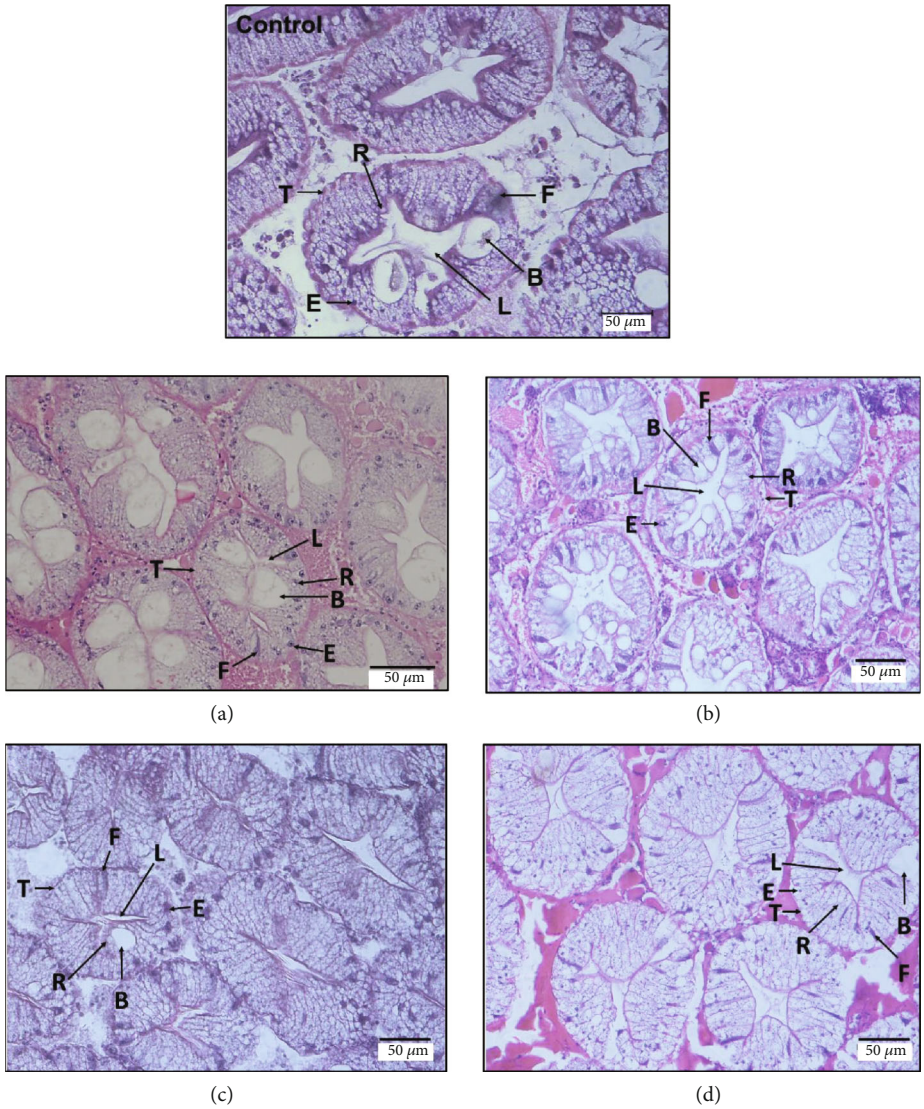

Figure 2: Histological section of hepatopancreas of the female orange mud crab, *S. olivacea*, fed graded lipid levels (control, (a) T1 = 60 g/kg, (b) T2 = 80 g/kg, (c) T3 = 100 g/kg, and (d) T4 = 120 g/kg) during 90 days of feeding trial using 20× magnification. B: B-cell, E: E-cell, F: F-cell, L: lumen, T: tubule, and R: r-cell. Bar = 50 μm.

