© 2020. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2020) 223, jeb212381. doi:10.1242/jeb.212381

RESEARCH ARTICLE

Induction of vitellogenesis, methyl farnesoate synthesis and ecdysteroidogenesis in two edible crabs by arachidonic acid and prostaglandins

CH. Swetha[1], B. P. Girish[1], M. Hemalatha[2] and P. Sreenivasula Reddy[2,*]

ABSTRACT

The present study investigated the effect of arachidonic acid (AA) and selected prostaglandins on the regulation of vitellogenesis, ecdysteroidogenesis and methyl farnesoate (MF) synthesis in the freshwater crab *Oziotelphusa senex senex* and the giant mud crab, *Scylla serrata*. Administration of AA and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) significantly increased ovarian index, oocyte diameter and ovarian vitellogenin levels and ecdysteroid and MF levels in the hemolymph of crabs. Secretions of MF and ecdysteroids from *in vitro* cultured mandibular organs (MO) and Y-organs (YO) isolated from intermolt crabs injected with AA, $PGF_{2\alpha}$ and PGE_2 were greater when compared with controls. In contrast, injection of prostaglandin D_2 (PGD_2) had no effect on vitellogenesis, ecdysteroid and MF levels in circulation. *In vitro* secretion of MF from MO explants isolated from avitellogenic crabs incubated with AA, $PGF_{2\alpha}$ and PGE_2 increased in a time-dependent manner. Conversely, incubation of YOs isolated from avitellogenic crabs with AA, $PGF_{2\alpha}$ and PGE_2 had no effect on secretion of ecdsyteroids. These results implicate prostaglandins in the regulation of reproduction by inducing the synthesis of MF and consequent ecdysteroid synthesis in brachyuran crabs, and provide an alternative molecular intervention mechanism to the traditional eyestalk ablation methodology to induce vitellogenesis and ovarian maturation in crustaceans.

KEY WORDS: Prostaglandins, Ecdysteroids, Methyl farnesoate, Ovarian development, Crustaceans

INTRODUCTION

In decapods, reproduction is mainly controlled by two antagonistically acting peptide hormones, i.e. gonad-inhibiting hormone (GIH), synthesized and secreted from the X-organ–sinus gland complex of eyestalks, which inhibits gonad development (Nagabhushnam et al., 1980; Rotllant et al., 1993), and gonad-stimulating hormone (GSH), secreted from the brain and thoracic ganglia, which stimulates gonad development (Otsu, 1963; Eastman-Reks and Fingerman, 1984). Later studies suggest that ecdysteroids secreted from Y-organs (YO) trigger reproduction in crustaceans (Chang, 1997; Laufer et al., 1998; Sumiya et al., 2014; Subramoniam, 2000, 2017; Gong et al., 2015, 2016; Medesani et al., 2015). In addition, methyl farnesoate (MF), a secretory product of the mandibular organ (MO), has also been implicated in inducing ovarian maturation in crustaceans (Reddy et al., 2004; Nagaraju, 2007; Kakaley et al., 2017; Li et al., 2019; Xie et al., 2018; Neelima and Reddy, 2019). The ovarian enhancement effect of MF may be indirect by stimulating the YO to synthesize and secrete ecdysteroids (Tamone and Chang, 1993).

Prostaglandins are oxygenated metabolites of arachidonic acid (AA) and two other polyunsaturated fatty acids, eicosatrienoic and eicosapentaenoic acids. Similar to vertebrates, prostaglandins play an important role in regulating several physiological processes in invertebrates including crustaceans (Stanley and Howard, 1998; Rowley et al., 2005; Di Costanzo et al., 2019). Prostaglandins such as PGE_2 and $PGF_{2\alpha}$ were discovered in several crustaceans and their involvement in the regulation of reproduction was also documented (Spaziani et al., 1993, 1995; Muriana et al., 1995; Raviv et al., 1999; Tahara and Yano, 2004; Meunpol et al., 2005; Huang et al., 2008; González-Félix et al., 2009; Preechaphol et al., 2010; Chansela et al., 2012; Wimuttisuk et al., 2013; Waiho et al., 2017; Di Costanzo et al., 2019). Supplementation of AA, through diet, promotes egg development and spawning in *Penaeus monodon* (Coman et al., 2011). Administration of PGE_2 stimulated ovarian maturation, oocyte proliferation and vitellogenin synthesis in the giant freshwater prawn, *Macrobrachium rosenbergii* (Sumpownon et al., 2015). Although we have also demonstrated that injection of PGE_2 and $PGF_{2\alpha}$ resulted in significant increase in ovarian index and oocyte diameter in a dose-dependent manner in the freshwater crab *Oziotelphusa senex senex* (Reddy et al., 2004), the interaction of prostaglandins with other crustacean reproductive hormones is not known. The aim of the present study was to determine the effect of prostaglandins in inducing vitellogenesis, ecdysteroidogenesis and MF synthesis in selected edible crustaceans. For the present study, we selected the freshwater rice field crab, *O. senex senex* and the giant mud crab, *Scylla serrata*, as test animals. *Oziotelphusa senex senex* is well known as 'poor man's protein' and is one of the important species for freshwater aquaculture; *S. serrata* is well known for its commercial importance, with the commencement, since the early 1980s, of live crab export from India.

MATERIALS AND METHODS

Collection and maintenance of animals

Intact adult crabs, *Oziotelphusa senex senex* (Fabricius 1798) (body mass of 30±3 g), were collected from paddy fields around Tirupati, India, and maintained at room temperature under a 12 h:12 h light: dark cycle. Crabs were housed 6–8 per glass aquaria (length:width: height=60:30:30 cm) with sufficient ambient medium (salinity: 0.5 ppt) and transferred to fresh medium every day. *Scylla serrata* (Forskål 1775) (150–170 g body mass) were collected from the Dugarajapatnam coast, PSR Nellore district, Andhra Pradesh, India, and maintained in filtered seawater (salinity 33 ppt) at room temperature under a 12 h:12 h light:dark cycle. The crabs were fed

[1]Department of Biotechnology, Sri Venkateswara University, Tirupati 517 502, India. [2]Department of Zoology, Sri Venkateswara University, Tirupati 517 502, India.

*Author for correspondence (psreddy1955@gmail.com)

CH.S., 0000-0001-6596-5362; P.S.R., 0000-0002-4174-719X

Received 14 August 2019; Accepted 9 January 2020

Journal of Experimental Biology (2020) 223, jeb212381. doi:10.1242/jeb.212381

with sheep meat *ad libitum* while in the laboratory and the ambient medium was changed daily. Female crabs in intermolt stage (stage C_4, according to the criteria described by Reddy, 1991; detailed description is given in Table S1) were selected for the present study.

Experimental design

The crabs were divided into six groups of 20 animals each. Animals in the first group served as initial controls and did not receive any treatment and were killed on the first day of the experiment. Animals in the second group served as concurrent controls and were injected with 10 µl of 5% ethanol in saline, and animals in groups 3, 4, 5 and 6 were injected with 1.0 µg AA, $PGF_{2\alpha}$, PGE_2 and PGD_2 per gram body mass, respectively, in 10 µl volume. Injections into groups 2–6 were administered on the 1st, 7th, 14th and 21st day and the crabs were killed on the 28th day. The injections were administered at the base of the coxa of the third walking leg. AA, $PGF_{2\alpha}$, PGE_2 and PGD_2 were purchased from Sigma-Aldrich, Mumbai, India, and test solutions were prepared fresh by dissolving AA, $PGF_{2\alpha}$, PGE_2 and PGD_2 in 5% ethanol in crustacean saline. Test doses were selected based on our dose–response studies in *O. senex senex* (Reddy et al., 2004). Ten crabs from each group were weighed, and the hemolymph and ovaries were isolated and used for further analysis. The remaining 10 crabs from each group were used for isolation of the MOs and YOs for determination of MF secretion and ecdysteroid secretion, respectively. No significant change in the body mass of crabs either within or between the six groups was observed during the experimental duration.

