Int. J. Aquat. Biol. (2019) 7(5): 245-253
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Detection and mode of action of retinoids on ovarian development in the mud crab, *Scylla serrata*

Kaduru Venkaiah, Daveedu Thathapudi, Sri Bhashyam Sainath*

Department of Biotechnology, Vikrama Simhapuri University, Nellore-524 320, AP, India.

Abstract: In the current study, the retinoic acid isomers such as 9-*cis* retinoic acid and all-*trans* retinoic acid were detected in the mature ovaries of mud crabs, *Scylla serrata* using HPLC analysis. Given the detection of retinoids in the ovaries, an attempt has been made to elucidate the possible role of retinoic acid in the regulation of reproduction in mud crabs. Injection of 9-*cis* retinoic acid induced ovarian maturation in intact mud crabs as evidenced by a significance elevation in the ovarian index (226.76%; $P<0.001$), and oocyte diameter (150.61%; $P<0.001$) accompanied by accumulation of yolk globules in the oocytes as compared to the untreated crabs. Further, a significant increase (258.63%; $P<0.0001$) in the circulatory ecdysteroid levels were also observed in 9-*cis* retinoic acid injected mud crabs over vehicle injected crabs. From the results, it can be postulated that retinoic acid-induced stimulation of ovarian maturation at least in part mediates ecdysteroids in the mud crabs.

Article history:
Received 17 July 2019
Accepted 13 August 2019
Available online 25 October 2019

Keywords:
Ecdysteroids
HPLC
Ovaries
Oocyte diameter
Retinoic acid

Introduction

It is well-known that the ovarian development in decapods is primarily under the control of two antagonistic hormones viz., gonad/vitellogenin inhibiting hormone (GIH or VIH) of the major neuro-endocrine system located in the eyestalks, X-organ-sinus gland complex (XO-SG) and gonad/vitellogenin stimulating hormone (GSH or VSH) secretion from the brain and the thoracic ganglia, respectively (Subramoniam, 2017). In addition, ecdysteroids of Y-organs and methyl farnesaote of mandibular organs also play a crucial role in the regulation of crustacean ovarian development (Swetha et al., 2011). However, their precise crosstalk during ovarian maturation is not well-defined (Rotllant et al., 2018). Therefore, understanding the molecules that coordinate the crosstalk between the endogenous hormones are instrumental to gain insights into the regulatory effects of peptides, steroids and the terpenoids during the ovarian development of decapod crustaceans (Sainath et al., 2013).

Vitamin A (retinol: ROL) is a multifaceted molecule with wide array of functions in vertebrates. The biologically active metabolite of vitamin A, retinoic acid (RA) acts as one of the signaling molecules in the coordination of reproductive regulators in vertebrates (Andre et al., 2014). In addition, the role of RA in the regulation of a range of physiological processes such as embryonic development and organogenesis, tissue homeostasis, cell proliferation, differentiation, embryonic growth and development and vision is well acknowledged (Theodosiou et al., 2010; Clagget-Dame and Knutson, 2011; Macejova et al., 2016). Most notably, the biological functions of RA are operated via genomic actions where RA through the ligation of its cognate nuclear receptors [wherein 9-*cis* retinoic acid (9CRA) exerts its genomic actions via retinoic acid X receptors (RXRs) and retinoic acid receptor (RAR), whereas all-*trans* retinoic acid (ATRA) exerts its genomic action via RARs]. After ligand bounded, the RAR/RXR or RXR/RXR specifically binds the retinoid response elements on the DNA and

*Correspondence: Sri Bhashyam Sainath with all authors equal contribution
E-mail: drsainath@simhapuriuniv.ac.in
DOI: https://doi.org/10.22034/ijab.v7i5.787

