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Effect of vertebrate steroid hormones on the ovarian maturation stages of orange mud crab, *Scylla olivacea* (Herbst, 1796)

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ABSTRACT

Orange mud crab, *Scylla olivacea* soughted as delicacy because their flesh is tasty and has high quality which boosted its commercial value in Malaysia. This studies focused on *S. olivacea* ovarian maturation after the introduction of steroid-based hormones i.e. 17α-hydroxyprogesterone (17α-OHP) and 17α-hydroxypregnenolone (17α-OHPL). The effects brought by these hormones were explored through external morphology, histology of oocyte and haemolymph hormone expressions. The crabs, *S. olivacea* were collected from Kuala Muda, Kedah on the West Coast of Peninsular Malaysia (5°39′N 100°19′E). After brief incubation, the crabs were introduced with treatments (through injection) of 95% alcohol (μl/g BW), 17α-OHP (0.01 μg/g BW and 0.1 μg/g BW) and 17α-OHPL (0.01 μg/g BW and 0.1 μg/g BW). During the treatment period, ovary coloration, gonad somatic index (GSI), oocyte diameter, oocyte structure and, levels of 17α-OHP as well as 17α-OHPL in haemolymph were collected every 10 days throughout the 60 days of treatment. Crabs injected with 95% alcohol showed negligible signs of ovarian maturation compared to those injected with hormones. However, crabs injected with 17α-OHPL showed increased ovarian maturity, produced the highest GSI (2.51 ± 0.72%) and produced large oocytes (diameter = 178.63 μm) with uniform development compared to crabs injected with 17α-OHP. In addition, decreased 17α-OHPL concentrations in the crab's haemolymph signifies utilization (of this hormone) to produce oestrogen. Hence, the findings obtained from this study depict fundamental biological information of the crab, *S. olivacea*. Nevertheless, the use of matured female *S. olivacea* is highly recommended for better results.

Statement of relevance:

This article is suitable with Aquaculture Journal because the findings will give a big contribution in mud crab aquaculture. Mud crab seedling especially Scylla olivacea is getting less landing every year, new approach needed in ensuring enough supplies of S. olivacea to be available. Using hormone in this study article would help the hatchery production of S. olivacea to increase.

1. Introduction

Currently, the mud crab aquaculture industry relies completely on the wild-caught seedlings and gravid female crabs, rendering the large-scale rearing process of mud crab as difficult (Keenan, 1999). In addition, due to decreased juveniles in the wild, the mud crab industry remains at a slower growth compared to other species (Keenan, 1999; Misieng, 2007). A constant availability of seedlings are required to overcome this problem (Ikhwanuddin et al., 2014). Unlike shrimp culture, mud crab culture lacks established techniques for gravid female production in captivity. In the wild, gravid female crabs usually burry themselves until egg spawning completes. During that time, female crabs rarely unearth themselves to feed (Phelan and Grubert, 2007). Therefore, the requirement of gravid female mud crab for hatchery spawning is essential and becomes an important aspect for future development of mud crab culture. By achieving this, the pressure posed by obtaining wild gravid female crabs are reduced and renders culture techniques successful to obtain sustained supply of mud crab seedlings and larvae.

As measure to meet market demands, ovarian maturation is sought as an effective approach in crustacean aquaculture. To do this, hormone introductions were preferred choices and such applications were proven effective especially in recent years. Hence, hormone injection is practical as alternative to induce gonad development. This method requires less handling and reduces stress of the crab (Ikhwanuddin et al., 2013). For example, ovaprim applications among catfish became a promising technique to accelerate the maturity of its ovary (Olubiyi et al., 2005). Yet, studies on ovarian maturation of the mud crab, *S. olivacea* using aid of hormones remain scarce and poorly elaborated. A number of steroids such as progesterone, 17α-hydroxyprogesterone (17α-OHP), 6β-hydroxyprogesterone, 20α-hydroxyprogesterone (20α-OHP), pregnenolone, 17α-hydroxypregnenolone (17α-OHPL), 17β-estradiol,

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estrone, testosterone, etc. were recognized as gonad stimulating hormones (Fingerman et al., 1993; Subramoniam, 2000; Tsukimura, 2001; Wilder et al., 2002; Oetken et al., 2004). Relatively, researchers working on pregnenolone, dehydroepiandrosterone, progesterone, 17α-OHP, testosterone, oestradiol and oestrone discovered their presence in the gonad whereas hormones like pregnenolone and 17α-OHPL were discovered in hepatic tissue.

The present study used 17α-OHP and 17α-OHPL to induce orange mud crab *S. olivacea* ovarian maturation. Therefore, by this attempt, possibilities and potential of steroid-based hormones are used to develop techniques and provide baseline information as future research reference in mud crab seed production. This will support commercial production of *S. olivacea* through sustained-development of mud crab for mass culture.

2. Methodology

2.1. Crab samples and culture condition

90 sexually immature female mud crab in intermolt stage samples were collected from Kuala Muda, Kedah on the West Coast of Peninsular Malaysia (Fig. 1). The mud crabs collected were in the range of 6–7 cm of carapace width (CW) and body weight (BW) of 70-80 g. All crabs were acclimatized for seven days in fibreglass culture tanks (3.0 × 1.0 × 1.0 m). Sand (depth approximately 5 cm) was added as bottom substrate. Several pieces of PVC tubes (7.6 cm diameter × 30 cm length) were also added as shelter and to reduce cannibalism. Filtered seawater (using 50-μm cotton filter bag) was added into the tank to depths between 50 and 60 cm, followed by continuous aeration at moderate intensity. Salinity was maintained at 30 ± 1 ppt and temperature at 28 ± 1 °C (using heaters) during acclimatization and treatment. The crabs were cultured for 60 days with ambience photoperiod and at stocking rate of three crabs per m^2. Since non-static treatment was deployed, complete water exchange was done at 10 days intervals. The crabs were fed with fresh blood cockles, *Anadara granosa* (approximately three blood cockles per crab) three times per day (0800 h, 1400 h and 2100 h). Unconsumed food is removed during the next feeding day to reduce contamination.

2.2. Test hormone

The hormones, 17α-hydroxyprogesterone (17α-OHP) and 17α-hydroxypregnenolone (17α-OHPL) (Sigma, USA) were prepared as 10 μg/ml. Dilution was prepared from 1 g of each hormone and 95% alcohol through 10-folds dilution to obtain 1 μg/ml and through 100-folds dilution to obtain 10 μg/ml. Both of the hormones levels were assess using kit from the brand of Cusabio bought from local company, Axon Scientific. 17α-OHP level assessment were done using ELISA kit 17-hydroxyprogesterone (17α-OHP also known as 17-hydroxyprogesterone) catalogue no. CSB-E17295Fh and 17α-OHPL were done using ELISA kit 17-hydroxypregnenolone (17α-OHPL also known as 17-hydroxypregnenolone) catalogue no. CSB-EQ027292FI(96 T).