Measurement of ovarian index and oocyte diameter

The animals from the control and experimental groups were weighed and the ovaries were excised, blotted on a filter paper and weighed wet to the nearest milligram using a Shimadzu electric balance. The ovarian index (OI) was determined using the following formula:

$$\mathrm{OI} = (M_{\mathrm{ovary}}/M_{\mathrm{crab}}) \times 100, \quad (1)$$

where M_{ovary} is the wet mass of the ovary (g) and M_{crab} is the mass of the crab (g). For oocyte diameter determination, the ovaries were placed in Bouin's fixative (picric acid:formaldehyde:acetic acid, 75:25:5) immediately after their isolation. After 24 h, they were washed thoroughly and dehydrated with an ascending alcohol series. Dehydrated ovaries were embedded in paraffin wax (melting point 56–58°C) after clearing in xylene, sectioned transversely at 5 µm and stained with hematoxylin and counter-stained with eosin. The maximum and minimum diameters of up to 50 randomly sectioned oocytes of each sectioned ovary were measured using an ocular micrometer under a compound microscope (Olympus, Model-BX41TF HB, Japan). The measurements were made on the longest and shortest axes of each oocyte, both dimensions were added, and the mean was taken as mean oocyte diameter.

Quantification of ovarian vitellogenin levels by ELISA

Vitellogenin (VtG) was isolated from the ovaries (100 mg) of animals using the protocol described by (Tsukimura et al., 2000). The ovarian VtG content was estimated by enzyme linked immunosorbent assay (ELISA). The robustness of the ELISA was assessed with the measurement of inter- and intra-assay variation, where the variation of intra-assay was measured by adding the same concentration of standard to one microtiter plate 12 times, and the inter-assay variation was measured by comparing various dilutions of the standard added to 12 different microtiter plates. The detection limit of the ELISA was calculated as the lowest standard with an absorbance at least three times higher than the standard deviation of the first value that was significantly different from the ELISA control wells. The data were analyzed with Kyplot (version 2.0). Sensitivity of the ELISA was as little as 5.0 ng of VtG; intra-assay variation was 4% to 6% (n=12) and the inter-assay variation was 9% to 10.6% (n=12). A typical standard curve using purified *O. senex senex* VtG was presented in our earlier paper (Girish et al., 2015).

Hemolymph collection

Hemolymph was collected from the sinuses at the base of the third walking leg with a syringe and mixed (1:2) with anticoagulant (containing 1.4% Na_2HPO_4, 1.3% KH_2PO_4, 3.2% EDTA, 2% dextrose and 0.25% sodium citrate). The hemolymph samples were stored at −40°C, after centrifugation for 10 min at 4000 ***g*** at 4°C.

Determination of secretory rates of MOs and YOs

MOs and YOs were isolated from control and experimental crabs and incubated in 2.0 ml culture medium at 18°C on a shaker (50 rpm) in a CO_2 incubator. The levels of MF and ecdysteroids were determined from culture medium after 48 h. The culture medium for *O. senex senex* endocrine glands was prepared by dissolving 10.5 g of dried 199 medium (containing Earle's salts, L-glutamine without sodium bicarbonate) in 1 liter of distilled water, and finally, sodium bicarbonate (2.2 g) was added to the medium after sterilization to obtain a pH of 7.0. Further, the medium was supplemented with fetal bovine serum (10%), penicillin-G (12.5 µg ml^{-1}) and streptomycin (12.5 µg ml^{-1}). The osmolarity of the medium used to culture the glands isolated from *O. senex senex* was standardized earlier (Nagaraju et al., 2005, 2006).

In vitro culture of MOs and YOs

Both MOs and YOs were dissected from ice-anesthetized intermolt (stage C_4) crabs in avitellogenic stage and washed in ice-cold culture medium for 10 min. The incubated glands were observed under a microscope and no significant changes were detected in the size and shape of the cells. The cells were apparently normal even after 72 h incubation in the culture medium. Incubation of MOs/YOs in culture medium resulted in MF/ecdysteroid secretion in a time-dependent manner up to 48 h, whereas incubation of glands up to 72 h resulted in no further increase in secretion (Nagaraju et al., 2006; Reddy and Reddy, 2006). Hence, we restricted our *in vitro* studies to 48 h.

The culture medium for *S. serrata* glands was prepared by adding Leibovitz's medium+crab saline (1:1), in which survivability and proliferation of testicular cells of *S. serrata* was at a maximum (Shashikumar and Desai, 2011). Leibovitz L-15 medium with L-glutamine powder purchased from Thermo Fisher Scientific was used. The medium as powder was reconstituted with sterile filtered (0.22 µm porosity filters) artificial seawater (salinity 29%). Crab (*S. serrata*) saline was constituted with NaCl (440 mmol l^{-1}), KCl (11.3 mmol l^{-1}), $CaCl_2$ (13.3 mmol l^{-1}), $MgCl_2$ (26 mmol l^{-1}), Na_2SO_4 (23 mmol l^{-1}) and Hepes (10 mmol l^{-1}). All media were filtered through 0.22 µm pore size cellulose membrane prior to use. Antibiotics were routinely used and included penicillin-G (12.5 µg ml^{-1}) and streptomycin (12.5 µg ml^{-1}). The osmolarity was adjusted to 1050 mOsm kg^{-1} with crab saline having poly vinyl pyrrolidone (15 mmol l^{-1}). The pH was adjusted to 7.4.

Individual MOs and YOs were subsequently cultured in 24-well tissue culture plates in 2 ml of the culture medium and either 10 µl of test chemical (30 ng) or 10 µl of 5% ethanol for up to 48 h at 18°C on shaker (50 rpm) in a CO_2 incubator. The secretory products of

Journal of Experimental Biology (2020) 223, jeb212381. doi:10.1242/jeb.212381

Table 1. Effect of injection of arachidonic acid (AA) and the prostaglandins $PGF_{2\alpha}$, PGE_2 and PGD_2 on ovarian index, oocyte diameter and ovarian vitellogenin levels in *Oziotelphusa senex senex*

Group	Ovarian index (g %)	Oocyte diameter (µm)	Vitellogenin levels (mg g^{-1} tissue)
Control	0.22±0.04[a]	21.04±3.2[a]	0.08±0.03[a]
Concurrent control	0.23±0.04[a] (4.55)	22.05±3.2[a] (4.80)	0.08±0.02[a] (–)
AA injected	0.75±0.07[b] (240.91)	72.12±5.4[b] (242.78)	0.72±0.06[b] (800)
$PGF_{2\alpha}$ injected	0.81±0.07[b] (268.18)	99.24±7.2[c] (371.67)	0.89±0.07[c] (1012)
PGE_2 injected	0.65±0.06[b] (195.46)	79.26±6.6[b] (276.71)	0.82±0.08[b] (925)
PGD_2 injected	0.22±0.03[a] (–)	22.42±3.4[a] (6.56)	0.09±0.03[a] (12.5)

Values are means±s.d. of 10 crabs. Values in parentheses are percent change from control. Values with the same superscript do not differ significantly from each other at $P<0.05$.