regulate the transcriptional expression of RA target genes (Andre et al., 2014). Although complex, it has been shown that RA through autocrine and paracrine activities and via its retinoid receptors regulate the ovarian functions, including the formation and development of postnatal follicles and a complex differentiation process during the ovarian development in vertebrates (Macejova et al., 2016). The information with respect to vitamin A metabolism and its signaling components are still at its infancy in decapods (Andre et al., 2014). However, traces of information is available with regard to the crustacean retinoid system which includes the occurrence of retinoids, including retinoic acid, retinoid transport proteins: cellular retinoic acid binding protein and the retinoid signaling proteins: retinoid X receptors (Gu et al., 2002; Asazuma et al., 2007; Hopkins et al., 2008; Techa et al., 2013; Tang et al., 2014). Surprisingly, the role of RXR in the crustacean reproduction is well acknowledged (Nagaraju et al., 2011; Cui et al., 2013; Gong et al., 2016). Given that the genomic actions of retinoic acid are crucial for vertebrate reproduction and the retinoic acid X receptors and the retinoic acid isomers occurs endogenously in crustaceans, the current study was investigate to address the probable role of retinoic acid on the ovarian development in mud crabs, *Scylla serrata*.

Scylla serrata is widely consumed all over the world and has a huge demand at the international markets. However, the mud crab culture is not expanding as expected due to the lack of sufficient seed supply. Although, ESX is common practice to induce seed in hatchery industry, because of its side effects such as mortality in the brood stock and inferior quality and quantity of seed, there is a need for the development of reliable strategies. Currently, the seed requirements for mud crab culture is sustained from the wild-caught seedlings and the gravid female crabs, consequently a reduction on their population in the wild. Therefore, an effective way of inducing ovarian maturation thereby seed in the mud crabs has long been sought, which in turn minimizes the intensive fishing of gravid female mud crabs in the wild.

Materials and Methods

Collection and maintenance of animals: Uninjured intermolt stage (C_4) mud crabs, *S. serrata* used in this study were collected from the Nellore coast, Andhra Pradesh, India. Female crabs (Body weight: 140±10 g; carapace width: 10-12 cm) were brought to the laboratory (approximately 7.5 km from the collection point) and separately maintained in large aquaria (3.0 x 1.0 x 1.0 m). The bottom of the aquaria was covered by sand (4 cm). All the crabs were acclimatized (10 days) to the laboratory conditions (constant salinity=30±1 ppt, pH=7.3±0.1, temperature= 25±3ºC) and continuous aeration with moderate intensity) before being used in the experiments. During their acclimatization, they were fed with fish flesh *ad libitum* (between 8 Am to 9 Am) and the ambient medium was removed during the next feeding day to reduce contamination.

Experimental design: In the present study, two experiments were designed wherein the first experiment was aimed to investigate the occurrence of retinoic acid (9-cis retinoic acid: 9CRA and all-trans retinoic acid: ATRA) in the ovaries of mud crabs and the second experiment was to evaluate the probable effect of RA on ovarian maturation in the mud crabs, *S. serrata*.

Experiment 1: The procedure followed in the identification of retinoid isomers (9CRA and ATRA) in mature ovaries of the crabs was based on the previously published reports (Hopkins et al., 2008; Kane and Napoli, 2010; Kim and Quadro, 2013). Briefly, the crabs were dissected under yellow light and the ovaries (mature stage or IV: Colour dark orange and oocytes are completely occupied by yolk globules), weighed, and immediately processed for the detection of RA isomers (9CRA and ATRA) under yellow light. To one aliquot (1 ml) of homogenate (obtained by homogenizing the ovarian tissue (30 mg) in 2 mL of phosphate buffered saline (pH=7.4) using hand homogenization with a ground glass homogenizer placed on ice) 3 ml of 0.025 M KOH in ethanol was added followed by vortexing for at least 10 s. After vortex, 10 ml of hexane was added to the mixture and again followed by vortexing (at least for

10 s). The mixture was centrifuged at 1000 x g and the resultant supernatant was taken into a disposable fresh glass tube followed by evaporation step under liquid nitrogen to get a yellow colour crystalline compound. Methanol (100 µl) was added to the resultant residue and subjected to HPLC analysis using the conditions similar to the detection of standards (see below). The HPLC chromatograms were interpreted using the computer software provided by the manufacturer.

Standard solutions of ATRA and 9CRA were prepared by dissolution in methanol (1 mg/ 1000 µl) followed by brief vortex. The following were the column conditions: Zodiac C_{18} column: 250 mm x 4.6 mm and i.d, 5 µm, total analysis time per sample: 20 min and the flow rate: 1 ml/min. The standard solutions of selected retinoids (20 µl) were injected into the column using the mobile phase (75% acetonitrile and 25% methanol). The corresponding absorbance (280 nm) and retention times for 9CRA and ATRA were recorded via agilent HPLC system (1100 series, Waldbronn, Germany). This served as identification parameters for 9CRA and ATRA from the mature ovaries of the crabs.