2.3. ELISA Analysis

The heamolymph collected were analysed with ELISA according to the manufacturer instruction. First, the haemolymph samples were diluted with carbonate buffer (pH 9.5–9.7) with the ration of 1:1 prior to ELISA assessment. 50 μl from each haemolymph diluted sample was added in the ELISA wells with replicates of three. 50 μl of Horseradish peroxide-conjugated (HRP-conjugated) and 50 μl of antibody (for 17α-OHP or 17α-OHPL) to each well. The colour of the mixture should be in blue colour. Then they were incubated for 1 h at 37 °C. After the incubation, each well was washed with 200 μl wash buffer three times.

The plate were shaken lightly for 10 s and removed for each time. Removing the liquid in this step is essential as it gives better performances for the kit to perform. The excess remaining liquid were removed by aspiration or decanting by inverting the plate and blots it against clean paper towels. After that, 50 μl of substrate A and 50 μl of substrate B were added to each well on the ELISA plate and incubated for 15 min at 37 °C in incubator. During this step the plate and the substrates A and B were kept in the dark and any temperature fluctuation were evaded. After the incubation, 50 μl of stop solution were added to each well. The plate was gently tapped or shakes to make sure the solution mixed well. The optical density (OD) of each well was determined using a

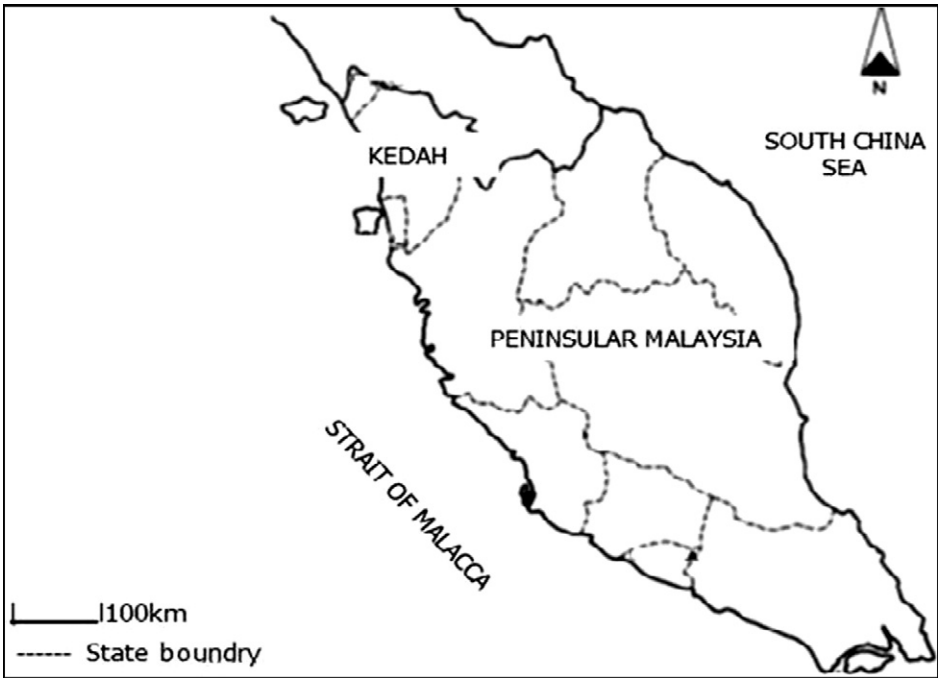

Fig. 1. Sampling site of mud crabs along Kuala Muda, Kedah coastal water, Peninsular Malaysian (5°39′N 100°19′E).

microplate reader (Thermo Scientific Multiscan FC) set to the wavelength of 450 nm (Cusabio Biotech CO., LTD).

2.4. Experimental design

The mud crabs were divided into five groups. The first group acted as control whereby this group did not receive any hormone injections. The next group (T1D1) is the treatment of 17α-hydroxy progesterone with concentrations of 0.01 μg/g BW. The next treatment (T1D2) is the treatment of 17α-hydroxy progesterone with concentrations of 0.1 μg/g BW. The next group (T2D1) is the treatment of 17α-hydroxy pregnenolone with the concentrations of 0.01 μg/g and the last treatment (T2D2) is the treatment of 17α-hydroxy pregnenolone with concentrations of 0.01 μg/g BW. The mud crabs received their injections according to their treatments at the beginning of the experiment and every interval of 10 days except on day 60.

The diluted hormone, 17α-OHP and 17α-OHPL were injected (with 27-gauge needle mounted onto 1 ml syringe) at the third walking leg of each crab. This step followed standardized procedures of Reddy et al. (2006) and Fatihah et al. (2014). Each crab was injected either, with concentrations of 0.01 μg/g BW or 0.1 μg/g BW. Then, evaluations were carried out at intervals of 10 days and covered hormone level estimations in the haemolymph, morphological observations and microscopic observations (through histology) of the ovary.

At interval of 10 days from 60 days of treatment, three mud crabs were removed from each treatment group and used as indicators. Firstly, haemolymph samples were extracted from the base of the third pereiopod using 1 mL syringe (27-gauge needle) that contained anti-coagulant (Saturated Tri-sodium citrate). About 0.1 ml of anti-coagulant was added to the syringe for every 1 ml of haemolymph obtained (1:10). Haemolymph samples were transferred into 1 ml micro centrifuge tube before centrifugation at 9000 rpm for 15 min at 3 °C. Centrifuged samples were stored at −80 °C and thawed on ice before analysis using Enzyme-Linked Immunosorbent Assay (ELISA).

After haemolymph collection, all mud crabs were executed by pithing before their ovaries removed and observed. Ovarian maturation was classified by coloration variations i.e. Stage-1: translucent to creamy white, Stage-2: yellowish, Stage-3: pale to dark orange and, Stage-4: dark orange to red-orange (Ikhwanuddin et al., 2014; Muhd-Farouk et al., 2014). Other information including, GSI was also recorded for each treatments.