MO (MF) and YO (ecdysteroids) were determined as specified below at different time points of incubation (6, 12, 24 and 48 h).

Determination of MF and ecdysteroid levels

MF was extracted from the medium (50 µl) by adding 2.5 ml of acetonitrile and 0.5 ml of 4% sodium chloride (w/v), centrifuged at 1000 ***g*** for 10 min at 4°C, and partitioned against 1 ml of hexane. The dried hexane extract was used for the determination of MF using HPLC. To determine circulatory MF levels, freshly drawn hemolymph samples (1.0 ml) were mixed with 2.5 ml of acetonitrile and 1.0 ml of 1% sodium chloride, and then extracted with hexane. In brief, samples containing MF were separated using a Bondapack C18 column (Waters, Milliford, MA, USA) with an isocratic elution of 70% acetonitrile in water (1.0 ml min^{-1}). Peaks were detected at 254 nm by UV absorption. Retention times of the peaks were compared with those of standard MF. The MF was also confirmed by co-chromatography with authentic all-*trans*-methyl farnesoate (a gift from Dr P. Ramachandra Reddy, Department of Biochemistry, Yogi Vemana University, Y.S.R. Kadapa District, Andhra Pradesh, India). MF levels were expressed as ng ml^{-1} and ng per MO.

To determine ecdysteroid secretion from YOs, 50 µl of the medium was removed from each well and extracted in 75% methanol. The dried methanolic extract was used for the determination of ecdysteroid level by radioimmunoassay. To determine circulating ecdysteroid levels, freshly drawn hemolymph samples (1.0 ml) were mixed with 2.5 ml of methanol, and the methanolic extract was used for the estimation of ecdysteroids. The radioimmunoassay was conducted as previously described (Chang and O'Connor, 1979) using the ecdysone antiserum (a generous gift from Dr W. E. Bollenbacher, University of North Carolina, Chapel Hill, NC, USA). The sensitivity of the assay was calculated as 0.3 ng and the intra-assay variation was found to be 6.2%. All of the samples were run at the same time to avoid inter-assay variation. Ecdysteroid concentrations were expressed as ng ml^{-1} and ng per YO.

Statistical analysis

Statistical analyses were performed by one-way ANOVA followed by Tukey's *post hoc* HSD test using SPSS.

RESULTS

Effect of AA or prostaglandins on ovarian index, oocyte diameter and ovarian vitellogenin levels

Following the 28 day treatment regimen, the mean ovarian index, oocyte diameter and ovarian vitellogenin levels in the crabs administered with AA, $PGF_{2\alpha}$ and PGE_2 were significantly ($P<0.05$) higher than in the control crabs, whereas mean ovarian index, oocyte diameter and ovarian vitellogenin levels in PGD_2 administered crabs were comparable with those in control crabs (Tables 1 and 2). The mean ovarian index, oocyte diameter in both control and concurrent control crabs were not significantly different following the 28 day experimental period.

Effect of AA or prostaglandins on MF and ecdysteroid levels in the hemolymph

The MF levels in the hemolymph of control *O. senex senex* (Fig. 1A) and *S. serrata* (Fig. 1B) were 0.25±0.04 and 0.32±0.05 ng ml^{-1}, respectively, and in control crabs following the 28 day experimental period the levels were 0.27±0.04 and 0.35±0.04 ng ml^{-1}. Administration of AA, $PGF_{2\alpha}$ and PGE_2 resulted in a significant increase ($P<0.05$) in the hemolymph MF levels in the experimental crabs when compared with control crabs. Conversely, administration of PGD_2 resulted in no significant changes in MF levels when compared with control crabs (Fig. 1).

The ecdysteroid levels in the hemolymph of control *O. senex senex* and *S. serrata* were 20.78±3.87 and 21.09±2.35 ng ml^{-1}, respectively (Fig. 2). The mean ecdysteroid levels in hemolymph of concurrent control crabs were comparable with control crabs following the 28 day experimental period. A significant increase ($P<0.05$) in ecdysteroid levels was observed in the hemolymph of AA, $PGF_{2\alpha}$ and PGE_2 injected animals when compared with controls, whereas no significant changes were observed in the ecdysteroid levels in PGD_2 administered crabs (Fig. 2).

MF and ecdysteroid secretion from MOs and YOs isolated from crabs injected with AA or prostaglandins

Injection of AA, $PGF_{2\alpha}$ and PGE_2 resulted in a significant ($P<0.05$) increase in MF secretion by MOs when compared with their

Table 2. Effect of injection of AA, $PGF_{2\alpha}$, PGE_2 and PGD_2 on ovarian index, oocyte diameter and ovarian vitellogenin levels in *Scylla serrata*

Group	Ovarian index (g %)	Oocyte diameter (µm)	Vitellogenin levels (mg g^{-1} tissue)
Control	0.31±0.04[a]	18.17±1.97[a]	0.11±0.04[a]
Concurrent control	0.33±0.04[a] (6.45)	20.06±2.11[a] (10.40)	0.13±0.03[a] (18.18)
AA injected	0.89±0.09[b] (187.10)	86.76±9.44[b] (377.49)	0.78±0.08[b] (609.09)
$PGF_{2\alpha}$ injected	0.88±0.09[b] (183.87)	98.71±9.14[b] (443.26)	0.81±0.09[b] (636.36)
PGE_2 injected	0.79±0.08[b] (154.84)	85.56±9.11[b] (370.88)	0.80±0.08[b] (627.27)
PGD_2 injected	0.36±0.03[a] (16.13)	21.01±2.92[a] (15.63)	0.10±0.03[a] (–9.09)

Values are means±s.d. of 10 crabs. Values in parentheses are percent change from control. Values with the same superscript do not differ significantly from each other at $P<0.05$.

Journal of Experimental Biology (2020) 223, jeb212381. doi:10.1242/jeb.212381

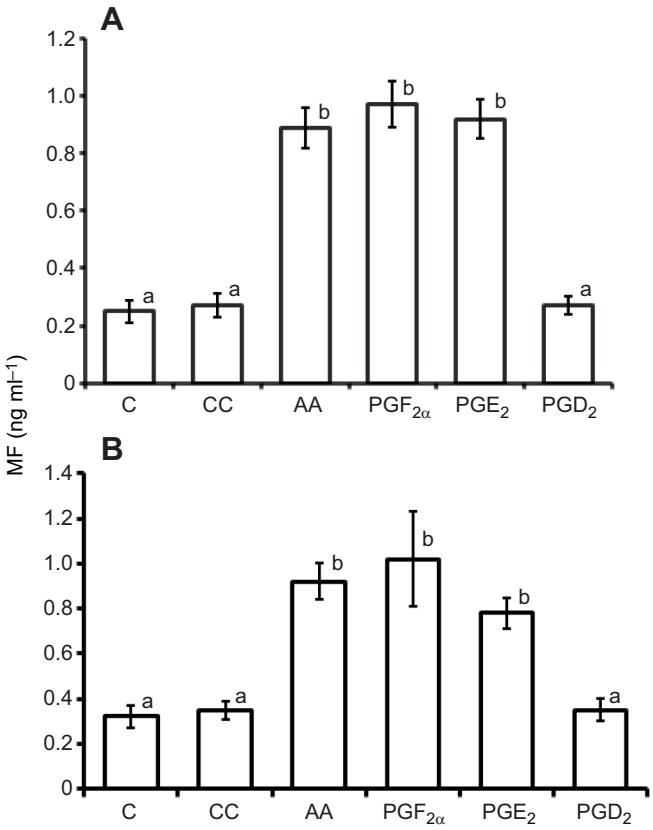

Fig. 1. Effect of administration of arachidonic acid (AA) and the prostaglandins $PGF_{2\alpha}$, PGE_2 and PGD_2 on methyl farnesoate (MF) levels in the hemolymph of crabs following the 28-day experimental period. (A) *Oziotelphusa senex senex*; (B) *Scylla serrata*. C and CC represent control and concurrent control values, respectively. Bars are means±s.d. of 10 crabs. Bars with different superscripts differ significantly at $P<0.05$.

