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Effect of ecdysteroids on oogenesis in the freshwater crab *Travancoriana schirnerae* Bott, 1969 (Crustacea: Gecarcinucidae)

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Abstract. This study evaluated the reproductive performance of female *Travancoriana schirnerae* Bott, 1969 (Crustacea: Gecarcinucidae) administered with 20-OH ecdysone during different phases of the oogenic cycle. The effect of administration was evaluated by comparing the mean gonadosomatic index, oocyte diameter, oocyte proportion values and histological features of the control and concurrent control ovaries with those of the experimentals. The results clearly indicated that 20-OH ecdysone can stimulate ovarian growth and maturation in all phases of the oogenic cycle, though it caused statistically significant effects only during the early and middle vitellogenic phases, evidenced from the accelerated gonadosomatic index, oocyte diameter and oocyte proportion values, occurrence of vitellogenic oocytes in avitellogenic and previtellogenic ovaries, increased degree of yolk deposition and proliferation of gonia in the experimental ovaries compared to the controls. The outcome of this study is quite promising in the aquaculture practice of this locally abundant edible freshwater crab which forms a cheap protein substitute for the malnourished tribes/poor people of Wayanad.

Keywords: 20-OH ecdysone; Freshwater crab; Oogenesis; ovarian growth and *Travancoriana schirnerae*.

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Introduction

Besides the prime function of moulting, embryogenesis and metamorphosis, ecdysteroids play pivotal roles in growth and maturation of the ovary in crustaceans (Adiyodi and Subramoniarn, 1983; Young et al., 1993; Subrarnoniam, 2000; Wongsawang et al., 2005; Brown et al., 2009). In crustaceans, the Y-organ or the moulting gland synthesizes and secretes ecdysteroids from dietary cholesterol (Chang, 1985). Yudin et al. (1980) reported a direct relation between the circulating titre of ecdysteroids and vitellogenesis in the blue crab *Callinectes sapidus*. In *Carcinus maenas*, Lachaise et al. (1981) demonstrated an enhancement in ecdysteroid titre with stage of

maturation of the ovary. In the amphipod *Orchestia gammarella*, removal of Y organ in postmoult stage restrained the commencement of vitellogenesis (Blanchet, 1982). In *Acanthonyx lunulatus* (Chaix and De Reggi, 1982) and *Macrobrachium rosenbergii* (Laufer et al., 1993), the hemolymph titre of ecdysteroids was found at its peak in females with ovaries in late maturation phase. Okumura et al. (1992) found a perceptible rise in the hemolymph titre of ecdysteroid with the stage of development of the ovary in the reproductive moult cycle of *Macrobrachium nipponense*. Mu et al. (2014) have shown high ecdysteroid receptor expression in the ovaries of the crab *Portunus trituberculatus* after mating, signifying its role in ovarian growth and maturation. Gong et al. (2015) described three isoforms of ecdysteroid receptors in *Scylla paramamosain* ovary which possibly suggest the involvement of ecdysteroids in inducing ovarian growth and maturation. In the freshwater crab *Barytelphusa cunicularis*, Kale (2017) observed elevated values for ovarian index and oocyte diameter following β-estradiol administration.

Though literature is plenty regarding the role of ecdysteroids on reproduction in brachyurans, their functional role in the control of reproduction is divisive and mode of action on vitellogenesis is till now not determined, especially in freshwater brachyurans. In this context, the current research on the effect of exogenous administration of 20-OH ecdysone on growth and development of the ovary in a locally available edible freshwater crab, *Travancoriana schirnerae* Bott, 1969 (Crustacea: Gecarcinucidae), is undertaken.

Methodology

Adult females (carapace width 4.5-5.0 cm) with ovaries in various developmental stages were collected monthly for a period of one year (2017-2018) from rice fields near Ondayangadi (11° 49’ 20.3” N and 76° 01’ 47.1” E) and allowed to accustom with the controlled laboratory conditions (temperature 25 °C ± 2 °C and a photoperiod of 12 h light and 12 h dark) for 3-4 days. Crabs were fed daily once with boiled egg, pulses and decaying aquatic vegetation. Their carapace width and moult stages were determined by noting down the changes in the pleopod setae and the carapace texture.

The ecdysteroid (20-OH ecdysone) used in this experiment (Sigma Chemicals, USA) was dissolved in 10% ethanol (1 mg/1 mL). Individuals were divided into three groups; Group I (n = 15) which formed the initial control, were sacrificed on day one of the experiment. Group II, which received 10% 10 µL ethanol/crab/injection formed the concurrent controls and Group III (n = 15 each) which received 40 ng 20-OH ecdysone/injection/crab on days 1, 7, 14, and 21 formed the experimentals. The injections were given through the arthrodial membrane of the coxa of the last walking leg. Both the concurrent controls and experimentals were sacrificed on the 28th day of the experiment. The body weights and wet weights of ovaries of control and experimental crabs were recorded to determine the Gonadosomatic Index (GSI).

For measurement of oocyte diameter, around 50 oocytes/ovary were chosen randomly and their diameters were measured using a calibrated ocular micrometer. The stage of maturation of the ovary was assessed from the colour of ovary, oocyte diameter and from microscopical examinations. Pieces of ovaries were fixed in Bouin’s fluid, dehydrated in ascending grades of alcohol, infiltered and embedded in paraffin wax, sectioned and stained in Heidenhain’s hematoxylin-eosin, observed and photographed under a Leica DM 500 Research Microscope. Data were presented as Mean±SD and

analysed statistically using ANOVA. A probability value equal to or less than 0.05 was considered statistically significant.