The calculation of GSI is as below.

$$\text{Gonad somatic index} = \frac{\text{Ovary weight } (g)}{\text{Body weight } (g)} \times 100\%$$

After external observations, samples were dissected (contain oocytes and clear projection of ovary structure) for microscopic preparations. Sample preparations followed standard laboratory protocols for Haematoxylin and Eosin staining. The dissected ovarian lobes were fixed in 10% buffered formalin solution for 11 h., dehydrated in 70% alcohol and lastly placed in tissue processor for 18 ± 1 h at 60 °C. After processing, samples were mounted onto their cassettes using paraffin wax, sectioned into 5 μm films using a microtome (Leica RM835), transferred into water bath (between 40 and 45 °C) for expansion before mounting onto slides (glycerol + egg white was used as adhesive). The slides were dried using a hot-plate (40 °C) overnight. Then, the slides were immersed into a series of xylenes, dehydrated in a series of ethanol, stained in a haematoxylin solution, decolorized in 1% acid alcohol, re-stained in 0.5% aqueous eosin and lastly, mounted with DPX to prepare a microscope slide. During observations, oocyte diameter was measured and number of oocytes were estimated (Leica Microsystem, Wetzlar GmbH, DM LB 2, Germany).

2.5. Data collection analysis

With this, important information in the present study include hormone concentration in the haemolymph, colouration of ovaries, Gonad Somatic index (GSI) as well as, oocyte size and structure. These were collected every 10 days during the 60 days of treatment. Interpretations of hormones in haemolymph, GSI and oocyte were made using Correlation matrix in GraphPad Prism (ver. 6.0) software.

3. Results

3.1. Hormone levels in the haemolymph

Overall, there were fluctuations of 17α-OHP and 17α-OHPL in the haemolymph of crabs regardless those from control or treatment groups. Interestingly, the levels of 17α-OHP in crabs from the control group appeared elevated compared to those from the treatment groups, 0.01 μg/g BW (T1D1) and 0.1 μg/g BW (T1D2) (Fig. 2) except on Day 30. By Day 40, 17α-OHP level was highest (4.66 ng/ml) among crabs from the control group. In the first exposure group, T1D1, 17α-OHP appeared low on Day 10 (2.00 ng/ml) but, it rose to 2.92 ng/ml by Day 30. During the following observations of T1D1, the steroid hormone appeared fluctuated in the crabs and increased slightly by Day 60. When compared to the second treatment dose (T1D2) steroid hormone levels increased by Day 7 but it decreased between Day 30 and Day 50 before regaining by Day 60. In addition, 17α-OHP levels in crabs from T1D2 were always lower than crabs from the control and T1D1 groups.

As for the next treatment group, 17α-OHPL, similar trends were also observed where hormone levels in the crab from control group appeared elevated than treatment groups, 0.01 μg/g BW (T2D1) and 0.1 μg/g BW (T2D2) (Fig. 3). But, 17α-OHPL level appeared highest in Day 30 (15.05 ng/ml) crabs. For T2D1, 17α-OHPL appeared elevated by Day 10 in the crab but it decreased from Days 7 to 40. However, this hormone regained by Day 50 and 60 in the crab. The highest 17α-OHPL level in T2D1 was 6.8 ng/ml which was lower than at the starting point of the experiment. As for T2D2, the steroid hormone followed a decreasing trend, continuously until termination of treatment. Control was low for the whole experimental period. T2D2 highest 17α-OHPL level was at Day 30 which was also lower than the starting point of the experiment similar to T1D1. T1D2 showed negatively strong correlation ($r = 0.30$) with the 17α-OHP levels while control and T1D1 had weak correlation. Although there was correlation in all of the 17α-OHP treatments, they are no significant ($p < 0.05$) result being obtain. However, for 17α-OHPL, weak correlation was established with control group but, strong negative correlation was established between the treatment groups, T2D1 and T2D2.

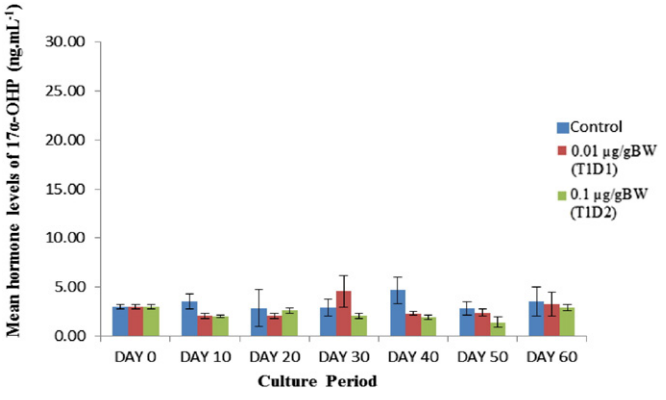

Fig. 2. Hormone (17α-OHP) levels in crab (ng mL^{-1}) for control and the treatment T1D1 (0.01 μg/g BW) and T1D2 (0.1 μg/g BW) groups within the 60 day treatment period.

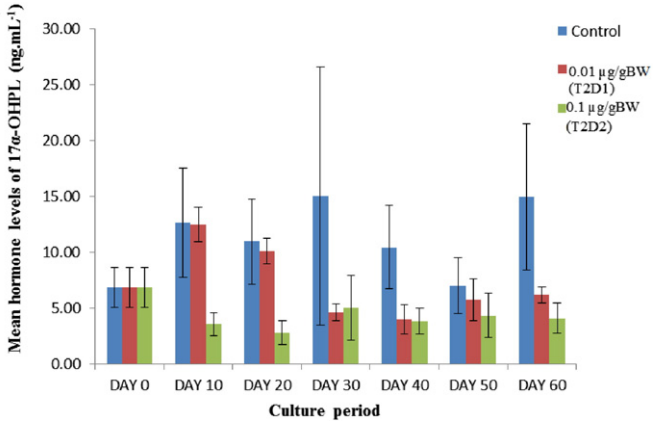

Fig. 3. Hormone (17α-OHPL) levels in crab (ng·mL^{-1}) for control and the treatment T2D1 (0.01 μg/g BW) and T2D2 (0.1 μg/g BW) groups within the 60 day treatment period.

3.2. External Morphology of Ovary

3.2.1. Ovary colour

Fig. 4 showed the four ovarian maturation stages that were used to distinguished the stage in the present study based on ovary coloration (Table 1), Control appeared to have ovaries maintained at Stage-1 i.e. white-translucent/creamy-white with ribbon-like structure. By Day 60, about 16.65% of crabs from T1D1 showed Stage-2 (yellow coloration) and Stage-3 (light-orange coloration) ovarian maturation while the remaining 83.36% only possessed Stage-1 ovaries (Table 1). As for T1D2, about 5.55% of crabs showed Stage-3 ovarian maturation while the remaining 95.45% possessed Stage-1 ovarian maturity (Table 1). Compared to the second hormone, about 27.78% of crabs from T2D1 possessed Stage 3 and Stage 4 (orange to dark orange coloration) ovaries whereas the remaining 72.9% only possessed Stage 1 (Table 1) ovaries. In the second treatment group, T2D2, about 16.67% crabs showed Stage 3 and Stage 4 ovarian maturation when compared to the other 83.33% which only possessed Stage-1 ovaries (Table 1).