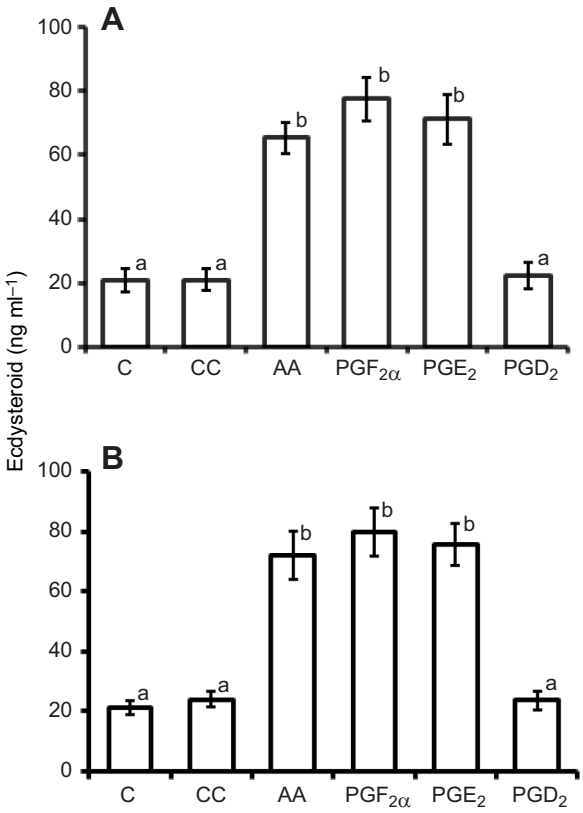

Fig. 2. Effect of administration of AA, $PGF_{2\alpha}$, PGE_2 and PGD_2 on ecdysteroid levels in the hemolymph of *crabs* following the 28-day experimental period. (A) *Oziotelphusa senex senex*; (B) *S. serrata*. C and CC represent control and concurrent control values, respectively. Bars are means ±s.d. of 10 crabs. Bars with different superscripts differ significantly at $P<0.05$.

respective controls (Fig. 3). Similarly, a significant ($P<0.05$) increase was observed in ecdysteroid secretion by YOs isolated from crabs injected with AA, $PGF_{2\alpha}$ and PGE_2 when compared with controls (Fig. 4). The MF secretion by MOs and ecdysteroid secretion by YOs isolated from PGD_2 administered crabs was comparable with that of control groups (Figs 3 and 4).

In vitro secretion of MF from MOs and ecdysteroid secretion from YOs isolated from avitellogenic crabs and incubated with or without AA or prostaglandins

Incubation of MOs isolated from avitellogenic crabs with AA, $PGF_{2\alpha}$ and PGE_2 resulted in a significant ($P<0.05$) increase in the MF secretions in a time-dependent manner when compared with their respective controls, whereas the secretion of MF from MO incubated with PGD_2 was comparable with controls (Tables 3 and 4). No significant change in ecdysteroid secretion was observed when YOs isolated from avitellogenic crabs were incubated with AA, $PGF_{2\alpha}$, PGE_2 and PGD_2 (Tables 3 and 4).

DISCUSSION

In crustaceans, maturation of the ovary can be identified based upon change in color, increase in the size of the ovary and increase in oocyte diameter (Nagaraju, 2011; Swetha et al., 2015). The changes in ovarian VtG levels were also used as a sensitive biochemical marker to determine the maturation of the ovary. The ovarian index

Table 3. *In vitro* secretion of methyl farnesoate (MF) from mandibular organs (MOs) and ecdysteroids from Y-organs (YOs) isolated from avitellogenic crabs (*Oziotelphusa senex senex*) incubated with or without AA, $PGF_{2\alpha}$, PGE_2 and PGD_2

Incubation time (h)	Control	Concurrent control	AA injected	$PGF_{2\alpha}$ injected	PGE_2 injected	PGD_2 injected
MF (ng per MO)						
6	12.22±1.42	12.51±1.17 (2.37)	18.81±1.76* (53.93)	20.14±2.34* (64.81)	16.74±2.01* (36.99)	12.79±2.06 (4.66)
12	17.02±2.17	17.61±2.82 (3.47)	29.09±3.41* (70.92)	35.63±4.02* (109.34)	29.97±3.56* (76.09)	18.09±2.17 (6.29)
24	22.78±2.86	23.24±3.02 (2.02)	48.04±4.26* (110.89)	52.34±4.87* (129.76)	44.57±5.03* (95.65)	23.25±3.41 (2.06)
48	28.87±3.12	29.29±2.58 (1.45)	51.22±4.07* (77.42)	57.06±3.38* (97.65)	50.88±4.85* (76.24)	27.37±3.44 (–5.19)
Ecdysteroid (ng per YO)						
6	2.57±0.52	2.41±0.31 (–6.23)	2.74±0.38 (6.62)	2.68±0.22 (4.28)	2.97±0.42 (15.56)	2.74±0.33 (6.61)
12	4.47±1.88	4.73±0.78 (5.82)	4.89±0.91 (9.39)	5.06±1.21 (13.19)	4.97±0.66 (11.19)	4.89±0.61 (9.40)
24	6.02±1.92	6.09±1.48 (1.16)	6.72±1.87 (11.63)	6.37±1.87 (5.81)	6.87±0.78 (14.12)	6.76±0.95 (12.29)
48	7.46±1.41	7.68±0.89 (2.95)	7.06±1.01 (–5.36)	7.04±1.11 (–5.63)	8.01±0.92 (7.37)	7.91±0.88 (6.03)

Endocrine glands were incubated in culture medium for 48 h. Medium was removed at each time point shown and assayed for secretory product. Values are means±s.d. of 6 samples. Values in parentheses are percent change from respective control. *Significantly different from respective control at $P<0.05$.