Experiment 2: Uninjured intact female crabs (C_4) were randomly divided into three groups. Crabs which did not receive any treatment served as initial control group (group 1; n=12) and sacrificed on day 1 of the experiment, whereas the crabs in group 2 (concurrent controls; n=12) received injections (100 µl) of crustacean saline containing ethanol (1 µl of ethanol was mixed in 99 µl of crustacean saline). On the other hand, crabs in group 3 (n=12) were independently injected (100 µl) with 10^{-7} mole/crab 9CRA (Cayman chemicals, Inc., Ann Arbor, MI). The required concentration of 9CRA was prepared freshly on the day of experimentation by dissolving in ethanol and diluted accordingly in crustacean saline (Pantin, 1934). The injections were given on days 1, 7, 14, and 21 and all the animals were sacrificed on 28^{th} day to analyze the selected ovarian maturation endpoints. The dose selection was based previous studies (Cui et al., 2013) and also our preliminary studies (data not shown).

The total duration of the experiment was 28 days. Before the start of the experimentation (one day) feeding was stopped. The vehicle and/or test chemicals were administered into the crabs through the arthrodial membrane at the base of one of the walking legs. All the crabs were ice anesthetized before handling. After completion of the experimental period, the crabs from the control and the experimental groups were sacrificed to analyze ovarian index, oocyte diameter, and histology of the ovary. The crabs from control, concurrent and 9CRA-injected groups were weighed and the ovaries were excised, cleaned in crustacean saline, blotted on filter paper, and weighed wet to the nearest milligram. The ovarian index was determined using the following formula:

Ovarian index = [Wet weight of the ovary (g)/ Weight of the crab (g)] X 100.

The diameter of 20 oocytes from each ovary was 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. The ovarian sections were prepared according to the method described by Bancraft and Stevens (1982) and they were visualized using the phase contrast microscope (Olympus, Model -BX41TF, Japan) and the hematoxylin and eosin stained ovarian sections were photographed.

Hemolymph collection and ecdysteroid assay: Ecdysteroids in the hemolymph was analyzed in crabs at different reproductive stages (Quinitio et al., 2007) and also during the experimental conditions using Enzyme-linked immunosorbent technique (Kingan, 1989; Abuhagr et al., 2014). The primary antibody and HRP-conjugated ecdysone was purchased from Dr. Timothy Kingan (timkingan@gamil.com) and the secondary goat anti-rabbit IgG antibody was purchased from Merck, India. For ecdysteroid analysis, crab hemolymph samples (100 µl) were collected at the base of the 3^{rd} walking leg using 1 ml syringe (27-guage needle). They were mixed with 300