3.2.2. Gonad Somatic Index (GSI)

The GSI of crabs in the control group remained low throughout the treatment period except on Day 40 (Figs. 5; 6). As for crabs from treatment groups, those in T1D1 showed increased GSI by Day 30 (0.79%) while those from T1D2 only exhibit increased GSI on Day 50 (1.44%). During other days, the crabs GSI from all groups were below 0.5%. Comparatively, in the second treatment group, T2D1, the crab's GSI remained low from Days 1 to 30 but it increased on Days 40 (1.75%) and 60 (2.51%). Crabs from T2D2 showed low GSI rates up to Day 40 with gradual increment by Day 50 and intense increment by Day 60 (1.74%). The interaction between GSI, control and T1D2 groups were negative unlike with T1D1 ($r = 0.35$), which was positive but weak. Comparatively, the interaction between GSI and treatment groups, T2D1($r = 0.71$) and T2D2 ($r = 0.62$) were positive and strongly correlated. Only the treatment, T2D1 ($p = 0.04$; <0.05) was found significant.

3.3. Ovary Histology Assessments

3.3.1. Oocyte morphological structure

The crabs from control group only had Stage-1 ovaries despite the termination of treatment after 60 days (Fig. 7). The ovaries of crabs from this group were filled with oogonia while primary oocytes possessed large nucleus, no yolk globule and abundant follicle cells. After the 17α-OHP treatment, crabs from T1D1 group possessed ovaries with Stages 1, 2, 3, and 4 development whereas crabs from T1D2 possessed ovaries from all development stages except Stage-2 (Figs. 8 and 9). Crabs treated with 17α-OHPL also possessed ovaries at advanced stage

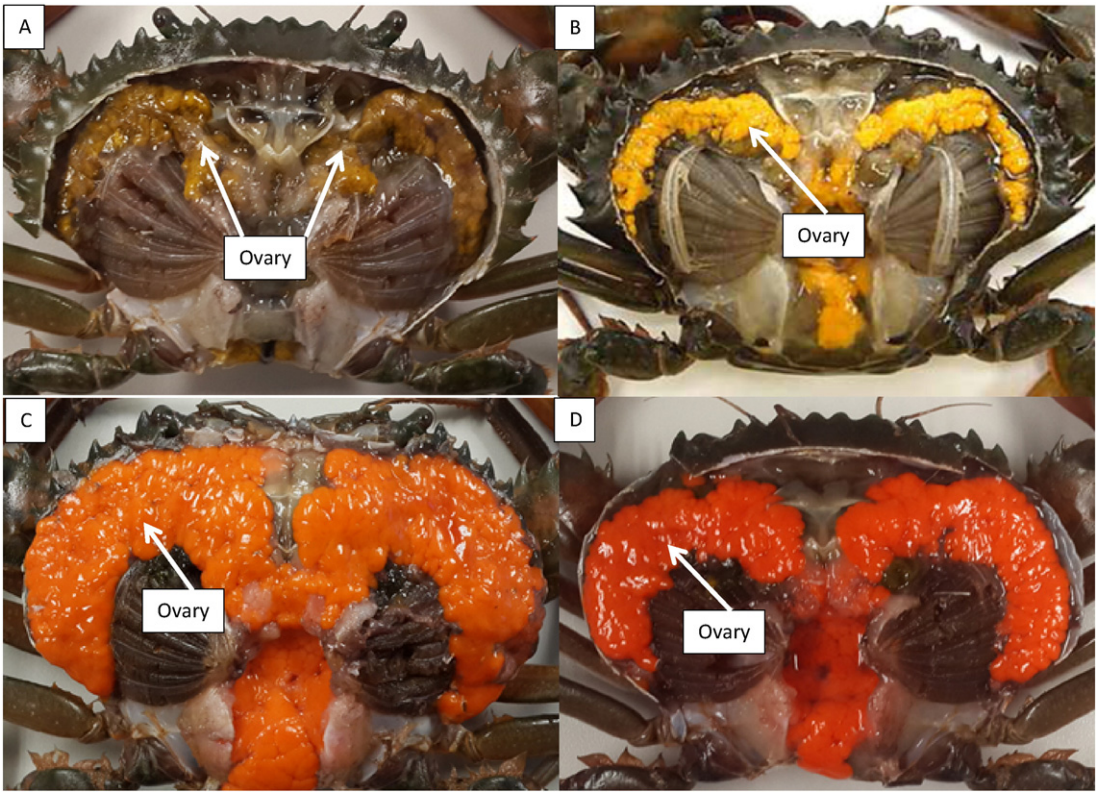

Fig. 4. Ovary Stages of *S. olivace.* (A) Stage-1 ovary (creamy white), (B) Early Stage-2 ovary (yellow), (C) Stage-3 ovary (orange) and (D) Stage-4 ovary (red orange).

Table 1
Ovarian maturation of *S. olivacea* by percentage (%) for each stage based on its coloration during the 60 day treatment covering control, 17α-hydroxyprogesterone and 17α-hydroxypregnenolone groups.

Hormone	Treatment	Ovary external morphology based on the ovary colouration			
		Immature stage ovary		Mature stage ovary	
		Creamy white (Stage 1)	Yellow (Stage 2)	Orange (Stage 3)	Red orange (Stage 4)
Control	95% alcohol μl/g BW	100%	–	–	
17α- hydroxyprogesterone	T1D1 0.01 μg/g BW	83.36%	5.55%	5.55%	5.55%
	T1D2 0.1 μg/g BW	94.45%	–	5.55%	–
17α- hydroxypregnenolone	T2D1 0.01 μg/g BW	72.22%	–	16.67%	11.11%
	T1D2 0.1 μg/g BW	83.36%	–	11.11%	5.55%

of development regardless of exposure dosage but, no crabs possessed ovaries from Stage-2 of development (Figs. 10; 11). Overall the oocyte morphological characteristics were same for all treatments (Table 2).

3.3.2. Oocyte diameter size

The mean oocyte diameter of crabs from the control group remained below 50 μm throughout the treatment period. However, in the treatment groups, T1D1, mean oocyte diameter increased and signified advanced developmental stages especially on days 10, 30 and 50 (Fig. 12). The largest oocyte diameter was recorded on Day 30 (82.97 μm) for this treatment group and signifies oocyte growth that occurs every seven days. In the dosage group T1D2, the crabs showed ovarian growth by day 10 but, its size was decreased by Day 7 and remained the same by days 30 and 40. Intense crab ovarian growth was observed on Day 50 (137.37 μm) by Day 60 the crab's mean oocyte diameter was found reduced to below 50 μm.

