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RESEARCH ARTICLE

SEROTONIN INDUCED OVARIAN MATURATION IN FEMALE MOLE CRAB *EMERITA EMERITUS*

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ABSTRACT

The present study demonstrates the impact of serotonin (5-HT) on ovarian development and lipid profiles in the mole crab *Emerita emeritus*. Exogenous 5 HT, at various concentrations ($1x10^{-6}$, $1x10^{-7}$ and $1x10^{-8}$ mol/crab) were injected into the crab at immature stage of ovarian development. Injection of serotonin significantly increased the gonadosomatic index, oocyte diameter and hepatosomatic index respectively. When compared to the control animals total lipid and triglyceride level increased in the ovary and hepatopancreas whereas the free fatty acid and lipase activity showed a marked decrease in hepatopancreas and ovarian tissue. Histological observations of the ovary, showed serotonin increased the ovarian maturation with regard to the arrangement and enlargement of oocytes in the ovary. Results clearly showed that 5HT, at $1x10^{-6}$ concentration has sufficiently enhanced the regulation of ovarian development with shorter duration in *E. emeritus*.

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INTRODUCTION

Reproduction is the most fundamental and indispensable physiological phenomenon in all living organisms to continue its race of their own kind. Eyestalk ablation induces ovarian maturation and spawning in penaeid shrimps (Panouse, 1943; Browdy, 1992). It has been reported that eyestalk ablation eliminates reproductive hormones including gonad-inhibiting hormone (GIH) and mandibular organ inhibiting hormone (MOIH) produced and secreted from the X-organ sinus gland complex (Huberman, 2000; Okumura and Aida, 2000). Eyestalk ablation suspends the controls on a large number of body functions. However, many alternative approaches have been proposed to replace eyestalk ablation, one of which is the endocrine manipulation. The gonadal development can be stimulated or inhibited by affecting the neurosecretory cells by many pharmacological agents and eyestalk ablation (Carlo *et al*., 2008; Coccia *et al*., 2010). It has been reported that neuro-regulators, including neurotransmitters and neuromodulators, mediate and control the release and physiological actions of neurohormones through different mechanisms (Kuo *et al*., 1995; Komali *et al*., 2005). Several neurotransmitter have been involved in the release of these reproductive hormones such as the biogenic amines (dopamine and 5-HT) and also shown to play important role in the synthesis and release of neurohormones in crustaceans (Kulkarni *et al.*, 1992; Sarojini *et al.*, 1995; Fingerman, 1997;Vaca and Alfaro, 2000; Aktas, 2005). Biogenic amines especially serotonin (5-HT) regulates synthesis and release of the reproductive regulator hormones, therefore it can affect on ovarian maturation and ovulation. Serotonin is derived from amino acid tryptophan in certain enzyme pathways distributed at high levels in optic ganglion, cerebral ganglion, circumoesophageal connectives, stomatogastric ganglion, and thoracic ganglia (Tinikul *et al*., 2008). Lipids play an important role during the development of decapod crustaceans, not only as energy source, but also as essential nutrients (Kanazawa *et al.*, 1985). Some essential fatty acids have also been shown to be of special significance for gonad maturation and brood quality (Soudant *et al*., 1996). In crustaceans, the hepatopancreas is generally regarded as a

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major lipid storage organ. In the case of female crustaceans, ovaries also contain higher levels of lipid than other organs and this suggests that lipids are important for maturation of crustacean ovaries. The fatty acid content and composition of the crustacean ovaries have a direct influence on reproduction, egg survival and embryonic development (Alava *et al.*, 1993). The mobilization and accumulation of lipid reserves in different tissues have been documented in several crustacean as maturation of oocytes is a time of intense protein and lipid synthesis and it is likely that the requirement for protein and lipid is higher at this time because lipids serve as the major energy reserves in crustaceans (Pillay and Nair, 1973; Palacios *et al.*, 2000; Barbara and Felder, 2006).

Hence in the present investigation attempts were made to enhance ovarian maturation without eyestalk abalation but by means of biogenic amine serotonin in the mole crab *E. emeritus* and analyse its effect on lipid content of the ovary and to confirm the enhancement of ovarian maturation in relation to the synthesis and mobilization of lipids from extra ovarian sites.

MATERIALS AND METHODS

Experimental animal

Mole crab *Emerita emeritus* was collected from the intertidal region of Elliot's beach at Besant Nagar, Chennai, India. Immature crabs of 17-18 mm CL weighing about 7-8 gms are selected for the study. The animals were maintained in plastic tanks with sufficient aeration with clean sand spread in slanting position. The sea water was changed daily and sand was changed once in a week.