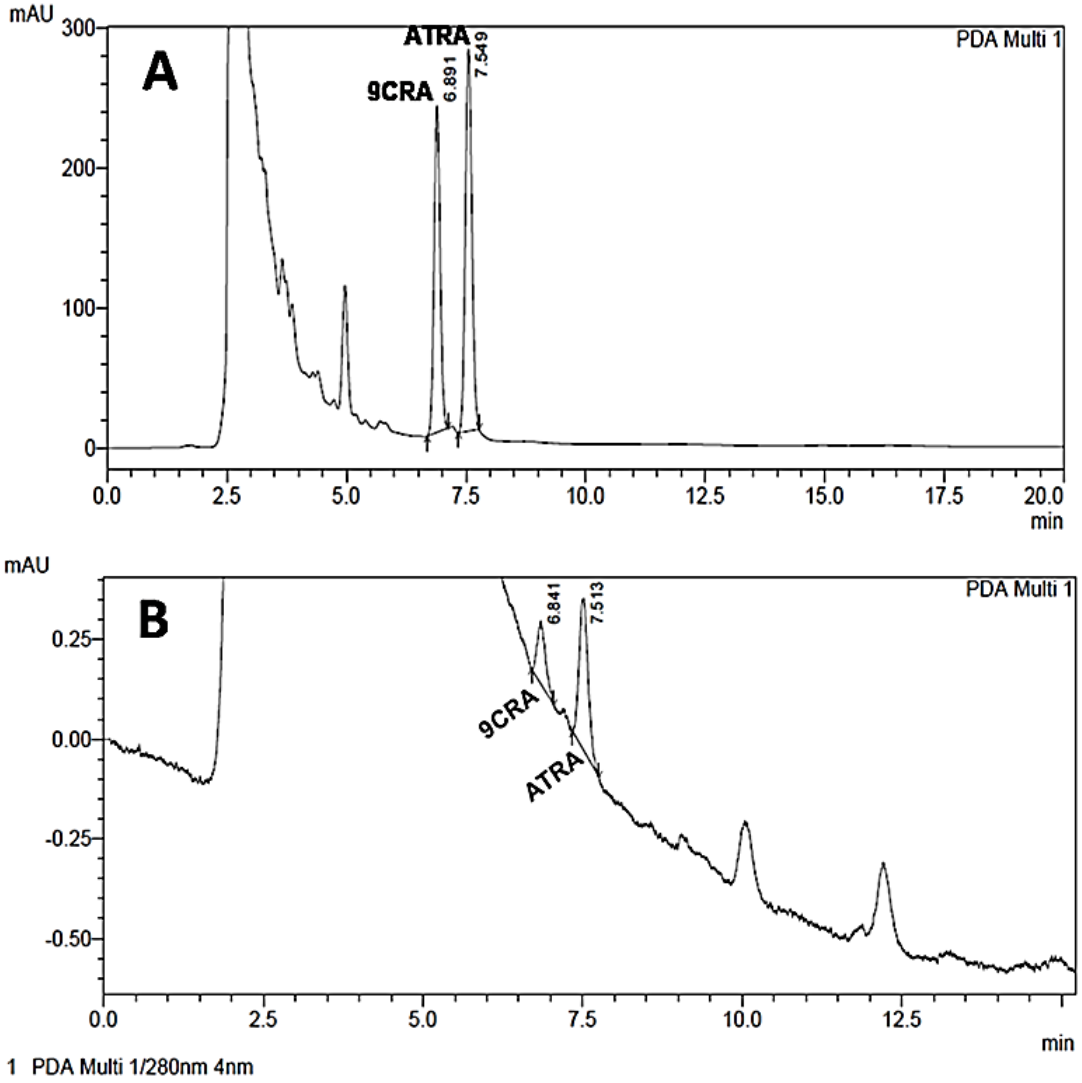

Figure 1. HPLC chromatogram of retinoic acid isomers (ATRA: all-trans retinoic acid; 9CRA: *9-cis* retinoic acid); A: standard mixture B: analysis in mature ovaries of mud crabs, *Scylla serrata*.

µl of methanol and the mixture was centrifuged for 10 min at 20,000 *g* at 4°C to remove precipitated proteins. The supernatants (10 µl) were evaporated to dryness using an evaporator (Thermo Scientific, Savant) and the samples were dissolved in 150 µl assay buffer containing the components [sodium phosphate (25 mmol; pH 7.5), NaCl (150 mmol), EDTA disodium dehydrate (1 mmol) and 0.1% bovine serum albumin] in one litre of autoclaved water. To the overnight incubated pre-coated ELISA plates (96-wells) with secondary antibody [0.5 µg in 90 µl of phosphate-buffered saline (pH 7.5) per well] assay buffer was added. This step was performed to block the unspecific sites. The plates were incubated for 2 h at room temperature followed by washing step three times with the assay buffer containing 0.05% Tween-20. After washing steps, the diluted hemolymph samples (50 µl) and standards of 20-hydroxyecdysone were incubated with 50 µl of primary antibody (1: 100,000) and 50 µl of 20E/HRP conjugate (1:10,000) were added to the wells and incubated at 4ºC overnight. The plates were washed thrice with PBS containing 0.05% Tween 20 and 100 µl of TMB substrate (tetramethylbenzidine solution and peroxidase solution: Thermo Scientifics) was added to the wells. The plates were incubated in dark for 20 min for colour development and the reaction was stopped by the addition of 100 µl of 1M phosphoric acid. The colour development was read at 450 nm using ELISA plate reader (Bio-Rad, USA).

Table 1. Effect of *in vivo* injection of 9-cis retinoic acid (9CRA) on ovarian index (WW%) and oocyte diameter (µm) in intact mud crabs.