In the second treatment group, T2D1, the crab's mean ovarian growth increased gradually and intensified by Days 40, 50 to its largest by Day 60 (178.63 μm) (Fig. 13). In the dosage group T2D2, the crab's ovarian mean growth was slow until Day 50. By Day 60, the ovarian growth was intensified as it achieved diameter of 158.19 μm. The relationship between control ($r = 0.68$), T1D2 ($r = 0.51$), T2D1 ($r = 0.89$), T2D2 ($r = 0.66$) and oocyte size diameter showed strong positive relationships except for T1D1 which was in moderation ($r = 0.43$). In spite of this, the only significant oocyte diameter was found in T2D1 ($p = 0.00$; <0.05).

4. Discussion

It was discovered that the treatment group, T2D1 i.e. 0.01 μg/g BW of 17α-hydroxypregnenolone most effectively induced ovarian maturation as the highest number of matured crabs and largest oocyte were found in this group. Immature crabs are able to produce 3 to 5 ng mL^{-1} of 17α-OHP and from 6.87 to 15.05 ng mL^{-1} of 17α-OHPL under natural conditions. After addition of the mentioned hormones to their blood, concentration of these hormones was greatly reduced in the crab's blood. Remarkably, crustaceans are able to reduce/inhibit hormone production when such hormones are present excessively (Kimball, 2013). However in vertebrates, their hormone production e.g. progesterone is reduced in the absence of fertilization (Kimball, 2013).

Upon receiving extra volumes of hormones (now more than in haemolymph of crabs in control group), the treated crabs could have excreted excess, an explanation to reduction in their blood. Since ovaries of these crabs showed signs of maturation, there is a possibility that excess hormone was channelled to their gonad to prepare them for reproduction. Unlike the male spiny lobster, *Panulirus polyphagus*, excess introductions of 17α-OHPL and 17α-OHP resulted to accumulations in their haemolymph (Fatihah et al., 2014). However, when female lobsters were injected with these steroid-based hormones, ovarian maturation is immediately induced. When contrasting both lobster genders, excess hormone in the haemolymph should amount to accumulation rather than reduction. Therefore, this coincides with the utilization of 17α-OHPL and 17α-OHP as physiological biosynthesis in female crab to stimulate ovarian maturation.

In terms of dosage, reduced amounts of 17α-OHP and 17α-OHPL were more effective for the crab's ovarian maturation compared to

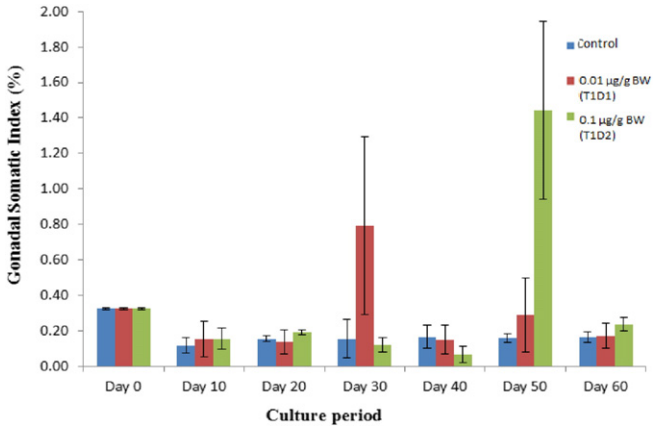

Fig. 5. Gonad Somatic Index (GSI) for crabs *S. olivacea* from the control and treatment groups (17α-OHP at 0.01 μg/g BW (T1D1) and 0.1 μg/g BW (T1D2)).

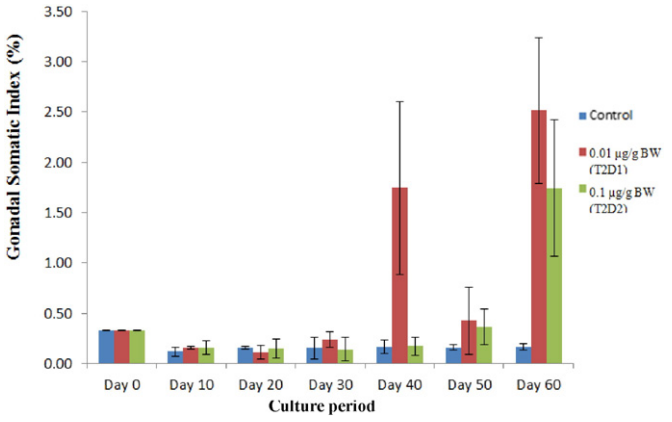

Fig. 6. Gonad Somatic Index (GSI) for crabs *S. olivacea* from the control and treatment groups (17α-OHPL at 0.01 μg/g BW (T2D1) and 0.1 μg/g BW (T2D2)).

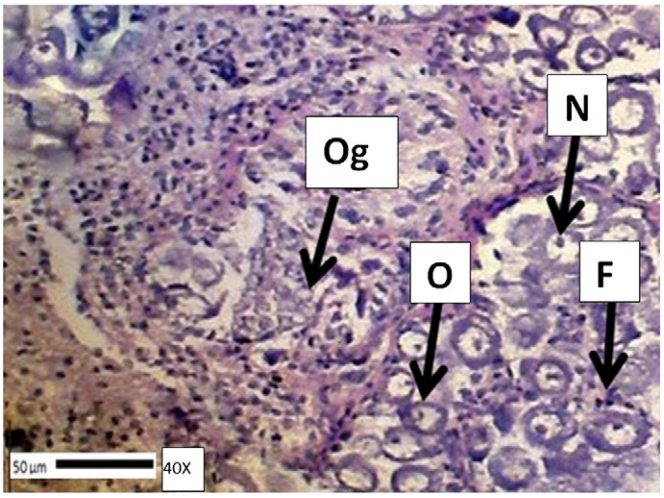

Fig. 7. Oocyte structure of *S. olivacea* from the control group. Structures — O: oocyte, F: follicle cell, N: nucleus, Og: oogonia.

higher doses. In fact, hormones function at optimum in lower volumes (Young, 2013). Hence, the effectiveness of both hormones were clearly projected in treatment groups T1D1 and T2D1. Induced amounts of steroid-based hormones to the crab's system activated its reproductive physiology unlike those of control group that had controlled amounts which were inadequate to trigger vitellogenesis. In addition, the presence of reproductive hormones (regardless concentration) sufficed to initiate vitellogenesis in invertebrates (Cruz et al., 2003). However, in larger invertebrate e.g. freshwater crab, *O. senex*, specific dosage of 17α-OHP was needed to induce ovarian maturation (Reddy et al., 2006).