Journal of Experimental Biology (2020) 223, jeb212381. doi:10.1242/jeb.212381

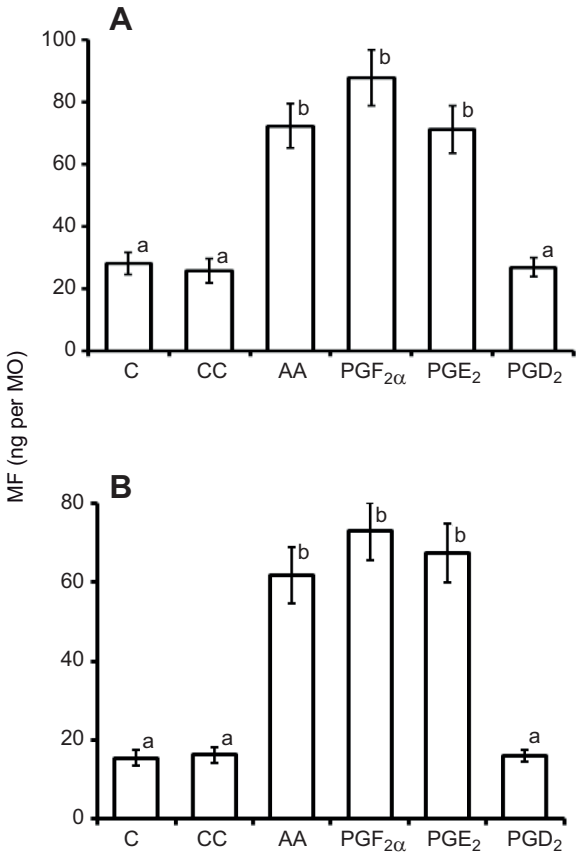

Fig. 3. Secretion of MF from mandibular organs (MOs) isolated from crabs injected with AA, $PGF_{2\alpha}$, PGE_2, and PGD_2 following the 28-day experimental period. (A) *Oziotelphusa senex senex*; (B) *S. serrata*. C and CC represent control and concurrent control values, respectively. Bars are means ±s.d. of 10 crabs. Bars with different superscripts differ significantly at $P<0.05$.

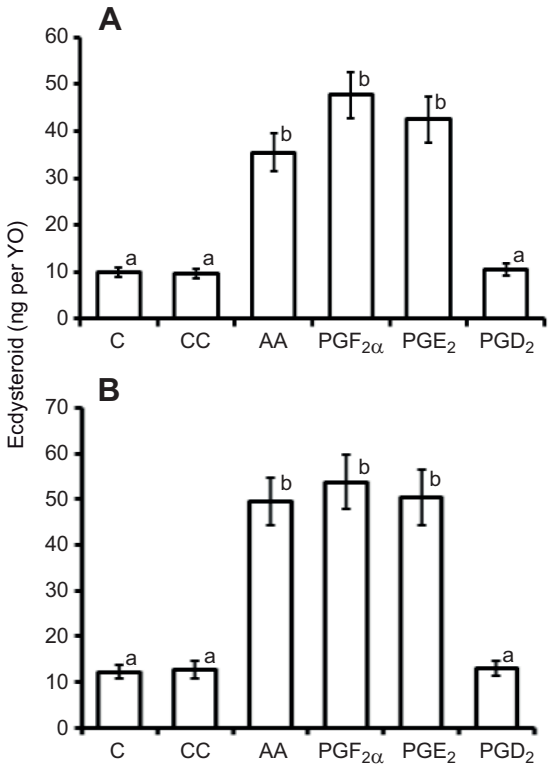

Fig. 4. Secretion of ecdysteroid from Y-organs (YOs) isolated from crabs injected with AA, $PGF_{2\alpha}$, PGE_2 and PGD_2 following the 28-day experimental period. (A) *Oziotelphusa senex senex*; (B) *S. serrata*. C and CC represent control and concurrent control values, respectively. Bars are means± s.d. of 10 crabs. Bars with different superscripts differ significantly at $P<0.05$.

at the immature stage was 0.367±0.035 (g %) and increased to 0.859±0.039, 1.650±0.060 and 3.238±0.082 (g %) during vitellogenic stages I, II and III, respectively, during the natural reproductive cycle of *O. senex senex*. Oocyte diameter also significantly increased ($P<0.0001$) from the immature stage to vitellogenic stage III (20.71±0.528 to 66.31±2.477 µm) during the natural reproductive cycle (Swetha et al., 2015). The ovarian VtG levels at pre-vitellogenic stage were 70±4.7 µg g^{-1} tissue, and the ovarian VtG levels gradually increased from vitellogenic stage I to vitellogenic stage III (240±16.2 to 690±21.3 µg g^{-1} tissue) (Girish et al., 2015). In the present study, injection of AA, $PGF_{2\alpha}$ and PGE_2 into avitellogenic female crabs resulted in significant increases over a 28 day treatment period in ovarian index, oocyte diameter and VtG levels in the freshwater rice field crab, *O. senex senex*, and in the mud crab, *S. serrata*. These results are in agreement with earlier results (Wouter et al., 2001; Glencross, 2009; Coman et al., 2011; Maheswarudu and Vineetha, 2013; Kangpanich et al., 2016; Prameswari et al., 2017; Xu et al., 2017). The results indicate that prostaglandins induce ovarian development, which could be (1) a direct action on the ovary or (2) an indirect action by blocking the eyestalk hormones or increased production of reproductive enhancing hormones (MF and ecdysteroids) and/or a combination of internal ovarian and external hormonal factors.

Table 4. *In vitro* secretion of MF from MOs and ecdysteroids from YOs isolated from avitellogenic crabs (*Scylla serrata*) incubated with or without AA, $PGF_{2\alpha}$, PGE_2 and PGD_2

Incubation time (h)	Control	Concurrent control	AA injected	$PGF_{2\alpha}$ injected	PGE_2 injected	PGD_2 injected
MF (ng per MO)						
6	5.46±0.08	5.88±0.65 (7.69)	9.01±1.12* (65.02)	11.21±1.33* (105.31)	7.89±1.01* (44.51)	6.02±0.09 (10.26)
12	8.51±1.01	8.89±0.87 (4.47)	24.31±3.02* (185.66)	22.33±2.71* (162.40)	18.54±1.87* (117.86)	9.02±0.09 (5.99)
24	14.07±1.38	13.76±1.65 (–2.20)	35.14±4.02* (149.75)	32.44±3.47* (130.56)	29.16±3.05* (107.25)	15.09±1.73 (7.25)
48	15.79±1.76	16.26±1.66 (2.98)	36.54±4.03* (131.41)	39.89±4.11* (152.63)	32.91±3.55* (108.42)	16.02±2.11 (1.46)
Ecdysteroid (ng per YO)						
6	5.06±0.82	5.77±0.65 (14.03)	5.14±0.71 (1.56)	5.11±0.83 (0.99)	5.19±0.68 (2.57)	5.09±0.73 (0.59)
12	7.41±1.00	8.34±0.79 (12.55)	7.56±0.84 (2.02)	7.62±0.95 (2.83)	8.87±1.12 (19.70)	8.02±1.01 (8.23)
24	10.04±1.11	9.87±1.02 (–1.69)	10.55±1.01 (5.08)	10.54±1.07 (4.98)	10.33±1.67 (5.88)	9.98±0.95 (–0.59)
48	13.52±1.25	14.11±1.78 (4.36)	13.32±1.11 (–1.48)	14.47±1.03 (7.03)	14.31±1.12 (5.84)	14.02±1.88 (3.70)

Endocrine glands were incubated in culture medium for 48 h. Medium was removed at each time point shown and assayed for secretory product. Values are means±s.d. of 6 samples. Values in parentheses are percent change from respective control. *Significantly different from respective control at $P<0.05$.