Groups	Ovarian index	Oocyte diameter
Control	$0.68^{a} \pm 0.064$	$49.13^{a} \pm 2.73$
Vehicle-injected	$0.71^{a} \pm 0.061$ (4.411)	$52.12^{a} \pm 3.68$ (6.085)
9CRA-injected	$2.32^{b} \pm 0.053$ (226.760)	$130.62^{b} \pm 7.92$ (150.61)

Values are mean ±S.D. of 12 individual crabs; values in the parentheses are percent change from that of control; for evaluation of statistical analysis and percent change, for vehicle injected crabs, crabs in initial control group served as controls and for 9CRA injected group crabs, crabs in vehicle injected group served as controls; values with same superscript in a column do not differ significantly from each other at $P<0.001$.

The intra- and inter-assay variations for this ELISA were found to be < 11 % and < 15 %, respectively.

Statistical analysis: The data were expressed as mean ±S.D. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-Hoc test (SPSS: Student version 7.5, SPSS Inc, Cherstsey., UK). Differences was considered significant at $P<0.05$.

Results

Detection of retinoic acid isomers (9CRA and ATRA) in mature ovaries of the mud crabs, *S. serrata*: A total of three HPLC separations were run using mature ovarian extracts of the crabs, where in two of the separations were done on the same mature ovarian extracts. Figure 1A and B shows the HPLC chromatogram of the standard and the test samples, respectively. The HPLC retention times of the standard retinoic acid isomers viz., 9CRA and ATRA were 6.891 minutes and 7.549 minutes, respectively. In the test samples, two peaks were noticeable with the HPLC retention times of 6.841 minutes and 7.513 minutes, which correspond to the standard HPLC retention times of 9CRA and ATRA, respectively suggesting the occurrence of endogenous retinoic acid isomers in mature ovaries of the mud crabs (Fig. 1).

Effect of 9CRA on ovarian index, oocyte diameter, and histology of the ovary in intact mud crabs, *S. serrata* during 28-day experimental period: The ovarian index and oocyte diameter in initial control and concurrent controls were 0.68±0.064 (WW%), 49.13±2.73 µm and 0.71±0.061 (WW%), and 52.12±3.82 µm, respectively in a 28 day experimental period (Table 1), suggesting no significant changes in the ovarian index and oocyte diameter in the concurrent controls as compared to initial controls. Injection of 9CRA significantly enhanced the ovarian index (226.76%) and oocyte diameter (150.61%) as compared to concurrent controls in a 28 day experimental period (Table 1). Histological observations of the ovaries in concurrent control (Fig. 2A) crabs indicated that the ovary was at the immature stage as evidenced by ovarian lobes completely occupied by oogonia surrounded by follicular cells. Whereas, the histological analysis of