compared with the wild-caught mud crab from Stage 4 was used as a control. Morphological analysis revealed that mud crabs from T1 showed the least BWG (1.38 ± 0.00 g) during the 90-day feeding trial compared to other treatments (T2 = 4.90 ± 0.91 g, T3 = 5.24 ± 0.96 g, and T4 = 14.43 ± 1.14 g). Following the data from BWG, the SGR disclosed the highest value in the mud crabs that were fed T4 ($0.10 \pm 0.01\%\text{da y}^{-1}$) from other treatments ($T1 = 0.04 \pm 0.01\%\text{day}^{-1}$, $T2 = 0.03 \pm 0.01\%\text{day}^{-1}$, and $T3 = 0.04 \pm 0.01\%\text{day}^{-1}$)($P < 0.05$). Meanwhile, high GSI was recorded in all treatments, indicating the maturity of ovaries in all crabs with the highest values noted in captive crabs compared to the wild-caught crabs, which recorded a GSI of 8.41 ± 1.13%. Among the treatments, the highest GSI were noted in crabs fed with T4 (14.51 ± 1.13%, $P < 0.05$) after 90 days of feeding trial. Similarly, higher HSI values were noted in mud crabs that were fed with T4 (5.23 ± 0.55%) compared to other treatments (T1 = 3.57 ± 0.82%, T2 = 4.46 ± 0.68%, and T3 = 2.88 ± 0.67 %; $P < 0.05$). At the same time, no significant difference was detected between HSI measured in crabs fed with T4 and crabs caught from the wild, even though the HSI in wild-caught mud crabs recorded a slightly higher value (5.27 ± 0.46%).

3.2. Histological Analysis of S. olivacea During Feeding Trial. The microscopic observations on histological sectioning of both hepatopancreas and ovary of captive mud crabs are shown in Figures 2 and 3, respectively. From Figure 2, it can be observed that the transverse section of hepatopancreatic tubules of the crabs consists of a single line of pseudostratified epithelium packed with E-, F-, B-, and R-cells that is arranged along the hepatopancreatic tubule.

At the same time, the cross-sections of the Stage 4 ovary were observed to be dominated by mature oocytes, enclosed within thin follicular cells, while the intercellular spaces