In the present study, administration of AA, $PGF_{2\alpha}$ and PGE_2 into female animals following a 28 day injection cycle resulted in a significant increase in MF and ecdysteroid levels in the hemolymph when compared with controls. MF and ecdysteroids are synthesized and secreted from the MO and YO, respectively. Circulatory MF levels in *O. senex senex* increased gradually from the pre-vitellogenic stage to vitellogenic stage III (16.3±3.1 to 56.7±6.2) during natural reproductive stages (Nagaraju et al., 2006). The data clearly indicate that MOs and YOs isolated from AA, $PGF_{2\alpha}$ and PGE_2 injected crabs showed a marked increase in secretory rates when compared with MOs and YOs isolated from control crabs. This indicates that prostaglandin-induced vitellogenesis might be due to enhanced ecdysteroidogenesis and MF synthesis from YOs and MOs, respectively. It is well established that the synthesis and secretion of MF and ecdysteroids are negatively regulated by mandibular organ inhibiting hormone (MOIH) and molt inhibiting hormone (MIH), respectively, produced in the X-organ–sinus gland complex of eyestalks. The elevated circulatory levels of MF and ecdysteroids and secretory rates of MOs and YOs in *O. senex senex* after administration of AA, $PGF_{2\alpha}$ and PGE_2 may be due to inhibition of release of MOIH and MIH from eyestalks or direct action of AA or PGs on MO and YO, or both.

Increased secretory rates of MOs and YOs isolated from AA, $PGF_{2\alpha}$ and PGE_2 injected crabs following a 28 day injection cycle motivated us to study the secretory rates of endocrine glands isolated from avitellogenic crabs incubated with AA, $PGF_{2\alpha}$ and PGE_2 *in vitro*. In the present study, incubation of MOs isolated from avitellogenic crabs with AA, $PGF_{2\alpha}$ and PGE_2 resulted in a significant increase in MF production in a time-dependent manner indicating the direct action of AA, $PGF_{2\alpha}$ and PGE_2 on MOs and induction of MF synthesis. Conversely, no significant change was observed in ecdysteroid secretion from YOs isolated from avitellogenic crabs and incubated with AA, $PGF_{2\alpha}$ and PGE_2. Tamone and Chang (1993) demonstrated that co-incubation of MOs with YOs isolated from *Cancer magister* stimulated ecdysteroid secretion from YOs *in vitro*. Elevated circulatory levels of ecdysteroids and increased secretions of ecdysteroids from YOs isolated from AA, $PGF_{2\alpha}$ and PGE_2 injected crabs in the present study may be due to an ecdysiotropic effect of MF, as suggested by Tamone and Chang (1993). It is well established that the circulatory levels of MF and ecdysteroids are at low levels prior to oocyte maturation and increase during vitellogenesis (Subramoniam, 2000; Gunamalai et al., 2004; Reddy et al., 2004). The induced vitellogenesis in crabs injected with AA, $PGF_{2\alpha}$ and PGE_2 is in accordance with the elevated circulatory levels of MF and ecdysteroids after AA and $PGF_{2\alpha}$ and PGE_2 administration. Conversely, PGD_2 has no effect on reproduction in *O. senex senex*. We have previously observed levels of $PGF_{2\alpha}$ and PGE_2 that increase in concert with oocyte maturation in *O. senex senex*, while levels of PGD_2 remain relatively low and constant (Reddy et al., 2004). The present data also reveal that PGD_2 is not involved in regulation of synthesis of MF and/or ecdysteroids. Piecing this evidence together, one may conclude that $PGF_{2\alpha}$ and PGE_2 are indeed involved in the induction of vitellogenesis, MF synthesis and ecdysteroidogenesis, whereas the involvement of PGD_2 in the regulation of reproduction is indefinable.

The present data suggest that PGD_2 is not involved in the regulation of MF or ecdysteroid synthesis, whereas $PGF_{2\alpha}$ and PGE_2 may mediate the induction of vitellogenesis by stimulating MF synthesis, and consequent ecdysteroid production. These are the first data showing an association between the prostaglandins and MF synthesis and ecdysteroidogenesis, and they provide an alternative molecular intervention mechanism to the traditional eyestalk ablation methodology to induce fecundity in aquaculturally important crustaceans.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: CH.S., B.P.G., P.S.R.; Methodology: CH.S., B.P.G., M.H., P.S.R.; Validation: CH.S., B.P.G., M.H.; Formal analysis: CH.S., B.P.G., M.H., P.S.R.; Investigation: CH.S., B.P.G.; Data curation: M.H., P.S.R.; Writing - original draft: CH.S., B.P.G., P.S.R.; Writing - review & editing: B.P.G., P.S.R.; Supervision: P.S.R.; Project administration: P.S.R.; Funding acquisition: P.S.R.

Funding

P.S.R. is grateful to the University Grants Commission, New Delhi, for awarding the Basic Science Research Faculty Fellowship. CH.S. and B.P.G. acknowledge the University Grants Commission (BSR-RFSMS) and Council of Scientific and Industrial Research (CSIR-SRF) respectively, for awarding research fellowships.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.212381.supplemental

References

Chang, E. S. (1997). Chemistry of crustacean hormones that regulate growth and reproduction. In *Recent Advances in Marine Biotechnology* (ed. M. Fingerman, R. Nagabhushanam and M. F. Thompson), pp. 163-178. New Delhi: Oxford and IBH Publishing, Co.

Chang, E. S. and O'Connor, J. D. (1979). Arthropod molting hormones. In *Methods of Radioimmunoassay* (ed. B. M. Jaffe and H. R. Behrman), pp. 797-814. New York: Academic Press.

Chansela, P., Goto-Inoue, N., Zaima, N., Hayasaka, T. and Sroyraya, M. (2012). Composition and localization of lipids in *Penaeus merguiensis* ovaries during the ovarian maturation cycle as revealed by imaging mass spectrometry. *PLoS ONE* **7**, 1-12. doi:10.1371/journal.pone.0033154

Coman, G. J., Arnold, S. J., Barclay, M. and Smith, D. M. (2011). Effect of arachidonic acid supplementation on reproductive performance of tank-domesticated *Penaeus monodon*. *Aquac. Nut.* **17**, 141-151. doi:10.1111/j.1365-2095.2009.00716.x

Di Costanzo, F., Di Dato, V., Ianora, A. and Romano, G. (2019). Prostaglandins in marine organisms: a review. *Marine Drugs* **17**, 428. doi:10.3390/md17070428

Eastman-Reks, S. and Fingerman, M. (l984). Effect of neuro-endocrine tissue and cyclic AMP on ovarian growth in *vivo* and *in vitro* in the fiddler crab, *Uca pugilator*. *Comp. Biochem. Physiol.* **79**, 679-684. doi:10.1016/0300-9629(84)90468-7

Girish, B. P., Swetha, C. H. and Reddy, P. S. (2015). Expression of *RXR*, *EcR*, *E75* and *VtG* mRNA levels in the hepatopancreas and ovary of the freshwater edible crab, *Oziothelphusa senex senex* (Fabricius, 1798) during different vitellogenic stages. *Sci. Nat.* **102**, 20. doi:10.1007/s00114-015-1272-9

Glencross, B. D. (2009). Exploring the nutritional demand for essential fatty acids by aquaculture species. *Rev. Aquacult.* **1**, 71-124. doi:10.1111/j.1753-5131.2009.01006.x

Gong, J., Ye, H., Xie, Y., Yang, Y., Huang, H., Li, S. and Zeng, C. (2015). Ecdysone receptor in the mud crab *Scylla paramamosain*: a possible role in promoting ovarian development. *J. Endocrinol.* **224**, 273-287. doi:10.1530/JOE-14-0526

Gong, J., Huang, C., Shu, L., Bao, C., Huang, H., Ye, H., Zheng, C. and Li, S. (2016). The retinoid X receptor from mud crab: new insights into its roles in ovarian development and related signaling pathway. *Sci. Rep.* **6**, 23654. doi:10.1038/srep23654