Study Design

Serotonin used in the experiment is obtained from Sigma Aldrich (USA). All test solutions are prepared fresh in degassed crustacean saline (Van Harreveld, 1936) before the start of each experiment to avoid oxidation. The biogenic amine was tested in various concentrations ranging from $1x10^{-6}$, $1x10^{-7}$ and $1x10^{-8}$ mol/crab respectively in a final volume of 20µl/crab. The test doses was selected based on the earlier dose response studies (Pushpalatha and Reddy, 2007; Sainath and Reddy, 2010). Immature female mole crabs are divided into five groups, Group I consisted of 6 crabs served as initial control and sacrificed on the 0 day of the experiment. Remaining four groups contained 48 crabs each. Group II served as concurrent control and Group III received serotonin at a concentration of $1x10^{-6}$ mol/crab, Group IV received $1x10^{-7}$ mol/crab, Group V received $1x10^{-8}$ mol/crab. For convenient analysis 48 animals in each group were further divided into four groups each consisting of 12 animals were observed on 1^{st}, 6^{th}, 12^{th} and 18^{th} day of the experiment. The first group served as control which did not receive any treatment. The second group served as control and received 20µl of physiological saline. Crabs of the third, fourth and fifth groups were injected with serotonin creatinine sulphate with a hypodermal syringe (1 ml 26 G1/2) and Precision Glide Needle through the arthrodial membrane of the last appendage. Gonadosomatic index, Hepatosomatic index, oocyte diameter, lipids, triglycerides, free fatty acids, lipase activity and histology of the ovary.

Gonadosomatic index (GSI)

Crabs were blotted dry with a filter paper and total weights recorded to the nearest milligram using an electronic balance. The gonads were carefully dissected and blotted with a filter paper to remove moisture. Total weight of the reproductive tissue was recorded to the nearest milligram. The gonadosomatic index was calculated using the following formula

GSI = Gonad weight / Body weight ×100.

Hepatosomatic index (HSI)

Crabs were blotted dry with a filter paper and total weights recorded to the nearest milligram using an electronic balance. The hepatopancreas was removed from each crab, blotted dry with a filter paper and weight was recorded to the nearest milligram. The hepatosomatic index of each crab was calculated by using the following formula,

HSI = Hepatopancreas weight/ Body weight ×100

Oocyte diameter

After 24 h fixation in aqueous Bouin's fluid, the ovaries were dehydrated through an alcohol series, cleared in xylene and embedded in paraffin wax. Subsequently 5 µm sections were cut, stained with hematoxylin-eosin and permanent slides prepared. The diameter of the oocytes from each ovary was measured using an ocular micrometer on a compound microscope in the following manner. Ocular micrometer, a glass disc with 100 equality spaced divisions, was put inside the eye piece and was calibrated by superimposing the graduations of ocular micrometer over graduations of the stage micrometer, having a known scale of 1mm. By determining the number of ocular micrometer divisions coinciding with the number of divisions on stage micrometer, the calibration factor for one ocular division was calculated for the objective and ocular lens by using the following formula

$$One\ ocular\ division(\mu m) = \frac{No.of\ division\ on\ ocular\ micrometer}{No.of\ division\ on\ stage\ micrometer} \times 100$$