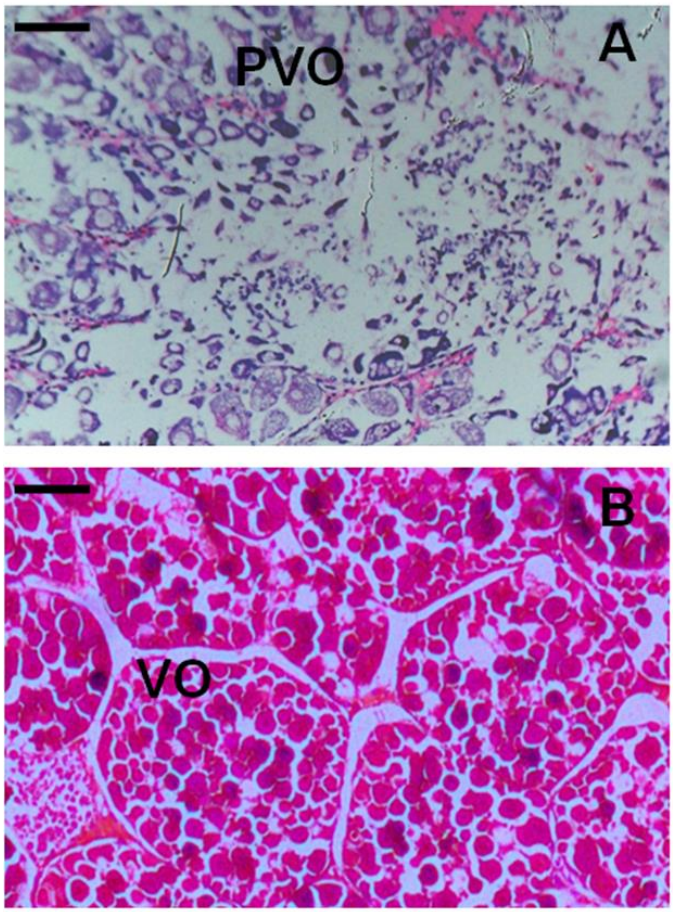

Figure 2. Transverse sections of ovary of vehicle (A), and 9CRA injected crabs (B) in a 28-day experimental period in the crab, *Scylla serrata*. PVO: Pre-vitellogenic oocyte; VO: vitellogenic oocyte. Scale line = 100 µm.

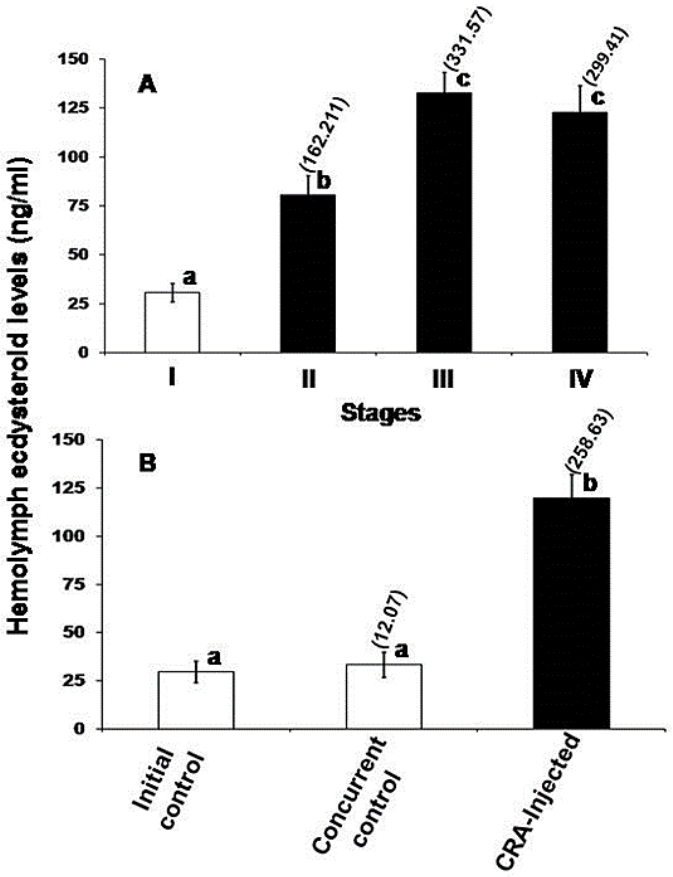

Figure 3. Changes in the ecdysteroid levels of hemolymph in mud crabs: at different reproductive stages (A) and injected with 9CRA (B). Bars are mean ±S.D. of 6 individual crabs (A) and 12 individual crabs (B); Values in the parentheses are percent change from that of control; For evaluation of statistical analysis and percent change, for crabs at stage II, III and IV, crabs at stage I served as controls (A) and for evaluation of statistical analysis and percent change, for vehicle injected crabs, crabs in initial control group served as controls and for 9CRA injected group crabs, crabs in vehicle injected group served as controls (B). Bars with same superscript in a column do not differ significantly from each other at $P<0.001$.

ovaries in 9CRA injected crabs indicated that the ovaries were at mature stage as evidenced by the accumulation of yolk globules in the oocytes (Fig. 2B).

Effect of 9CRA on haemolymph ecdysteroid levels in the intact mud crabs during 28-day experimental period: Significant increase in the hemolymph ecdysteroid levels was noticed during ovarian development as the crabs shift from immature stage (stage I) to late mature stage (stage III) (Fig. 3). However, no significant changes were observed in circulatory levels of ecdysteroids in crabs at stage III as compared to the crabs at stage IV (complete mature stage). No significant changes were observed in the hemolymph ecdysteroid levels in vehicle alone injected crabs as compared to initial controls. Whereas, injection of 9CRA showed a significant elevation (258.63%; $P<0.0001$) in the circulatory levels of ecdysteroids in crabs as compared to concurrent control crabs over a period of 28-days (Fig. 3).