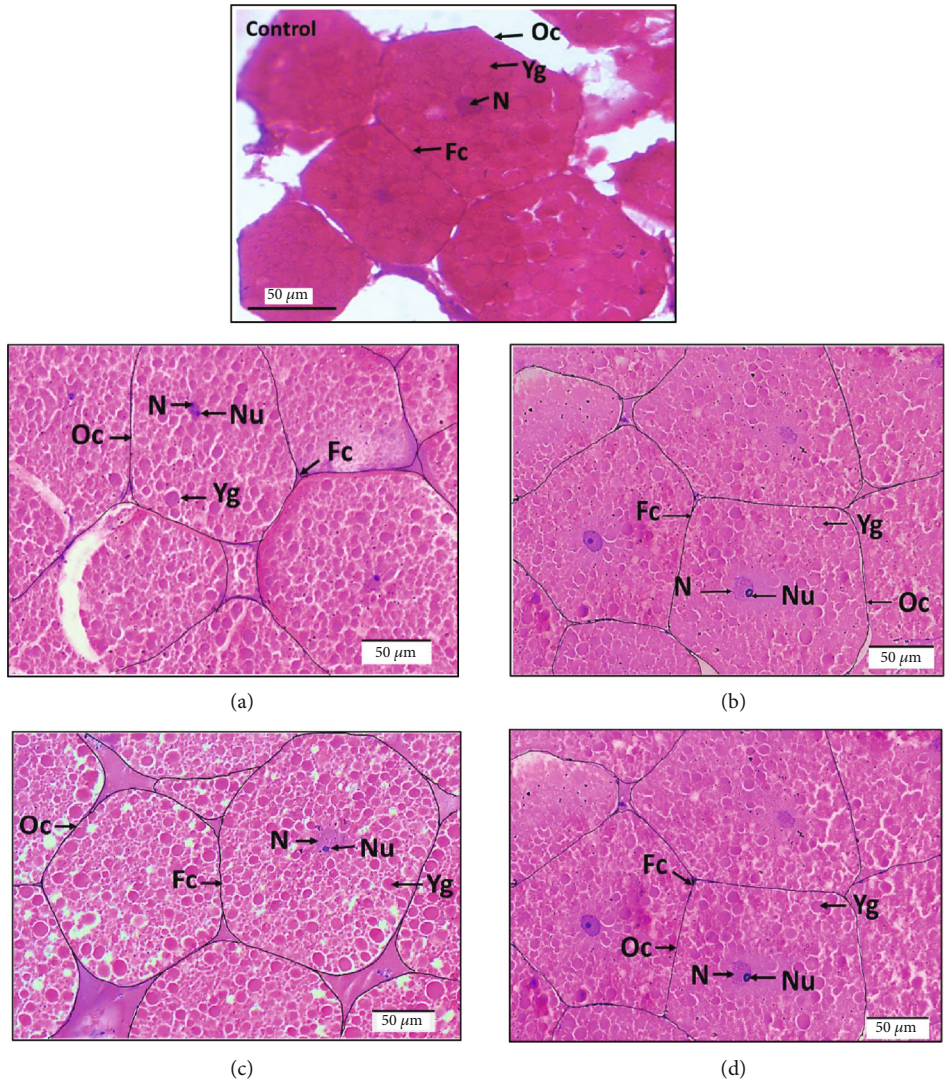

Figure 3: Histological section of ovary of the female orange mud crab, *S. olivacea*, fed graded lipid levels (control, (a) T1 = 60 g/kg, (b) T 2 = 80 g/kg, (c) T3 = 100 g/kg, and (d) T4 = 120 g/kg) during 90 days of feeding trial using 20× magnification. Fc: follicle cells; N: nucleus; Nu: nucleolus; Oc: oocyte; Og: oogonium; and Yg: yolk globule. Bar = 50 μm.

between oocytes showed minimal voids (Figure 3). Several cells could be distinguished, such as the follicle cells (Fc), nucleus (N), nucleolus (Nu), oocyte (Oc), oogonium (Og), and yolk globule (Yg). The oocytes showed irregular shapes that appeared granulated due to the high concentration of yolk globules. At this stage, the yolk granules were highly fused. The oocyte cells revealed an intense eosin staining with bright red color, showing highly acidophilic cells with evident yolk globules. No major differences were noted in the histological features of both hepatopancreas and ovary sectioning in the wild and captive *S. olivacea*; hence, only results related to the diameter were presented (Figure 4).

In Figure 4, the tubule diameter for all crabs was inversely proportional to the lipid levels where the crabs fed with the highest lipid level had the smallest tubule diameter (T4 = 135.10 ± 22.12 μm, T3 = 143.73 ± 29.84 μm, T2 = 159.68 ± 23.64 μm, and T1 = 183.19 ± 38.59 μm; $P \leq 0.00$). This was consistent with the hepatopancreas cross-section from Figure 2 where *S. olivacea* that were fed with T1 (60 g/kg lipid) showed the highest tubule diameter compared to other treatments. However, for mud crabs fed with T3, hyperplasia and dilation were noticed in their tubular lumen. In contrast, the diameter of the oocyte in the mud crabs was higher with the increase in lipid levels as crabs fed T1 revealed the smallest diameter in comparison to other treatments (T1 = 177.53 ± 14.29 μm, T2 = 188.82 ± 19.27 μm, T3 = 187.83 ± 18.39 μm, and T4 = 192.80 ± 21.93 μm; $P \leq 0.00$). The correlation (r) and regression (R^2) analysis showed a strong relationship between oocyte diameter and lipid levels such that the oocyte diameter was directly proportional to the lipid levels ($r = 0.91$; $R^2 = 0.8346$).