Yet, there was uncertainty whether the given dosage of 17α-OHP and 17α-OHPL, although the lowest, was optimum and did not interrupt the natural hormone production. Drawback includes absence of correlation relationships between ovarian development stages and the dosage of steroid-based hormones. However, the functions of hormones vary from species to species depending on expression pathways present in that individual. For instance, hormones like estradiol-17β and progesterone significantly induced ovarian development of tiger prawn, *Penaeus monodon* (Quinitio et al., 1994) but, not to ridgeback shrimp, *Sicyonia ingentis* and white shrimp, *Penaeus japonicus* (Summavielle et al., 1995).

Different development stages of ovaries and oocytes were easily identified through *S. olivacea* ovarian external morphological observations and it was based on colour variations (Ikhwanuddin et al., 2014; Muhd-Farouk et al., 2014). Such substantial information (based on coloration of ovaries by carotenoid pigments) was also extracted from other crustaceans (George, 1963; Heegaard, 1963; Farmer, 1974; Fyhn and Costlow, 1977). With this, carotenoid pigments are produced after vitelline production as lipo-glycol-carotene protein complex was found in oocyte cytoplasm (Harrison, 1990; Chang, 1993). Interestingly, the presence of different structures also give-rise to different ovarian coloration i.e. presence of fatty yolk in Stage-3 produce yellow coloration (King, 1948). Hence, the presence of different structures that attach to the ovaries of mud crab e.g. yolk, carotenoid, cytoplasmic protein give-rise to its coloration and signifies its development stage.

The introduction of additional hormones to an individual induces physiological functions regulated by that hormone. This be the case of 17α-hydroxyprogesterone which affects crab ovaries (Reddy et al., 2006). Comparatively, oestrogen introductions to the shrimp *M. rosenbergii*, triggers lipogenic activity in its ovaries (Ghosh and Ray, 1994). Likewise, when steroid-based hormones are introduced to the sword prawn, *Parapenaeopsis hardwicki* where such hormones accelerate its ovaries to develop for reproduction (Kulkarni et al., 1979).

The GSI of mud crab increased with its ovarian development/maturation but it was in lower values that previous attempts. For instance, increased GSI rates were achieved after 17α-OHP or 17α-OHPL was introduced to *S. olivacea* (Islam et al., 2010). Moreover, increased

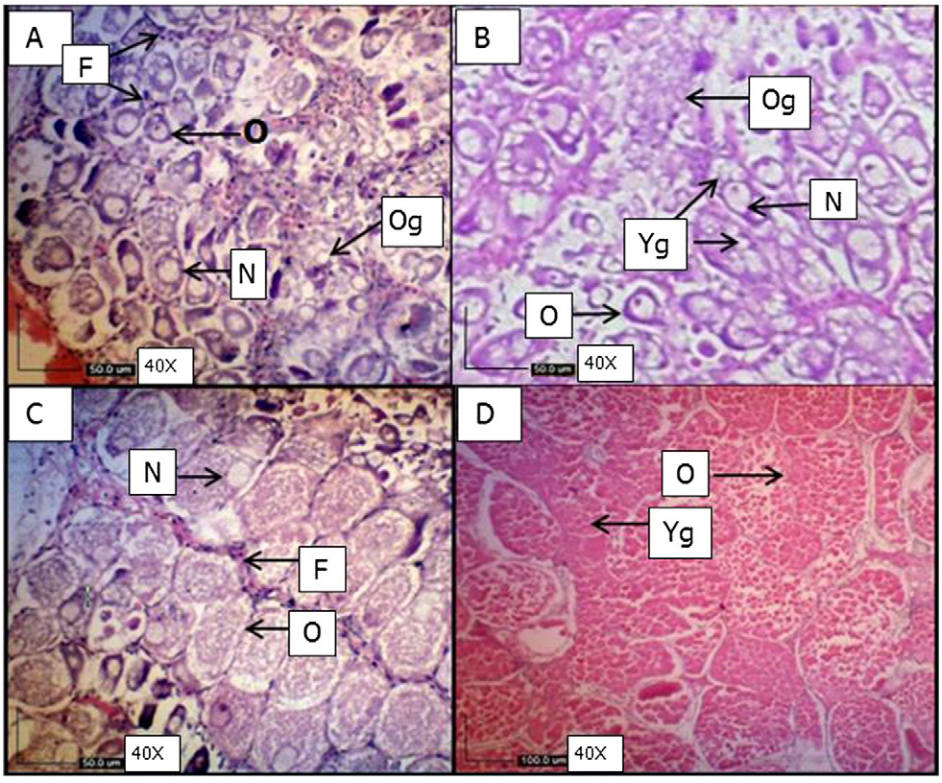

Fig. 8. Oocyte structure of *S. olivacea* from the T1D1 treatment of 17α-OHP. (A) Stage-1 oocyte, (B) Early Stage-2 oocyte, (C) Stage-3 oocyte and, (D) Stage-4 oocyte. Structures — O: oocyte, F: follicle cell, N: nucleus, Og: oogonia, Yg: yolk globule.

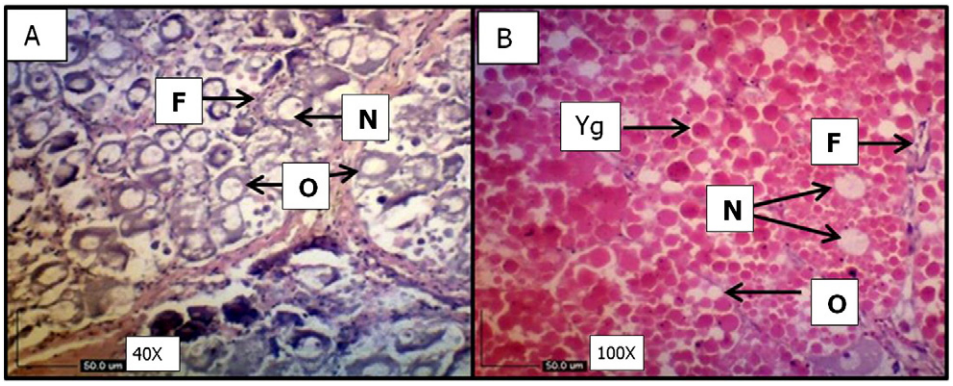

Fig. 9. Oocyte structure of *S. olivacea* from the T1D2 treatment of 17α-OHP. (A) Stage-1 oocyte, (B) Stage-3 oocyte. Structures — O: oocyte, F: follicle cell, N: nucleus, Yg: yolk globule.

GSI rates were also exhibited by the freshwater rice field crab, *Oziotelphusa senex senex* upon ovarian maturation after induced with 17α-OHP (Reddy et al., 2006).