Results

This study evaluated the reproductive performance of female *T. schirnerae* administered with 20-OH ecdysone during different phases of oogenesis. The effect of administration was evaluated by comparing the GSI, oocyte diameter, oocyte proportion values and histology of the ovaries of control and concurrent controls with those of the experimentals.

Effect of 20-OH ecdysone administration during avitellogenic phase

Though 20-OH ecdysone administration caused a spurt in GSI (0.238 µm ± 0.02 µm) and mean oocyte diameter (81.68 µm ± 1.95 µm) values than the initial controls (0.214 µm ± 0.02 µm and 76.14 µm ± 5.17 µm) and concurrent controls (0.223 µm ± 0.02 µm and 79.49 µm ± 3.05 µm, respectively), these values were not significant statistically (Table 1). Both control and treated ovaries showed normal development with oogonia in the germinal zone and the chromatin nucleolus (CN) and perinuclear (PN) stage oocytes towards the periphery. However, the histological architecture of injected crabs demonstrated the presence of primary (early) vitellogenic (PV) oocytes (5%) indicating signs of vitellogenesis in avitellogenic ovaries, a reduction in the proportion of shrunken follicles and atretic oocytes and a mild increase in proliferating oocytes (oogonia) (56%) in comparison to the control groups (53% and 54%, respectively) (Figures 1A, 2A-F).

Table 1. Comparison of GSI and mean oocyte diameter of control and treated *T. schirnerae* during various phases of development of the ovary.

Phases of oogenesis	Group	Gonadosomatic index		Mean oocyte diameter (µm)	
		Mean ± SE	F value	Mean ± SE	F value
Avitellogenic (April-May)	Control	0.214±0.02		76.14±5.17	
	Concurrent control	0.223±0.02	0.072	79.49±3.05	0.142
	20E treated	0.238±0.02	0.764	81.68±1.95	1.097
Previtellogenic (Jun-Sep)	Control	0.297±0.09		391.01±2.86	
	Concurrent control	0.302±0.05	0.002	394.57±5.58	0.612
	20E treated	0.328±0.05	0.059	398.12±12.30	1.345
Early vitellogenic (Oct- Nov)	Control	0.453±0.02		491.98±1.37	
	Concurrent control	0.462±0.01	0.069	500.76±12.45	1.429
	20E treated	0.534±0.02	7.783*	506.36±2.89	24.136*
Middle vitellogenic (Dec-Feb)	Control	1.127±0.08		780.30±33.04	
	Concurrent control	1.206±0.07	0.454	801.006±20.03	0.230
	20E treated	1.326±0.09	4.615*	958.84±35.34	13.477*
Late vitellogenic (Mar)	Control	4.243±0.26		1401.79±40.63	
	Concurrent control	4.329±0.37	0.038	1423.68±17.52	0.173
	20E treated	4.415±0.33	0.149	1469.29±13.22	1.695

Level of significance *P < 0.05.