The analysis of epithelial cell distribution in the hepatopancreatic tubules is shown in Figure 5. Particular focus was given to B- and R-cells that mainly function in secretory and storage of nutrients, respectively. From the figure, *S. olivacea* fed with T2 had the highest count of B-cells (8.71 ± 2.14 cells

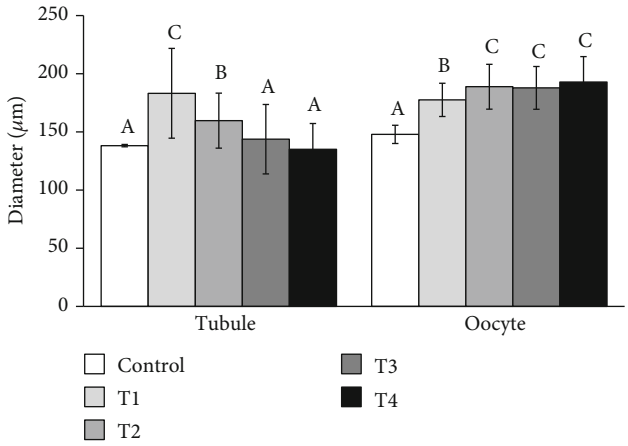

FIGURE 4: Tubule and oocyte diameter (μm) of mud crabs fed different dietary lipid (T1 = 60 g/kg, T2 = 80 g/kg, T3 = 100 g/kg, and T4 = 120 g/kg) during 90 days of feeding trials. ($n = 80$ tubules and $n = 80$ oocytes per treatment). ^{a,b,c}Means with different superscripts indicate significant differences ($P < 0.05$).

per tubule) compared to other treatments. However, no significant difference was detected between the treatments except for mud crabs fed with T4 (2.67 ± 0.58 cells per tubule; $P \leq 0.00$). On the other hand, R-cells in mud crabs fed with T3 showed the lowest counts (338.00 ± 45.66 cells per tubule) in comparison to other treatments (T1 = 415.20 ± 54.87, T2 = 406.86 ± 83.39, and T4 = 345.33 ± 59.37 cells per tubule). Compared to B-cells, the R-cells in *S. olivacea* were found abundantly in the cytoplasm of the tubule. Overall feeding trial revealed sound connective tissue ultrastructure with high lipid vacuolation in R-cells and hypertrophy of B-cells compared to the wild-caught mud crab.

3.3. Steroid Hormones Analysis of S. olivacea During the Feeding Trial. The variations of Pg and E_2 concentrations in the hemolymph and ovary of captive female orang mud crab, *S. olivacea*, during feeding trials are documented in Table 2. As the table indicates, there were increasing hormonal concentrations noted in the hemolymph from the beginning of the feeding trial, but only with slight differences. Inconsistent results were recorded in both hormones concentrations which the highest Pg concentrations were reported in mud crabs fed with T2 (+0.049 ng/mL) while the highest E_2 was reported in mud crabs fed with T1 (+0.072 ng/mL).

In ovary, E_2 presented the highest concentrations in mud crabs that were fed with T3 with a value of 12.65 ± 0.14 ng/mg, which was significantly different from other treatments (T1 = 8.78 ± 0.40 ng/mg, T2 = 7.41 ± 2.14 ng/mg, and T4 = 9.17 ± 2.06 ng/mg) ($P < 0.05$). On the contrary, Pg concentrations during the feeding trial showed the highest concentrations in *S. olivacea* fed with T4 (19.44 ± 0.33 ng/mg), followed by mud crabs fed with T3 (18.39 ± 2.91 ng/mg), T1 (18.11 ± 0.16 ng/mg), and T2 (16.05 ± 2.70 ng/mg). Yet, no significant difference was detected between the treatments. Simultaneously, Pg levels in captive *S. olivacea* revealed huge differences between wild-caught *S. olivacea* such that the Pg level in the ovary of the mud crabs kept in captivity (16.05–19.44 ng/mg) was lower compared to the wild-caught (146.36 ng/mg).