Under natural conditions, non-mating and immature crabs do not produce sufficient hormones to initiate ovarian development cum vitellogenesis (Cruz et al., 2003). When sufficient hormone is introduced, the crab's reproductive functions are activated to prepare itself for mating. For this reason, immature crabs were used as test subjects in the present study. This will justify the function of the induced hormone i.e. the female crab achieving its sexual maturity. Usually, after moulting, the shell of female mud crab is still soft. During that time, male crabs mate with their female crabs through spermatophore transfer which then triggers the ovarian maturation process. Comparatively, immature crabs need to undergo moulting first before mating while sexually prepared crabs are readily available for mating.

The effects of 17α-OHP and 17α-OHPL on the female crabs were only visible after 30 and 40 days of treatment, respectively. The delayed reaction rate of 17α-OHPL was attributed to its function as precursor for 17α-OHP although both hormones are precursors for many other steroid hormones which in the end lead to the production of oestrogen. The steroid-based hormones, 17α-hydroxyprogesterone (17α-OHP) and 17α-hydroxypregnenolone (17α-OHPL) originate from pregnenolone. 17α-OHPL is directly synthesized from pregnenolone whereas 17α-OHP is synthesized from 17α-OHPL. In addition, 17α-OHPL serves as precursor for, dehydroepiandrosterone (DHEA). Hence, together, 17α-OHP and DHEA are precursor for reproductive hormones such as cortisol, oestrogen, testosterone, etc. (Gilep et al., 2004; Lisurek and Bernhardt, 2004). Perhaps stress from the hormone injection and trans-configuration of both hormones contributed to fluctuating GSI. Hence, this brought changes to the reproductive organs of the crab where oocytes were found matured i.e. contained yolk globules - a significant appearance of vitellogenesis caused by oestrogen.

However, observing the effect from these steroid hormones only from the external morphology of the ovary would only give ambiguous

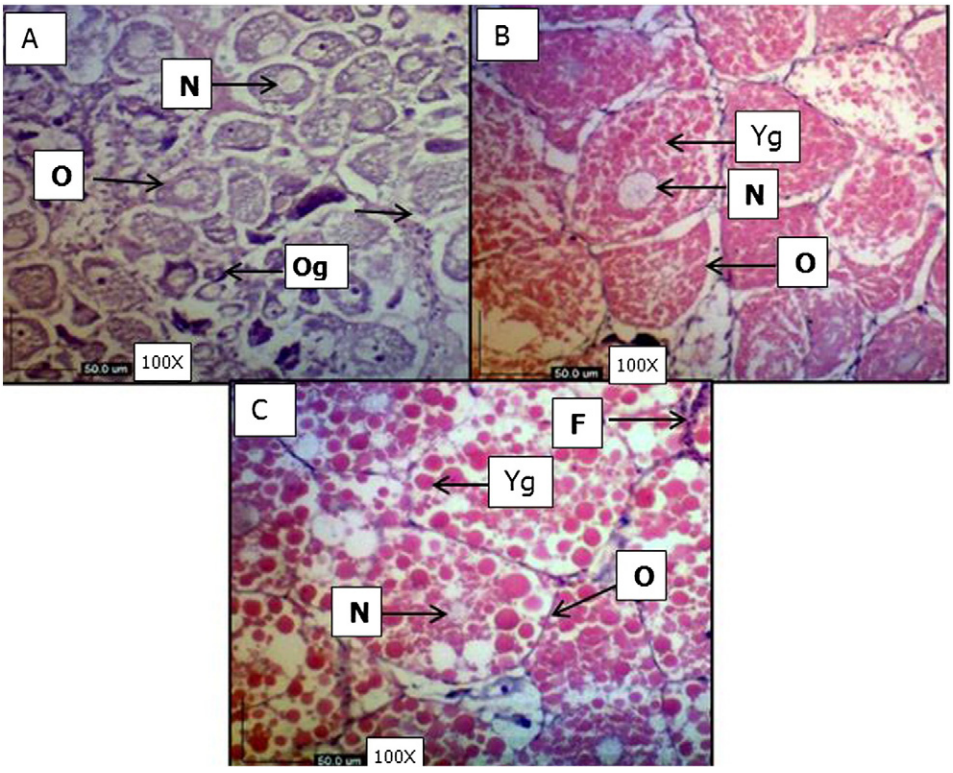

Fig. 10. Oocyte structure of *S. olivacea* from the T2D1 treatment of 17α-OHPL. (A) Stage-1 oocyte, (B) Stage-3 oocyte and, (C) Stage-4 oocyte. Structures — O: oocyte, F: follicle cell, N: nucleus, Og: oogonia, Yg: yolk globule.

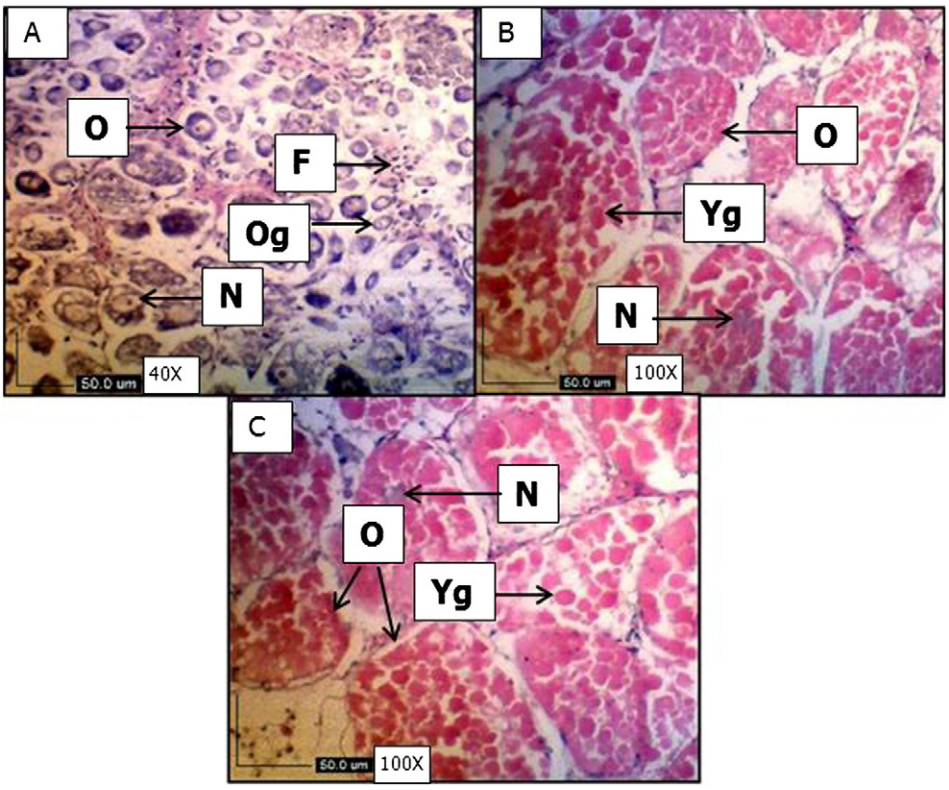

Fig. 11. Oocytes structure of *S. olivacea* from the T2D2 treatment of 17α-OHP. (A) Stage-1 oocyte, (B) Stage-3 oocyte and, (C) Stage-4 oocyte. Structures — O: oocyte, F: follicle cell, N: nucleus, Og: oogonia, Yg: yolk globule.