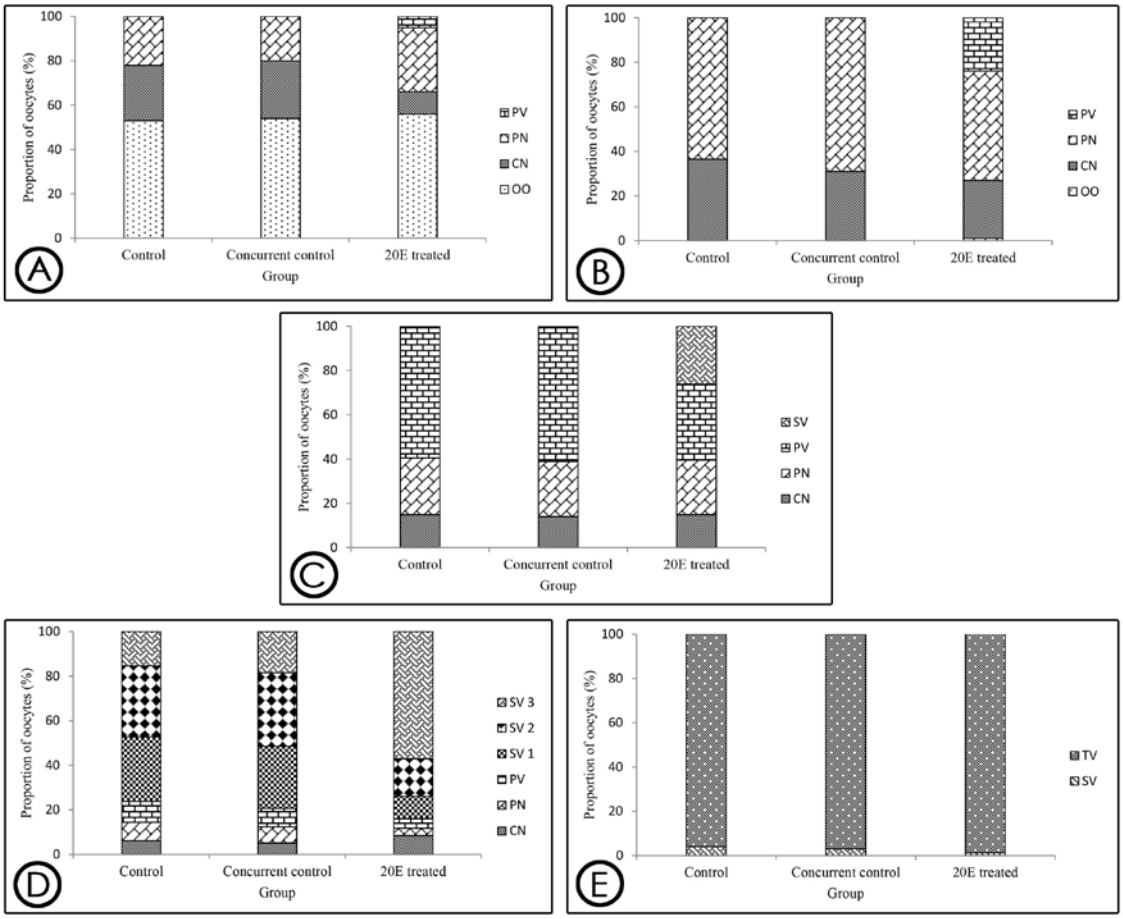

Figure 1. Effect of 20-OH ecdysone administration on oocyte proportion during various stages of maturation of the ovary in *T. schirnerae*. (A) Avitellogenic phase (B) Previtellogenic phase (C) Early vitellogenic phase (D) Middle vitellogenic phase (E) Late vitellogenic phase.

Effect of administration of 20-OH ecdysone during previtellogenic phase

Administration of 20-OH ecdysone during this phase did not cause a significant rise in GSI and mean oocyte diameter values (0.328 µm ± 0.05 µm and 398.12 µm ± 12.30 µm, respectively) with respect to the corresponding values of control (0.297 µm ± 0.09 µm and 391.01 µm ± 2.86 µm, respectively) and concurrent control crabs (0.302 µm ± 0.05 µm and 394.57 µm ± 5.58 µm, respectively) (Table 1). Nevertheless, histological analyses of injected ovaries revealed the presence of a large number of peripherally arranged primary vitellogenic oocytes (24%) along with the CN (26%) and PN stage oocytes (49%) and proliferating gonia (1%) arranged towards the centre of the ovary (Figures 1B). In addition, a follicular epithelium was noticed around the perinuclear and vitellogenic oocytes of treated crabs (Figures 3A-F). On the contrary, the control and concurrent control ovaries remained in the same stage of maturation till the end of the experimental period and contained only PN (64% and 69%, respectively) and CN oocytes (36% and 31%, respectively).