3.4. Relationship Between Steroid Hormones and Oocyte Diameter. Figure 6 showed the linear regression (R^2) and correlation (r) analysis that was carried out to distinguish the relationship between the oocyte diameter and Pg on the ovary of mud crabs fed with different dietary lipid levels. From the figure, a positive correlation between the oocyte diameter and Pg concentration was detected for all treatments, in which mud crabs fed with T4 showed the strongest correlation to the oocyte diameter and Pg with $y = 1.0905x + 170.09$ and $r = 0.73$. This was followed by mud crabs fed with T3 ($y = 1.3203x + 159.53$; $r = 0.65$) and T2 ($y = 1.393x + 158.63$; $r = 0.64$). However, T1 showed poor correlation with $y = 0.4672x + 169.71$ and $r = 0.12$. Regression analysis on the effect of lipid on the oocyte diameter and E_2 concentrations could not be performed as the data was inconsistent.

4. Discussion

Investigations on the ovarian and hepatopancreatic lipid concentration during reproduction have been extensively reported in several crustacean species studies such as red claw crayfish, *Cherax quadricarinatus* [44]; mud crab, *Scylla olivacea* [45]; and mantis shrimp, *Oratosquilla oratoria* [46]. It is widely accepted that lipid levels in crustaceans increase with their maturation stages [47]. In many cases, the ovarian lipid concentration increased during the reproductive phases, while the lipid content in the hepatopancreas significantly decreased [44–46]. These occurrences could be attributed to the mobilization of lipids during vitellogenesis [1, 31, 38], Tantikitti et al. [48]. In consideration of this, present studies have evaluated the effect of dietary lipid levels on the reproduction of mud crabs, *S. olivacea*, via morphological and histological examinations as well as hormonal concentrations. From the proximate analysis, it is noticed that the lipid levels in diets are increased by increasing the level of fish oil, which subsequently resulted in a higher level of HUFA, particularly ARA, EPA, and DHA. Reportedly, Aaqillah-Amr et al. [26, 47] documented an increase in ARA, EPA, and DHA in the diets developed for *S. olivacea* broodstock fed with 120 g/kg lipids (ARA = 0.81 mg/g, EPA = 1.27 mg/g, and DHA = 1.20 mg/g) as compared to that fed with 60 g/kg lipids (ARA = 0.47 mg/g, EPA = 0.32 mg/g, and DHA = 0.26 mg/g). These findings indicated that the increase in HUFA contents increased the lipid level of the crustacean diets, which is important when it comes to signaling pathways and growth performance of decapod crustaceans [49].

The morphological observations demonstrated that the lipid levels significantly influenced GSI in the captive *S. olivacea*. Present results indicate that crabs fed with T4 showed the highest GSI ($14.51 \pm 2.53\%$) and HSI ($4.76 \pm 1.43\%$) ($P < 0.05$) values compared to other treatments during the feeding trial and this could be mainly attributed to the high

Table 2: Levels of progesterone (Pg) and estradiol (E_2) in the hemolymph of captive female orange mud crab, *Scylla olivacea*, fed different dietary lipid levels (T1 = 60 g/kg, T2 = 80 g/kg, T3 = 100 g/kg, and T4 = 120 g/kg) during 90 days of feeding trials ($n = 6 \pm$ S.D.).

Treatment	Hemolymph (final day –day 1)		Ovary	
	Progesterone, Pg (ng/mL)	Estradiol, E_2 (ng/mL)	Progesterone, Pg (ng/mg)	Estradiol, E_2 (ng/mg)
Control	—	—	146.36 ± 18.92^{b}	9.72 ± 0.06^{ab}
T1	+0.028	+0.072	18.11 ± 0.16^{a}	8.78 ± 0.40^{a}
T2	+0.049	+0.009	16.05 ± 2.70^{a}	7.41 ± 2.14^{a}
T3	+0.027	+0.020	18.39 ± 2.91^{a}	12.65 ± 0.14^{b}
T4	+0.017	+0.028	19.44 ± 0.33^{a}	9.17 ± 2.06^{a}

[a,b]Means with different superscripts indicate significant differences ($P < 0.05$). The analysis of significant difference was made between treatments.