González-Félix, G. M., Perez-Velazquez, M. and Quintero-Alvarez, J. (2009). Effect of various dietary levels of docosahexanoic and arachidonic acids and different n-3/n-6 ratios on biological performance of pacific white shrimp, *Litopenaeus vannamei* raised in low salinity. *J. World Aquacult. Soc.* **40**, 194-206. doi:10.1111/j.1749-7345.2009.00242.x

Gunamalai, V., Kirubagaran, R. and Subramoniam, T. (2004). Hormonal coordination of molting and female reproduction by ecdysteroids in the mole crab *Emerita asiatica* (Milne Edwards). *Gen. Comp. Endocrinol.* **138**, 128-138. doi:10.1016/j.ygcen.2004.06.002

Huang, J.-H., Jiang, S.-G., Lin, H.-Z., Zhou, F.-L. and Ye, L. (2008). Effects of dietary highly unsaturated fatty acids and astaxanthin on the fecundity and lipid content of pond-reared *Penaeus monodon* (Fabricius) brood stock. *Aquacult. Res.* **39**, 240-251. doi:10.1111/j.1365-2109.2007.01868.x

Kakaley, E. K. M., Wang, H. Y. and LeBlanc, G. A. (2017). Agonist-mediated assembly of the crustacean methyl farnesoate receptor. *Sci. Rep.* **7**, 45071. doi:10.1038/srep45071

Kangpanich, C., Pratoomyot, J., Siranonthana, N. and Senanan, W. (2016). Effects of arachidonic acid supplementation in maturation diet on female

reproductive performance and larval quality of giant river prawn, *Macrobrachium rosenbergii*. *Peer J.* **4**, e2735. doi:10.7717/peerj.2735

Laufer, H., Biggers, W. J. and Ahl, J. S. B. (1998). Stimulation of ovarian maturation in the crayfish *Procambarus clarkii* by methyl farnesoate. *Gen. Comp. Endocrinol.* **111**, 113-118. doi:10.1006/gcen.1998.7109

Li, K., Jia, Q. Q. and Li, S. (2019). Juvenile hormone signaling – a mini review. *Insect Sci.* **26**, 600-606. doi:10.1111/1744-7917.12614

Maheswarudu, G. and Vineetha, A. (2013). Littoral Oligochaete *Pontodrilus bermudensis* Beddard: a potential source for arachidonic acid that stimulates maturation in penaeid shrimp. *J. Vet. Sci.* **114**, 290-300.

Medesani, D. A., Ferré, L. E., Canosa, L. S., Silveyra, G. R. and Rodriguez, E. M. (2015). Induction of vitellogenesis by 17-hydroxyprogesterone and methyl farnesoate during post-reproductive period, in the estuarine crab, *Neohelice granulate*. *Invert. Reprod. Dev.* **59**, 104-110. doi:10.1080/07924259.2015.1019014

Meunpol, O., Meejing, P. and Piyatiratitivorakul, S. (2005). Maturation diet based on fatty acid content for male *Penaeus monodon* (Fabricius) brood stock. *Aquacult. Res.* **36**, 1216-1225. doi:10.1111/j.1365-2109.2005.01342.x

Muriana, F. J., Gutierrez, V., Blaya, J. A. and Bolufer, J. (1995). Phospholipid fatty acid composition of hepatopancreatic brush-border membrane vesicles from the prawn *Penaeus japonicus*. *Biochimie* **77**, 190-193. doi:10.1016/0300-9084(96)88124-7

Nagabhushnam, R., Joshi, P. K. and Kulkarni, G. K. (1980). Induced spawning in prawn, *Parapenaeopsis stylifera* using a steroid hormone 17 hydroxy progesterone. *Indian J. Mar. Sci.* **9**, 227.

Nagaraju, G. P. C. (2007). Is methyl farnesoate a crustacean hormone? *Aquaculture.* **272**, 39-54. doi:10.1016/j.aquaculture.2007.05.014

Nagaraju, G. P. C. (2011). Reproductive regulators in decapod crustaceans: an overview. *J. Exp. Biol.* **214**, 3-16.

Nagaraju, G. P. C., Prasad, G. L. and Reddy, P. S. (2005). Isolation and characterization of mandibular organ-inhibiting hormone from the eyestalks of freshwater crab *Oziotelphusa senex senex*. *Int. J. Appl. Sci. Eng.* **3**, 61-68. doi:10.1016/s0044-8486(03)00519-2

Nagaraju, G. P. C., Reddy, P. R. and Reddy, P. S. (2006). *In vitro* methyl farnesoate secretion by mandibular organs isolated from different molt and reproductive stages of the crab *Oziotelphusa senex senex*. *Fish. Sci.* **72**, 410-414. doi:10.1111/j.1444-2906.2006.01164.x

Neelima, H. and Reddy, P. R. (2019). Effect of Methyl farnesoate in stimulation of moulting in the female crab *Oziothelphusa senex senex* (Fabricius, 1798). *J. Mar. Biol. Ass. India* **60**, 110-114. doi:10.6024/jmbai.2018.60.2.2046-16

Otsu, T. (1963). Bihormonal control of sexual cycle in the freshwater crab, *Potamon dehaani*. *Embryology* **8**, 1-20. doi:10.1111/j.1440-169X.1963.tb00183.x

Prameswari, K., Hemalatha, M., Kishori, B. and Reddy, P. S. (2017). Role of arachidonic acid and COX inhibitors in the regulation of reproduction in freshwater crab *Oziothelphusa senex senex*. *J. Aquac. Res. Dev.* **8**, 490. doi:10.4172/2155-9546.1000490

Preechaphol, R., Klinbunga, S., Khamnamtong, B. and Menasveta, P. (2010). Isolation and characterization of genes functionally involved in ovarian development of the giant tiger shrimp *Penaeus monodon* by suppression subtractive hybridization (SSH). *Genet. Mol. Biol.* **33**, 676-685. doi:10.1590/S1415-47572010000400014

Raviv, T., Tietz, A., Khayat, M., Boehm, E. and Michelis, R. (1999). Lipid accumulation in the ovaries of a marine shrimp *Penaeus semisulcatus* (De Haan). *Indian J. Exp. Biol.* **202**, 1819-1829.

Reddy, P. S. (1991). Neuroendocrine control of molting in the fresh water field crab *Oziotelphusa senex senex* Fabricius. *Trop. Freshwater Biol.* **2**, 213-222.