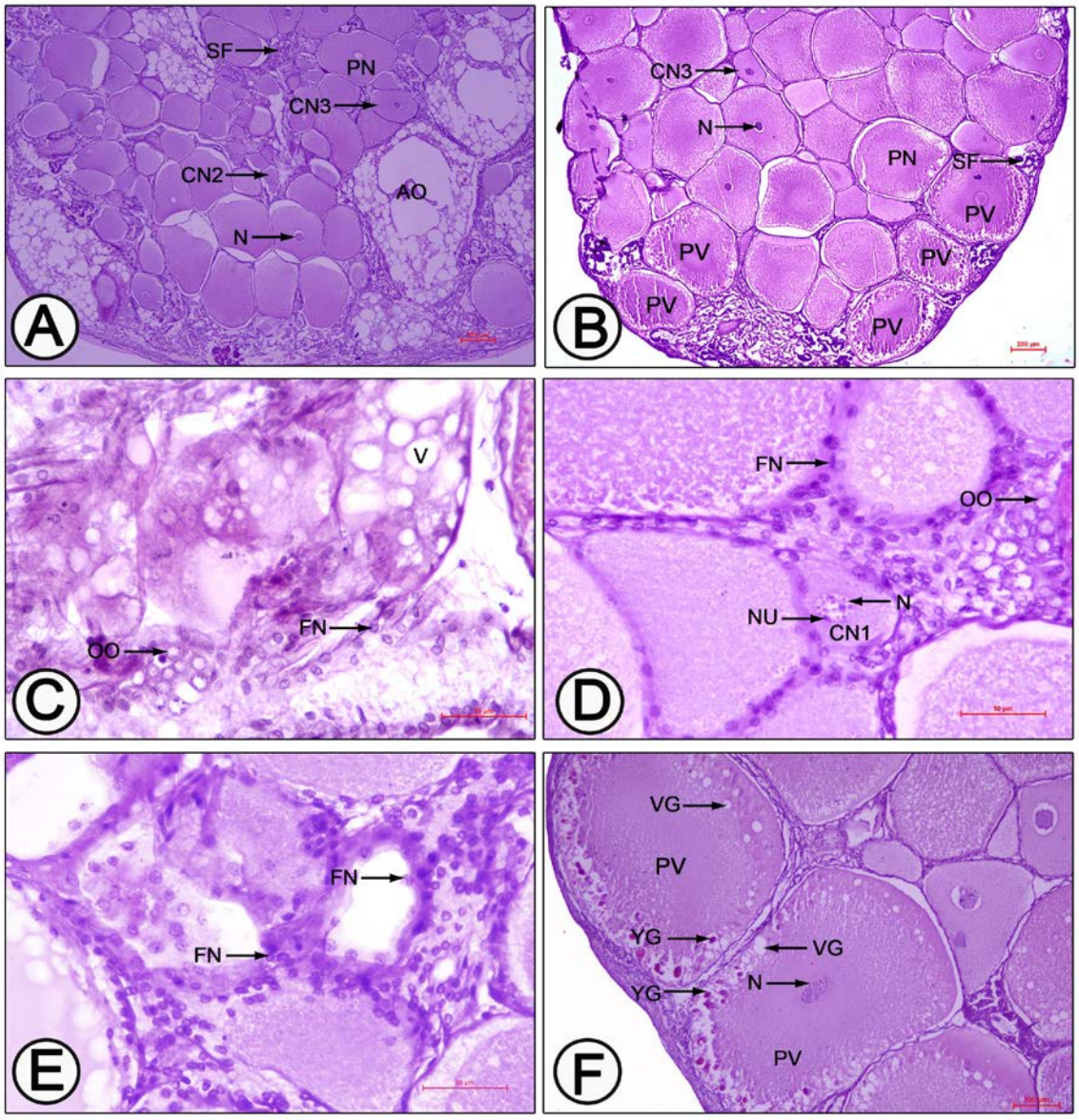

Figure 2. Longitudinal sections of control and treated ovaries in avitellogenic phase. (A) Control ovary depicting CN and PN oocytes, shrunken follicles and atretic oocytes (B) Injected ovary illustrating reduction in atretic oocytes and shrunken follicles (C-D) Ovaries of control and treated crabs portraying oogonial proliferation (E) Experimental ovary demonstrating follicle cell proliferation (F) Cortical alveoli in perinuclear stage oocytes of treated crabs. AO: Atretic oocyte; CN1: Chromatin nucleolus stage 1 oocyte; CN2: Chromatin nucleolus stage 2 oocyte; CN3: Chromatin nucleolus stage 3 oocyte; FN: Follicle nucleus; N: Nucleus; NU: Nucleolus; OO: Oogonia; PN: Perinuclear stage oocyte; PV: Primary vitellogenic oocyte; SF: Shrunken follicle; V: Vacuole; VG: Vacuolated globule; YG: Yolk globule.

Effect of administration of 20-OH ecdysone during early vitellogenic phase

Both GSI and mean oocyte diameter values escalated significantly (0.534 µm ± 0.02 µm and 506.36 µm ± 2.89 µm, respectively) ($P < 0.05$) in crabs administered with 20-OH ecdysone during this phase. The initial and concurrent controls had the GSI and oocyte diameter values 0.453 µm ± 0.02 µm and 491.98 µm ± 1.37 µm and 0.462

µm ± 0.01 µm and 500.76 µm ± 12.45 µm, respectively (Table 1). The most prominent feature of treated ovaries were the occurrence of many secondary (middle) vitellogenic (SV) oocytes (26%) while the initial control and concurrent control ovaries were dominated by primary (early) vitellogenic oocytes (60% and 61%, respectively) with small percentages of PN (25% each) and CN stages (15% and 14%, respectively) (Figures 1C). Another important feature of injected crabs was the incidence of oogonial nests in the germinal zone. Histological analyses of the ovaries of experimentals displayed more number of vacuolated globules (15.00 µm-28.70 µm) and large, highly basophilic yolk granules (12.50 µm-20.50 µm) in primary oocytes than their control groups (12.80 µm -23.50 µm and 8.05 µm -13.25 µm, respectively) (Figures 4A-E).

Effect of administration of 20-OH ecdysone during middle vitellogenic phase

The GSI and oocyte diameter values were significantly high ($P < 0.05$) in injected group (1.326 µm ± 0.09 µm and 958.84 µm ± 35.34 µm, respectively) compared to the initial (1.127 µm ± 0.08 µm and 780.30 µm ± 33.04 µm, respectively) and concurrent controls (1.206 µm ± 0.07 µm and 801.006 µm ± 20.03 µm, respectively) indicating that 20-OH ecdysone injection during this period can stimulate ovarian growth (Table 1). Both the control (76% and 79%) and treated ovaries (84%) were dominated by secondary vitellogenic oocytes, however, treated ovaries had a significantly higher percentage of secondary vitellgenic stage 3 (SV3) oocytes (57%) (with large vacuolated globules and yolk platelets) than the control groups (15% and 19% respectively). Proliferation of gonia and development of young oocytes (CN and PN stage oocyte) were perceptible in the germinal zone of ovaries of injected crabs (Figures 1D, 5A-D).

Effect of 20-OH ecdysone administration during late vitellogenic phase

Administration of 20-OH ecdysone in late vitellogenic phase did not cause a significant rise neither in the GSI (4.415 µm ± 0.33 µm) nor in the mean oocyte diameter (1469.29 µm ± 13.22 µm) values compared to the initial (4.243 µm ± 0.26 µm and 1401.79 µm ± 40.63 µm, respectively) and concurrent control values (4.329 µm ± 0.37 µm and 1423.68 µm ± 17.52 µm, respectively) (Table 1). Furthermore, there was not much difference in the histological appearance of the ovaries of control and experimental animals; both the control (96% and 97%, respectively) and experimental ovaries (99%) were dominated by tertiary vitellogenic oocytes with a minor proportion of secondary vitellogenic oocytes (Figures 1E, 6A-D).