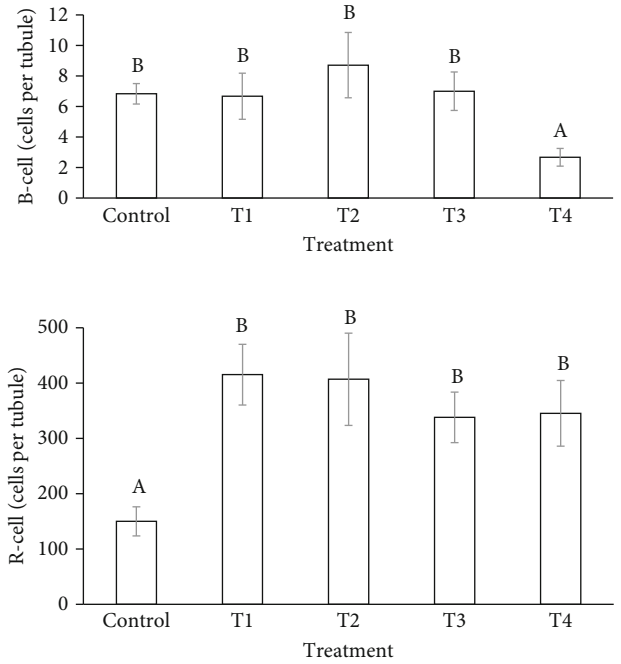

Figure 5: Average number of hepatopancreatic cells (B-cell and R-cell) of mud crabs fed different dietary lipid levels (T1 = 60 g/kg, T2 = 80 g/kg, T3 = 100 g/kg, and T4 = 120 g/kg). [a,b,c]Means with different superscripts indicate significant differences ($P < 0.05$).

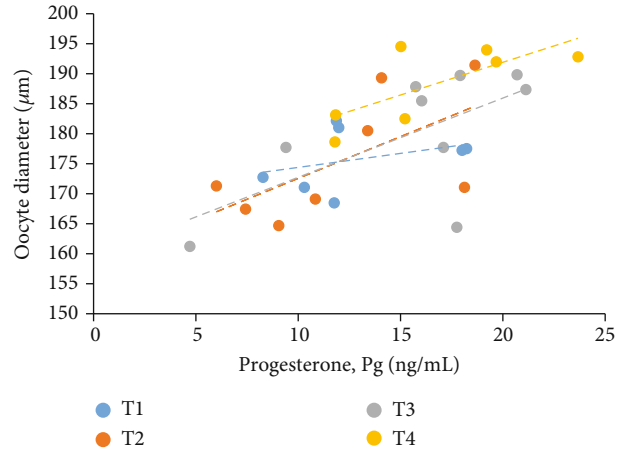

Figure 6: Correlation and linear regression analyses between progesterone and Pg levels in the ovary with oocyte diameter of captive mud crabs, *S. olivacea*, fed different lipid levels (T1 = 60 g/kg, T2 = 80 g/kg, T3 = 100 g/kg, and T4 = 120 g/kg).

percentage of lipid inclusion. As the hepatopancreas absorb and store nutrients, especially lipid, this organ provides a considerable amount of energy to the ovary during reproduction in crustaceans [50]. The high lipid level supplied in the T4 (120 g/kg) resulted in higher HSI and GSI values in the mud crabs than in other treatments. This study shared similarities with Xu et al. [51], who reported that HSI increased in proportion to dietary lipid levels (60 g/kg, 90 g/kg, and 120 g/kg) in the diet of Pacific white shrimp, *Litopenaeus vannamei*. Moreover, Rodríguez-González et al. [52] obtained higher GSI (6.04%) and HSI (7.96%) values in red claw crayfish when they were fed with 120 g/kg lipid, which explains the accumulation of nutrients in both organs. These findings signify that ovarian maturation progressed rapidly when the crabs were fed with high lipid levels. Such data is supported by a recent study carried out by Aaqillah-Amr et al. [1], in which increasing fatty acid concentration has been detected in the ovary of *S. olivacea,* with a decrease in fatty acid concentration in hepatopancreas during the maturation, suggesting that nutrients get transferred from the hepatopancreas to the ovary to support reproduction. Meanwhile, the higher HSI values in the wild-caught mud crabs ($5.27 \pm 0.46\%$) may be due to the wide accessibility to various food sources they have compared to the crabs kept in captivity [53]. Djunaidah et al. [54] mentioned that broodstock nutrition directly affects the maturation in crabs and food limitations can seriously inhibit ovarian maturation. Azra and Ikhwanuddin [55] added that mixed diets could improve the reproductive performance of adult crabs.