Reddy, P. R. and Reddy, P. S. (2006). Isolation of peptide hormones with pleiotropic activities in the freshwater crab, *Oziotelphusa senex senex*. *Aquaculture.* **259**, 424-431. doi:10.1016/j.aquaculture.2006.05.019

Reddy, P. S., Reddy, P. R. and Nagaraju, G. P. (2004). The synthesis and effects of prostaglandins on the ovary of the crab *Oziotelphusa senex senex*. *Gen. Comp. Endocrinol.* **135**, 35-41. doi:10.1016/j.ygcen.2003.08.002

Rotllant, G., De Kleijne, D., Charmantier-Daures, M., Charmantier, G. and Van Herp, F. (1993). Localization of crustacean hyperglycemic hormone (CHH) and gonad-inhibiting hormone (GIH) in the eyestalk of *Homarus gammarus* larvae by immuno cytochemistry and *in situ* hybridization. *Cell Tissue Res.* **271**, 507-512. doi:10.1007/BF02913734

Rowley, A. F., Vogan, C. L., Taylor, G. W. and Clare, A. S. (2005). Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems. *J. Exp. Biol.* **208**, 3-14. doi:10.1242/jeb.01275

Shashikumar, A. and Desai, P. V. (2011). Development of cell line from the testicular tissues of crab *Scylla Serrata*. *Cytotechnology* **63**, 473-480. doi:10.1007/s10616-011-9365-6

Spaziani, E. P., Hinsch, G. W. and Edwards, S. C. (1993). Changes in prostaglandin E2 and F2 alpha during vitellogenesis in the Florida crayfish *Procambarus paeninsulanus*. *J. Comp. Physiol. B.* **163**, 541-545. doi:10.1007/BF00302112

Spaziani, E. P., Hinsch, G. W. and Edwards, S. C. (1995). The effect of prostaglandin E2 and prostaglandin F2 alpha on ovarian tissue in the Florida crayfish *Procambarus paeninsulanus*. *Prostaglandins* **50**, 189-200. doi:10.1016/0090-6980(95)00124-7

Stanley, D. and Howard, R. W. (1998). The biology of prostaglandins and related eicosanoids in invertebrates: cellular, organismal and ecological actions. *Am. Zool.* **38**, 369-381. doi:10.1093/icb/38.2.369

Subramoniam, T. (2000). Crustacean ecdysteroids in reproduction and embryogenesis. *Comp. Biochem. Physiol.* **125**, 135-156. doi:10.1016/S0742-8413(99)00098-5

Subramoniam, T. (2017). Steroidal control of vitellogenesis in crustacea: a new understanding for improving shrimp hatchery production. *Proc. Indian Natn. Sci. Acad.* **83**, 595-610. doi:10.16943/ptinsa/2017/48969

Sumiya, E., Ogino, Y., Miyakawa, H., Hiruta, C., Toyota, K., Miyagawa, S. and Iguchi, T. (2014). Roles of ecdysteroids for progression of reproductive cycle in the fresh water crustacean *Daphnia magna*. *Front. Zool.* **11**, 60. doi:10.1186/s12983-014-0060-2

Sumpownon, C., Engsusophon, A., Siangcham, T., Sugiyama, E., Soonklang, N., Meeratana, P., Wanichanon, C., Hanna, P. J., Setou, M. and Sobhon, P. (2015). Variation of prostaglandin E2 concentrations in ovaries and its effects on ovarian maturation and oocyte proliferation in the giant freshwater prawn, *Macrobrachium rosenbergii*. *Gen. Comp. Endocrinol.* **223**, 129-138. doi:10.1016/j.ygcen.2015.04.019

Swetha, C. H., Girish, B. P. and Reddy, P. S. (2015). Reproductive cycle and fecundity in natural population of edible freshwater crab, *Oziothelphusa senex senex* (Fabricius, 1798) (Decapoda: Brachyura). *J. Aqua. Res. Dev.* **6**, 7. doi:10.4172/2155-9546.1000349

Tahara, D. and Yano, I. (2004). Maturation-related variations in prostaglandin and fatty acid content of ovary in the kuruma prawn (*Marsupenaeus japonicus*). *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **137**, 631-637. doi:10.1016/j.cbpb.2003.12.005

Tamone, S. L. and Chang, E. S. (1993). Methyl farnesoate stimulates ecdysteroid secretion from crab Y-organs *in vitro*. *Gen. Comp. Endocrinol.* **89**, 425-432. doi:10.1006/gcen.1993.1050

Tsukimura, B., Bender, J. S. and Linder, C. J. (2000). Development of an anti-vitellin ELISA for the assessment of reproduction in the ridgeback shrimp, *Sicyonia ingentis*. *Comp. Biochem. Physiol.* **127**, 215-224. doi:10.1016/S1095-6433(00)00255-5

Waiho, K., Fazhan, H., Shahreza, M. S., Moh, J. H. Z., Noorbaiduri, S., Wong, L. L., Saranya, S. and Ikhwanuddin, M. (2017). Transcriptome analysis and differential gene expression on the testis of orange mud crab, *Scylla olivacea*, during Sexual maturation. *PLoS ONE* **12**, e0171095. doi:10.1371/journal.pone.0171095

Wimuttisuk, W., Tobwor, P., Deenarn, P., Danwisetkanjana, K., Pinkaew, D., Kirtikara, K. and Vichai, V. (2013). Insights into prostanoid pathway in the ovary development of the penaeid shrimp, *Penaeus monodon*. *PLoS ONE* **8**, 1-15. doi:10.1371/journal.pone.0076934

Wouter, R., Lavens, P., Nieto, J. and Sorgeloos, P. (2001). Penaeid shrimp broodstock nutrition: an update review on research and development. *Aquaculture* **202**, 1-21. doi:10.1016/S0044-8486(01)00570-1

Xie, X., Liu, M., Jiang, Q., Zheng, H., Zheng, L. and Zhu, D. (2018). Role of Kruppel homolog 1 (Kr-h1) in methyl farnesoate-mediated vitellogenesis in the swimming crab *Portunus trituberculatus*. *Gene* **679**, 260-265. doi:10.1016/j.gene.2018.08.046

Xu, H., Zhang, Y., Luo, K., Meng, X., Luan, S., Cao, B., Chen, B., Liang, M. and Kong, J. (2017). Arachidonic acid in diets for early maturation stages enhances the final reproductive performances of Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture* **479**, 556-563. doi:10.1016/j.aquaculture.2017.06.037

Table S1. The characteristic features of molt cycle in *Oziotelphusa senex senex.*

Molt stage	**Sub-stage**	**Exoskeleton**	**Epidermis**	**Diagnostic characters**
Postmolt (A and B)	A_1	Soft and shiny	Transparent	Animal is extremely quiescent; exoskeleton is soft membrane (parchment-like). Legs are not functional; animal does not feed.
	A_2	Soft	Granular	Animal is inactive and non-feeding; moves with difficulty; is unable to lift its body on its legs
	B_1	Hardening	Granular	Progastric area has become hard; other areas are firm and easily depressible; propodite and meropodite can be bent without cracking.
	B_2	Hardening	Granular	Mesogastric and urogastric areas attain rigidity; corpus and propodus assume uniform hardness; meropodite and propodite crack if bent; feeding commences
Intermolt (C)	C_1	Hard	Granular	Complete integument is hard except in the cardiac area
	C_2	Hard	Granular	Entire carapace is hard; appendages are uniformly hard. The animal feeds actively.
	C_3& C_4	Hard	Granular dense	No clear cut demarcation is observed in this crab, between stages C_3 and C_4; locomotion and feeding are normal.
Premolt (D)	D_0	No new cuticle yet	Apolysis begins	No setal development was observed
	D_1	Appearance of new pigmented layer.	Invaginates	Formation of new setae is observed in the mastigobranch of III maxillipede*.
	D_2	Gap formation between old and new cuticle	Invaginates	New pigmented layer is secreted under the old one
	D_3	Thinning of old cuticle	Invaginates	Feeding ceases; new integument is completely detached from the overlying old one; the line of dehiscence would be conspicuous under finger pressure; cardiac area cracks if pressed too hard; blood is milky
	D_4	Paper-like old cuticle	Completion of invagination	Carapace breaks open along the line of dehiscence; body can be compressed easily with fingers
Ecdysis	E	Old cuticle is shed	Transparent	Exuviation

*This phenomenon is readily observed under the binocular microscope or with a hand lens and is of great importance in determining incipience of premolt.