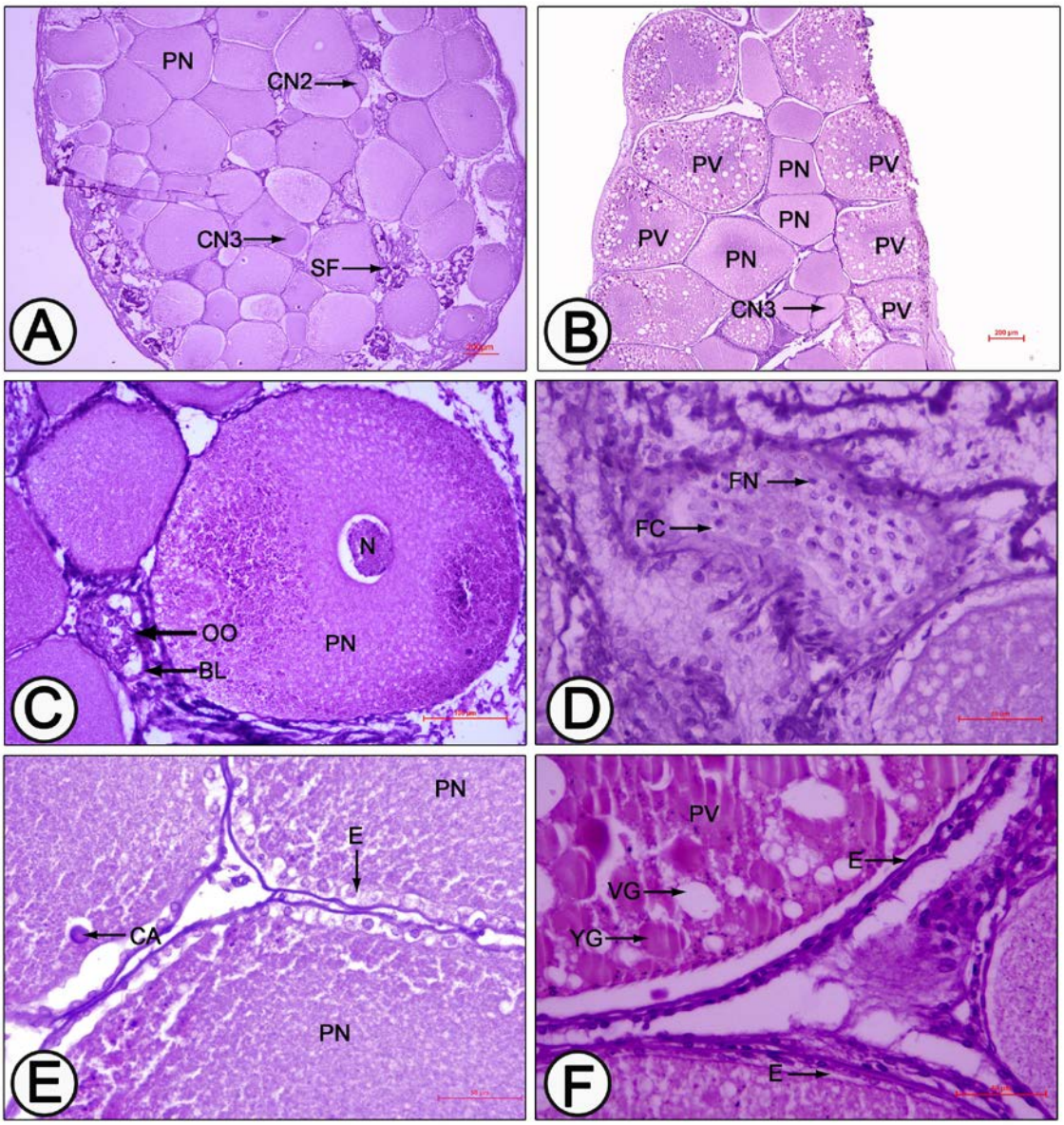

Figure 3. Light micrograph of ovary of control and 20-OH ecdysone administered crab during previtellogenic phase. (A) Ovary of control crabs populated by chromatin nucleolus and perinuclear stage oocytes (B) Primary vitellogenic oocytes in ovary of injected crab (C) Germinative islets in the ovary of treated crab (D) Treated ovary showing follicle cell proliferation (E) Follicular epithelium around the perinuclear oocytes in injected crab (F) Vitellogenic oocytes of experimental crab encircled by a bilayered follicular epithelium. BL: Basal lamina; CAA: Cortical alveoli; CN2: Chromatin nucleolus stage 2 oocyte; CN3: Chromatin nucleolus stage 3 oocyte; E: Epithelium; FC: Follicle cell; FN: Follicle nucleus; N: Nucleus; OO: Oogonia; PN: Perinuclear stage oocyte; PV: Primary vitellogenic oocyte; SF: Shrunken follicle; VG: Vacuolated globule; YG: Yolk globule.