For histological assessments, the experiment conducted on captive crabs fed with different lipid levels has been proved to enhance vitellogenesis by providing the crabs with the necessary energy [1]. The discovery of higher oocyte diameters (T1 = 177.53 ± 14.29 μm, T2 = 188.82 ± 19.27 μm, 258 T3 = 187.83 ± 18.39 μm, and T4 = 192.80 ± 21.93 μm) in comparison to the tubule diameters (T1 = 251 183.19 ± 38.59 μm, T2 = 159.68 ± 23.64 μm, T3 = 143.73 ± 29.84 μm, and T4 = 135.10 ± 22.12 μm) in all treatments throughout the feeding trials supported the statement that

hepatopancreas act as a storage organ that distributes nutrients to ovary during its maturation. The results demonstrated that lipid levels showed a parallel trend in the tubule diameter as the dietary lipid level increased. This study observed the lowest tubule diameters ($135.10 \pm 22.12\ \mu$m), lowest R-cell count (345.33 ± 59.37 cells per tubule), and the highest oocyte diameters ($192.80 \pm 21.93\ \mu$m) in mud crabs fed with T4, suggesting that these crabs fully optimized the nutrient content for reproduction compared to other treatments. According to Štrus et al. [56], the content of lipid droplets in the R-cells depends on their nutritional availability such that the nutrients reserved are mobilized during vitellogenesis as reported in the lobster *Panulirus* sp. This study as well as a previous study by Simon [57] on the spiny lobster, *Jasus edwardsii*, indicate that the dietary lipids were paralleled with the growth performance and higher lipid provisions resulted in higher growth. Zheng et al. [58] added that lipid metabolism is essential for steroid production during vitellogenesis, mainly as an energy provider in *E. sinensis*. At the same time, captive mud crabs had prominent R- and B-cells with a larger tubule diameter in all treatments compared to the wild-caught mud crabs, thus signifying the effectiveness of the feed formulation to the crab performance. The roles of these epithelial cells are well documented in crustaceans. As such, the presence of B-cells suggested their function in the intracellular digestion and absorption of nutrients [59]. According to Hidir et al. [38], the accumulation of B-cells during the late stage of ovarian maturation is closely related to the secretion of the digestive enzymes in the mud crabs, *S. olivacea*. The final product of this intracellular digestion will be further assimilated by the R-cells for metabolism and energy storage, either in the form of lipid or glycogen [60–62].