Discussions

Previously, retinoids such as all-*trans* RA, RAL, 13-*cis*-RAL and retinoic acid isomers such as all-*trans*RA and 9-*cis*RA using HPLC coupled with GC/MS in the blastemas of regenerating limbs in the fiddler crab, *U. pugilator* has been reported (Hopkins et al., 2008). Moreover, studies of Liñán-Cabello et al. (2003) reported the RAL in the ovary of shrimp, *Litopenaeus vannamei* using diode array spectrophotometer. Liñán-Cabello and Paniagua (2004) also discovered the retinoids such as 13-*cis*RAL, all-*trans*RAL and 13-*cis*ROL in the ovaries at maturation stage IV and all-*trans*RAL in the eyestalks of the shrimp, *L. vannamei*. In this study, we detected the RA isomers such as 9CRA and ATRA in mature ovaries (stage IV) of mud crabs, *S. serrata* using HPLC analysis. Retinoic acid is the active metabolite of vitamin A. Thus, identification of retinoic acid isomers in mature ovaries might suggest the occurrence of vitamin A metabolism in crabs. Interestingly, enzymes that convert retinaldehydes to RA have been identified in the extracts of *Uca pugilator* blastemas (Hopkins, 2001) and, carotene 15 -15 monooxygenase cDNAs have been identified in an EST library of crab blastema tissues (Durica et al., 2006).

Vitellogenesis is a key step through which the developing oocytes are nourished via yolk globules and is under the control of vitellogenin gene in crustaceans (Warrier and Subramoniam, 2002; Jia et al., 2013). In this study, we found a significant increase in the ovarian index and oocyte diameter associated with the accumulation of the oocytes in ovary of mud crabs injected with 9CRA. This may suggest the stimulatory effect of 9CRA on

vitellogenesis. Recently, it has been shown that the exogenous injection of 13CRA could lead to concurrent up-regulation of vitellogenin gene expression from the hepatopancreas of crabs, *O. senex senex* (Girish et al., 2018). The results also indicated that the injection of 9CRA also enhanced the circulatory levels of ecdysteroids in intact mud crabs, suggesting that the 9CRA at least in part mediates ecdysone signaling (Girish et al., 2018). It is well-known that the process of vitellogenesis is regulated by different hormones, including the ecdysteroids (Subramoniam, 2017). The ecdysteroid levels were also significantly elevated as the crabs shift from the immature stage to the mature stage (Gong et al., 2015). It has been shown that the genomic actions of ecdysone signaling occur via ecdysteroid receptors (EcR), a member that belongs to the nuclear receptor family in crustaceans. It was reported that the promoter region of vitellogenin gene contains several transcription binding sites, including the dimer partners, EcR and the ultra spiracle that are required to execute the genomic actions of ecdysteroids (Martin et al., 2001). Interestingly, injection of 13CRA also enhanced the expression levels of EcR and RXR mRNA in the hepatopancreas (the site for vitellogenesis) in freshwater crabs, *O. senex senex*. Thus, ligand bound EcR combines with the RXR, which is also known as ultra-spiracle in insects to form a heterodimer complex. This complex (ligand bounded EcR/RXR) attaches to its respective response elements on the promoter region of vitellogenin thereby facilitates its transcription (Tiu et al., 2010).

To summarize, accumulation of yolk globules in the oocytes of 9CRA-injected mud crabs may be attributed to the stimulation of vitellogenesis mediating ecdysone signaling. The second plausible reason could be attributed to the ability of 9CRA to inhibit the release of GIH from the XO-SG complex which eventually leads to the release of GSH from the brain and thoracic ganglia thereby ovarian maturation. Nevertheless, the detection of retinoic acid in the mature ovaries of crabs and stimulation of ovarian growth after 9CRA injection suggests, at least in part, a selective mode of action of retinoic acid. Further, studies to elucidate the mechanism(s) of action of retinoic acid and its crosstalk with other endogenous hormones in the regulation of ovarian maturation might be helpful to develop reliable strategies for captive breeding in crustacean aquaculture.

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