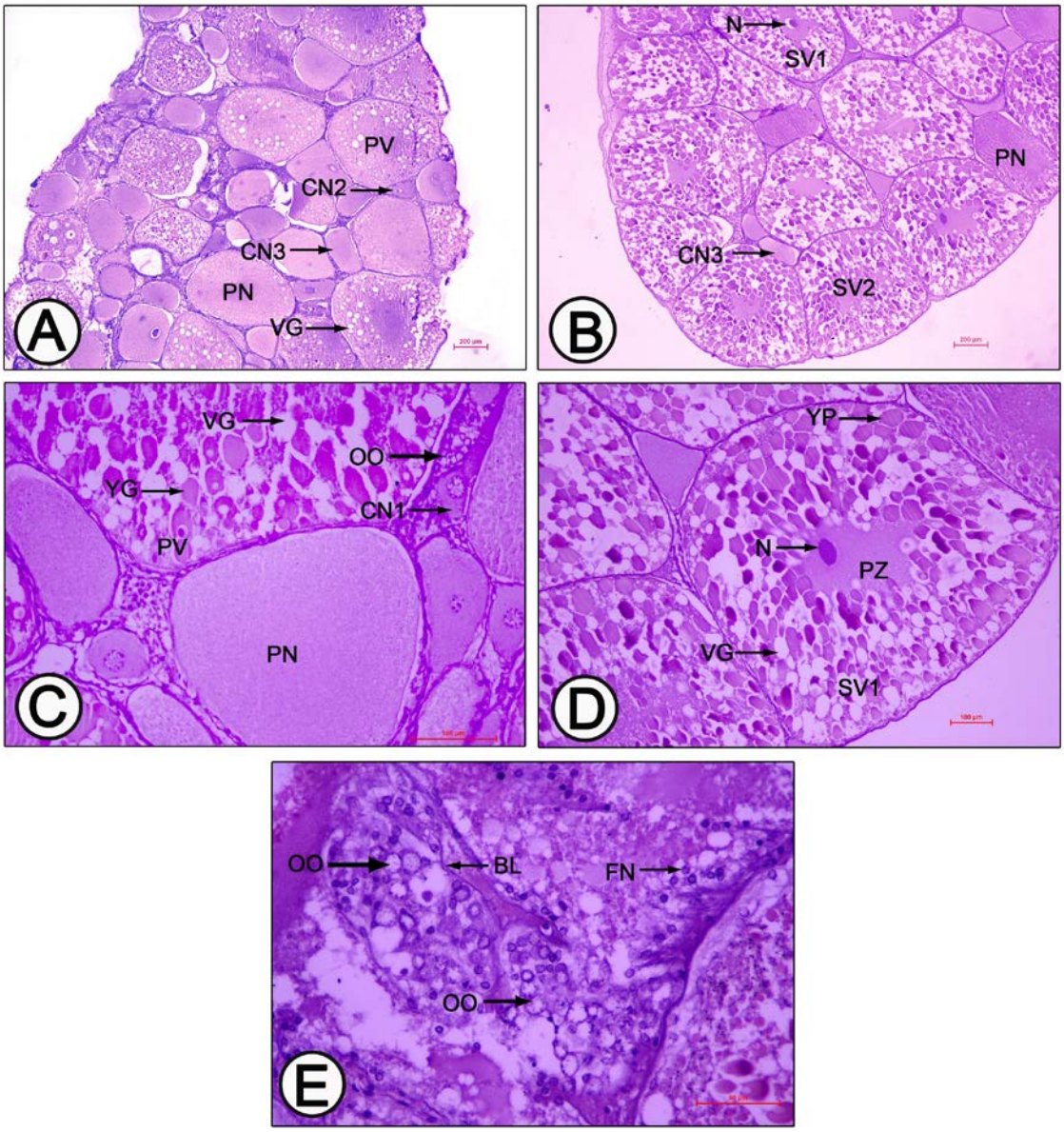

Figure 4. Ovaries of control and 20-OH ecdysone administered crabs during early vitellogenic phase. (A) Control ovary dominated by primary vitellogenic oocytes with vacuolated globules and yolk granules in the cortical region (B) Treated ovary dominated by secondary vitellogenic oocytes (C) Primary oocytes of experimentals with vacuolated globules, yolk globules and young oocytes (D) Vacuolated globules and yolk platelets in secondary oocytes of injected crab (E) Experimental ovary showing oogonial nest. BL: Basal lamina; CN1: Chromatin nucleolus stage 1 oocyte; CN2: Chromatin nucleolus stage 2 oocyte; CN3: Chromatin nucleolus stage 3 oocyte; FN: Follicle nucleus; N: Nucleus; OO: Oogonia; PN: Perinuclear stage oocyte; PV: Primary vitellogenic oocyte; PZ: Perinuclear zone; SV1: Secondary vitellogenic stage 1 oocyte; SV2: Secondary vitellogenic stage 2 oocyte; VG: Vacuolated globule; YG: Yolk globule; YP: Yolk platelet.