information further study need to be observed through the histological analysis. From the histological analysis, the steroid-based hormones successfully induced oocyte development in the crab but, produced inconsistent oocyte size and arrangement. For instance, in increased dosage of 17α-OHP, the group T1D2 mostly had crabs with Stage-3 and moderately with Stage-2 ovaries and oocyte development. Such poor oocyte uniformity was also present among the banana shrimp, *Fenneropenaeus merguiensis* after serotonin introductions (Ikhwanuddin et al., 2012). In the present study, 17α-OHP and 17α-OHPL induced *S. olivacea* ovarian and oocyte growth, an explanation for the appearance of reduced ovary sizes and absence of oocytes among crabs from the control group after the 60 days of treatment.

In the present study, the size of *S. olivacea* ovaries after hormonal treatment coincides with findings of Ikhwanuddin et al. (2014). Other than varying duration for the hormones to express effects on the crab's oocyte development, other considerations include susceptibility of the crab itself towards the introduced hormone. In vertebrates, 17α-OHPL is the precursor for 17α-OHP and both hormones are precursors for oestrogen. However, operational mechanisms of both hormones in crustaceans still remains unknown (Sahay and Sahay, 2012). Interestingly, as previously mentioned, 17α-OHP appear induced ovarian maturation much earlier than 17α-OHPL. Since 17α-OHP is readily available whereas 17α-OHPL needs time to convert to 17α-OHP, these facts influenced oestrogen production and hence, made 17α-OHP treatments (T1D1 and T1D2) induce ovarian maturation at an earlier rate.

Only immature female *S. olivacea* treated with hormones produced advanced stages (Stages 2–4) of ovarian and oocyte development within 60 days of treatment. The scarcity of studies that used 17α-OHPL to induce crustacean reproduction organ development makes the present findings valuable to the field of aquaculture. In fact, other pioneering work only examined effects of the hormone on male spiny lobster *P. polyphagus* (Fatihah et al., 2014) and effects of 17α-OHP on ovarian maturation of the freshwater crabs, *Barytelphusa cunicularis* (Kale et al., 2011). Since both studies showed promising results and when

Table 2
Oocyte structure characteristics of *S. olivacea* from the control and all treatment groups (T1D1, T1D2, T2D1 and T2D2).

Ovarian maturation stages	Microscopic observations after histology staining
1	Ovary filled with oogonia, primary oocytes and follicle cells. Primary oocyte possesses large nuclei and is surrounded by follicle cells. Oocyte diameter: 31.56 μm ± 12.65.
2	Oocyte diameter: 69.50 μm ± 3.71. Small yolk globules develop in the cytoplasm of larger oocytes (Fig. 2). Visible primary oocyte and follicle cells
3	Oocyte diameter: 1563 μm ± 18.39. Visible oocyte nucleus with large yolk globule formation in the cytoplasm. Follicle cells appear flattened.
4	Oocyte's diameter: 167.39 μm ± 37.72. Cytoplasm filled with very large oocytes and yolk globules. Yolk globules appear attached to each other. Poor visibility of follicle cells and nucleus.

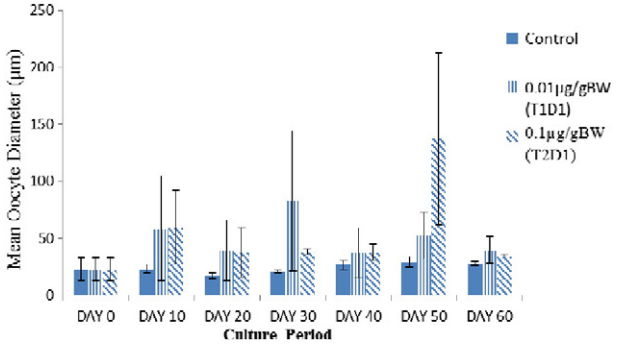

Fig. 12. Mean oocyte diameter (μm) of *S. olivacea* from the control, T1D1 and T1D2 treatments groups of 17α-OHP from days 0 to 60.

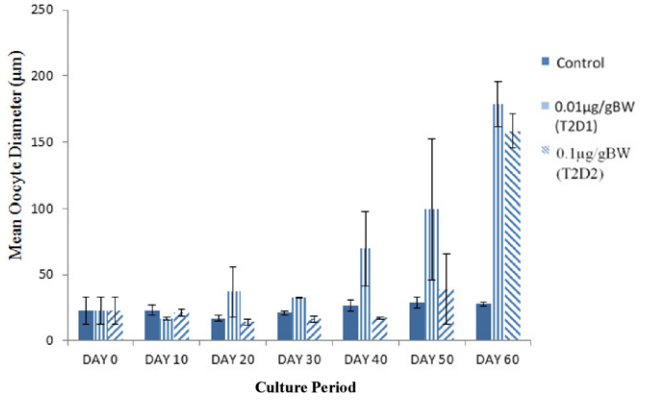

Fig. 13. Mean oocyte diameter (µm) of *S. olivacea* from the control, T2D1 and T2D2 treatments groups of 17α-OHPL from Days 0 to 60.

considering the findings of the present study, it is therefore proven that 17α-OHPL was successful to induce crustacean ovary maturation.

5. Conclusion

Findings from the present study show that T1D2 treatment i.e. 0.01 µg/g BW of 17α-OHPL was the most suitable hormone at optimum concentration to stimulate *S. olivacea* ovarian maturation and, produce the highest GSI as well as large oocytes. The use of ovarian examination (by coloration) and microscopic observations (histology) also confirmed the above findings. This is because, crabs injected with 17α-OHP produced ovaries of different colours, depending on development stages whereas, the number and size of oocyte as well as the size of yolk globules and follicle cells also varied at each ovary development stage. Hence, by incorporating these findings, both hormones i.e. 17α-OHP and 17α-OHPL induce vitellogenesis in the mud crab by becoming the precursor to many of the reproductive hormones. Simultaneously, the provision of these hormones to the crab sped ovarian development in combination with maturation because in absence (based on ambient concentration in crab–control) during 60 day experimental periods was insufficient to prepare female *S. olivacea* for mating.

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