The vertebrate steroid hormones, particularly Pg and E_2, are widely considered to affect the reproductive activities in the decapod crustacean [6]. Pg hormone which is synthesized by cholesterol also acts as a precursor to other steroid hormones, including 17a-hydroxyprogesterone and E_2 [63]. The presence of these hormones in the ovary and hemolymph is well recorded in many crustaceans, with the levels changing as the maturation progressed [9, 64]. The analysis of the steroid concentrations in the hemolymph during feeding trials on *S. olivacea* showed an increasing trend, indicating that the crabs have utilized the lipid provisions from the feed consumption. As dietary lipids could serve as a source of essential fatty acids, their transport from the hepatopancreas to the ovaries could help improving lipid deposition and energy utilization [32]. As different dietary lipid levels have been reported to differ between species, qualitative analysis is thus required to confirm the specific requirement in *S. olivacea*. The high concentrations of steroid hormones in ovaries partly result from their lipid levels. Investigation of *S. olivacea* verified that providing the crabs with 120 g/kg lipid level (T4) resulted in the highest oocyte diameter and Pg concentrations while crabs fed with 80 g/kg lipids (T2) had the lowest Pg concentrations throughout the experiments. As diets from T4 consisted of high levels of lipids, the high steroid concentrations recognized in the ovaries could be because of the high lipid provision in the diet compared to other diets. Evidence indicated that Pg played a vital role in the ovarian development in *S. olivacea* and that the concentrations were affected by the provision of lipid in feed formulation. According to Meunpol et al. [65], the success in reproductive performance of black tiger prawn that was fed a natural diet (polychaetes) resulted from the presence of arachidonic fatty acids and the other hormones in the diets. At the same time, the analysis of Pg in the ovary showed a higher concentration than that of E_2. This finding is similar to what has been reported in *S. serrata*, which showed a higher Pg concentration compared to E_2, especially in the late vitellogenic stage [66]. According to Ye et al. [63], the changes in Pg concentrations correlate with different ovarian maturation stages in crustaceans, suggesting that Pg may control vitellogenesis. On the contrary, Okumura and Sakiyama [67] reported a negative relationship between ovarian maturation and hemolymph levels of steroids in *M. japonicus*. Other studies have noted a positive relationship between Vg levels in hemolymph as well as circulatory levels of both Pg and E_2 in shrimp *P. monodon* [68], prawns [69], and crabs [70]. These differences suggested a dichotomous role in Vg between the hormones [8]. We have yet to identify the factors that cause vertebrate steroid changes due to environmental factors, such as food, the surrounding environment, or those caused by internal synthesis. Further explanation is also needed to clarify the roles of these hormones [5]. In addition, this chapter revealed an interesting finding that the level of Pg in the wild-caught mud crabs (146.36 ng/mg) was much higher than that of those kept in captivity (6.71–19.44 ng/mg). These huge differences may be related to their surrounding environments, i.e., mating and prey-predator interactions that affect the crabs' social behavior. Mud crabs, per se, have wide access to various food sources and other social interactions, such as prey-predator interactions and partner mating, which may trigger the dynamic changes in the vertebrate steroid level of these crabs. In contrast, *S. olivacea* kept in captivity showed lower Pg levels possibly because of their confinement and non-exposure to mate partners. According to Lenz and Hartline [71], the presence of mechanoreceptors in crabs is vital to their social behavior, such as mating and prey-predator interaction, which relate to the fluctuations in their hormonal changes. As a future study, it would be interesting to explore the overall mechanisms of crabs within their natural environment.

5. Conclusion

Results of the present study indicate that oocyte diameter, tubule diameter, and steroid concentrations were significantly affected by dietary lipid levels. This study demonstrated that mud crabs fed with T4 showed the best performance in terms of oocyte development and steroid concentrations and has confirmed the effects of lipid inclusion to directly affect the reproduction and the endocrine of crabs. It is suggested that 120 g/kg lipid is the most suitable lipid level to be used in diet formulations as a positive correlation was noticed between the oocyte diameter and steroid hormone, particularly the Pg concentration in ovarian tissue with the dietary lipid levels for *S. olivacea*. Further

experiment is expected to be carried out in the future to determine the nutrient-dose response as 120 g/kg lipid may not be the optimum lipid level for reproductive performance in *S. olivacea*.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Aaqillah Amr Mohd Amran designed the study, accumulated data, and drafted the paper. Hidir Ariffin drafted the paper. Noordiyana Mat Noordin designed the study and was involved in funding acquisition and supervision. Muyassar H. Abualreesh and Hongyu Ma were involved in data validation. Teoh Hong Peng was engaged in funding acquisition. Khor Waiho, Hanafiah Fazhan, Ahmad Ideris Abdul Rahim, and Muhamad Zulhilmi Ramlee were engaged in conceptualization. Mhd Ikhwanuddin designed the study and was involved in funding acquisition, project administration, and supervision.

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