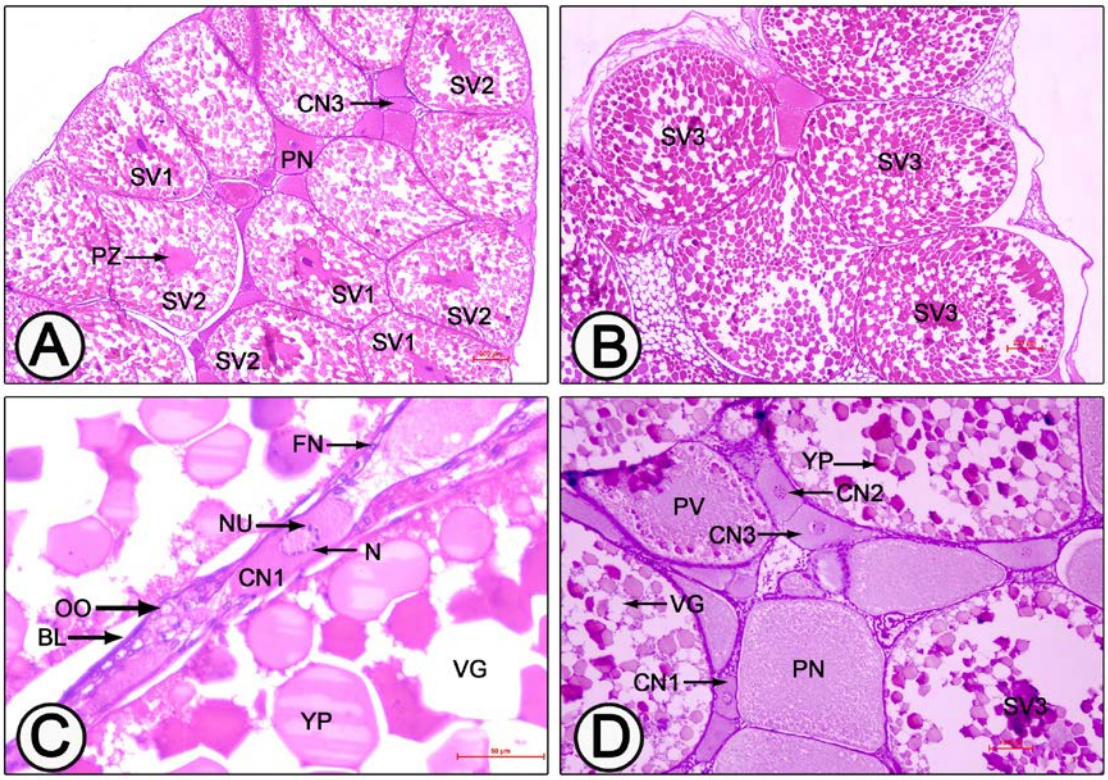

Figure 5. Light micrograph of ovary of control and 20-OH ecdysone administered crab during middle vitellogenic phase. (A) Control ovary dominated by SV1 and SV2 oocytes (B) Ovary of treated crab dominated by SV3 oocytes (C) Experimental ovary with large yolk platelets, vacuolated globules and proliferation of gonia in the germinal zone (D) Experimental vary showing development of young oocytes (CN and PN) from oogonia. BL: Basal lamina; CN1: Chromatin nucleolus stage 1 oocyte; CN2: Chromatin nucleolus stage 2 oocyte; CN3: Chromatin nucleolus stage 3 oocyte; FN: Follicle nucleus; N: Nucleus; NU: Nucleolus; OO: Oogonia; PN: Perinuclear stage oocyte; PV: Primary vitellogenic oocyte; PZ: Perinuclear zone; SV1: Secondary vitellogenic stage 1 oocyte; SV2: Secondary vitellogenic stage 2 oocyte; SV3: Secondary vitellogenic stage 3 oocyte; VG: Vacuolated globule; YP: Yolk platelet.

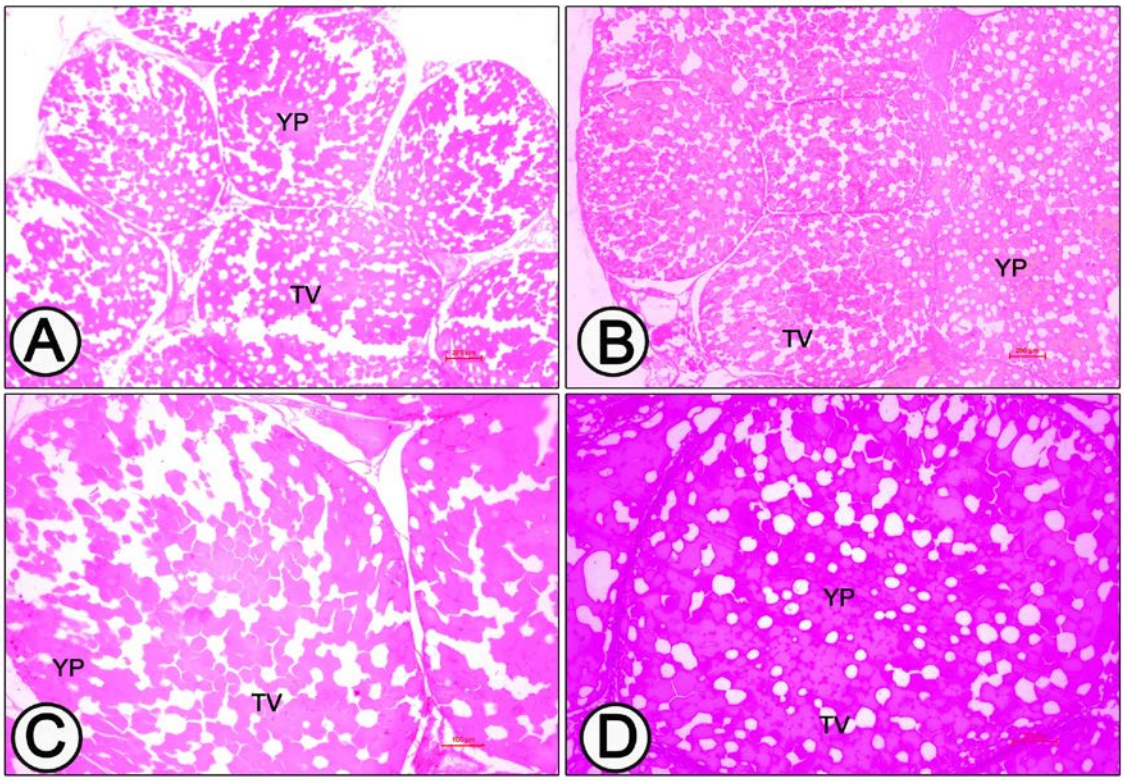

Figure 6. Ovaries of control and 20-OH ecdysone administered *T. schirnerae* during late vitellogenic phase. (A-B) Control and treated ovary packed with tertiary vitellogenic oocytes (C-D) Tertiary vitellogenic oocytes at higher magnification in control and treated crabs. TV: Tertiary vitellogenic oocyte; YP: Yolk platelet.

Discussion

Our observations present enough evidence that 20-OH ecdysone administration accelerated ovarian growth in all phases of development of the ovary except during the late vitellogenic phase as evidenced from the elevated gonadosomatic index, mean oocyte diameter and oocyte proportion values, presence of vitellogenic oocytes in prereproductive ovaries and the degree of yolk deposition in vitellogenic oocytes of experimental crabs over the controls.

The present study observed an increase in gonadosomatic index, oocyte diameter and oocyte proportion values in crabs treated with 20-OH ecdysone. Incongruous observations were made by scientists regarding the role of ecdysteroids on reproduction in crustaceans. Several authors suggested steroid (17α-OH progesterone and estradiol) induced enhancement in ovarian indices and oocyte diameters in *Litopenaeus vannamei*, *Procambarus clarkii* and *Oziotelphusa senex senex* (Tsukimura and Kamemoto, 1988; Rodriguez et al., 2002; Sujathamma and Dayakar, 2015; Reddy et al., 2006, 2016; Swetha et al., 2016). Administration of 17α-OH pregnenolone as well as 17α-OH progesterone stimulated ovarian index, oocyte diameter and ovarian maturity in *S. olivacea* (Muhd-Farouk et al., 2016). The increase in GSI and oocyte diameter in 20-OH ecdysone injected crabs compared to the controls in the present study is suggestive of the increased yolk deposition enticed by 20-OH ecdysone.

The involvement of vertebrate type steroid hormones in boosting up ovarian growth have been suggested by

several authors (Nagabhushanam et al., 1987; Sarojini et al., 1990; Zapata et al., 2003; Kale et al., 2008). Couch et al. (1987) noticed a high level of 17β-estradiol in developing ovaries of crustaceans. Progesterone, estrone or 17β-estradiol stimulated vitellogenesis was observed in both freshwater (Sarojini et al., 1985, 1986) and marine penaeids (Kulkarni et al., 1979; Nagabhushanam et al., 1980; Yano, 1987; Van Herp and Payen, 1991). Reddy et al. (2006) and Kale (2017) described the 17 α-OH progesterone and β-estradiol influenced increase in ovarian index, oocyte diameter, ovarian growth and vitellogenin synthesis in the freshwater crabs *O. senex senex* and *B.cunicularis*. In *S. olivacea*, Muhd-Farouk et al. (2014) reported enhanced growth of ovary on administration of 17α-OH progesterone and 17α-OH pregnenolone. Medesani et al. (2015) observed significantly increased ovarian indices and vitellogenin levels in female *Neohelice granulata* fed with 17α-OH progesterone pelleted feed. In the tiger prawn *Penaeus monodon*, in vivo and in vitro administration of 17β-estradiol and 17α-OH progesterone persuaded vitellogenesis and ovarian maturation (Merlin et al., 2015). Oogenesis was hastened in *Parapenaeopsis stylifera* and *Chasmagnathus granulata* administered with steroid hormones (Nagabhushanam et al., 1987: Zapata et al., 2003). On the other hand, Rodriguez et al. (2002) and Okumura and Sakiyama (2004) observed a negative correlation between ovarian maturation and vertebrate steroids in *P. clarkii* and *Marsupenaeus japonicus*.

In the present study, 20-OH ecdysone treatment caused the production of vitellogenic oocytes in avitellogenic and previtellogenic ovaries and secondary vitellogenic oocytes in early vitellogenic ovaries. In other words, 20-OH ecdysone treatment stimulated avitellogenic and previtellogenic ovaries to grow into vitellogenic ovaries. Moreover, the degree of yolk deposition was high in vitellogenic oocytes of treated crabs than their corresponding controls. Similar research has been carried out by Swetha et al. (2016) in *O. senex senex* wherein previtellogenic females reached vitellogenic stage III when administered with 17β-estradiol and progesterone. In *L. vannamei*, Chan (1995) noticed a high 20-OH ecdysone titre in the early stages of development of the ovary. Lachaise and Hoffmann (1981) identified three ecdysteroids: ecdysone, 20-OH ecdysone and ponasterone A in *C. maenas* ovary during early maturation stages. The hemolymph vitellogenin level was found parallel to the ecdysteroid titre during vitellogenesis in isopods and amphipods suggesting its role in vitellogenin synthesis (Steel and Vafopoulou, 1998). Conversely, De Meusy (1962) demonstrated a total non-intervention of ecdysteroids in vitellogenesis in *C. maenas* where Y-organectomy could not cease vitellogenesis. Crompton (1967) noticed a reticence in the production of 20-OH ecdysone during maturation phase and accrual of other ecdysteroids during immature phase ovary.Laufer et al. (1988) and Young et al. (1993) observed a reduction in hemolymph ecdysteroid titre during vitellogenesis in *Libinia emarginata* and *P. monodon*. Gunamalai et al. (2003) described the exclusion of ecdysteroids by ovary during vitellogenesis in *Emerita asiatica.*

The current study also observed an increase in the proliferation of oogonia and follicle cells following 20-OH ecdysone administration. In crustaceans, progesterone induced the development of germ cells and gonia was reprted by Brandau (1970) and Joshi (1980). In *C. maenas*, Arvy et al. (1954) established the involvement of ecdysteroids in the proliferation of young oocytes. Chaix and De Reggi (1982) suggested the possible involvement of ecdysteroids in the continuation of oocyte meiosis in *A. lunulatus* and *Palaemon serratus* (Lanot and Cledon, 1989). On the contrary, in *M. rosenbergii*, the level of ecdysteroid was kept minimum during

the development of the ovary (Okumura and Aida, 2000).

Conclusions

Though the traditional practice of eyestalk ablation can induce precocious growth and maturation of ovary and spawning in crustaceans, it is often associated with high mortality, inferior quality seed and poor hatchability. An alternate to this is the use of several non-surgical procedures like the administration of steroid hormones or stimulatory neurotransmitters either as injections or as feed supplements. The outcome of the present study is quite promising which can be exploited in the aquaculture practice of this edible crab.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest.

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