No. of divisions on stage micrometer

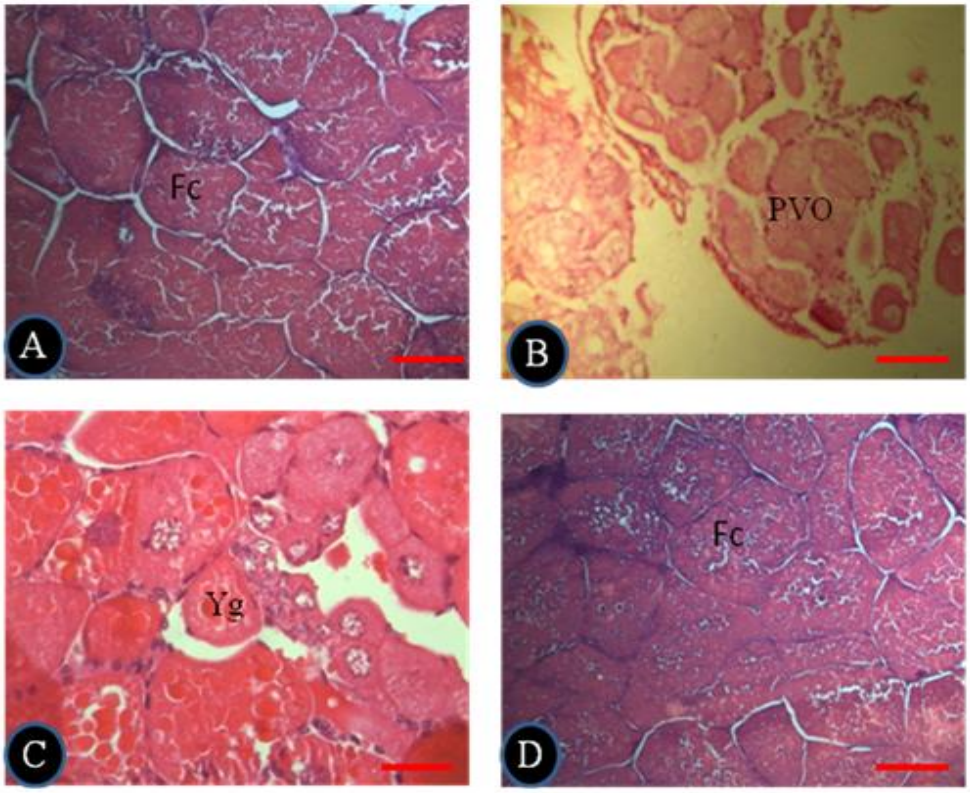
Lipid extraction and estimation

Total lipid content was extracted by the method of Folch *et al*. (1957) and Triglycerides was estimated by the method of Rice (1970). Free fatty acids was estimated by the method of Horn and Menahen (1981) with the colour reagent of Itaya (1977) and Lipase activity was estimated by the method of Winkler and Stuckmann (1979) with p-nitrophenyl palmitate (PNPP) as the substrate. The data collected on the different parameters of the experimental study were subjected to statistical analysis by Mean±SD (Snedecor and Cochran, 1989). The statistical significance of differences among means was examined using two way analysis of variance (ANOVA).

RESULTS

In the present investigation, gonadosomatic index, hepatosomatic index and oocyte diameter of females treated with serotonin exhibited significant increase compared to the control depending upon the dosage. The ovarian index and oocyte diameter in initial control crabs and concurrent control crabs are 0.92±0.06 (WW%), 64.13±0.99µm and 0.92±0.05 (WW%), 64.14±1.71µm, respectively (Table 1). The ovarian index and oocyte diameter significantly increased ($P<0.05$) from 0-18th day of the experiment in the control groups. The GSI values showed no much variation in the first day of the experiment. In 1x10^{-6} mol/crab serotonin injected group, the GSI value increased to 3.16±0.14% after 6 days exposure. Whereas in the lower concentration of 1x10^{-8} mol/crab serotonin injected group, the GSI increased to 3.58±0.17% after 18 days of the exposure. Similarly at lower concentration of serotonin, the oocyte diameter did not show much variation compared to control.

At higher concentration of serotonin, the oocyte diameter drastically increased to 297.7±1.48µm on 6th day of the experiment indicating that the oocytes are in vitellogenic stage of development. The occurrence of minimum size of oocyte on 12th day of the experiment at higher concentration revealed that the crab had undergone spawning, having proliferating immature oocytes, whereas the control crabs spawned only on the 22nd day of the experiment (Table 1). In the moderate concentration of 1x10^{-7}mol/crab the immature oocytes could be seen only on 18th day of the experiment. On the same time at low concentration of serotonin (1x10^{-8} mol/crab), vitellogenic oocytes could be seen only on the 18th day of experiment. No mortality of the crabs was observed in either experimental groups or in the control groups throughout the study. Histological observation was prominent in the ovary of the crab treated with 1x10^{-6} mol/crab of serotonin. On the 6th day of the experiment the ovary showed normal development with vitellogenic oocytes containing distinct ooplasm filled with yolk granules.



(A) Ovary of *E.emeritus* on 6th day of the experiment showing vitellogenic oocytes with fully occupied large yolk granules along with flattened and attached follicle cells (Fc).

(B) Ovary of *E. emeritus* on 12th day of the experiment showing pre-vitellogenic oocytes (PVO) with germarium and follicle cells.

(C) Ovary of *E. emeritus* on 12th day spawned crabs on 18th day of the experiment showing yolk granules (YG) deposited at the periphery with prominent nucleus.

(D) Ovary of *E. emeritus* in control crab on 18th day of the experiment showing vitellogenic oocytes with flattened and attached follicle cells (Fc)

Figure 1. Photomicrographs of the sections of ovary of crabs injected with serotonin (1x 10^{-6} mol/crab)

Table 1. Effect of various doses of serotonin injection on GSI, HSI and Oocyte diameter in the mole crab *E.emeritus*

Treatment groups	Gonadosomatic index (GSI) %					Hepatosomatic index (HSI) %					Oocyte diameter (µm)				
	0 day	1st day	6th day	12th day	18th day	0 day	1st day	6th day	12th day	18th day	0 day	1st day	6th day	12th day	18th day
Control	0.92± 0.06	0.92± 0.05	1.00± 0.04	2.45± 0.09	3.36± 0.14	1.33± 0.05	1.33± 0.03	1.21± 0.03	1.12± 0.08	1.03± 0.04	64.33± 0.99	64.14± 1.71	122.4±1.91	219.17±1.85	302.98±2.39
1×10^{-6}	0.92± 0.05	0.96± 0.02	3.16± 0.14	1.27± 0.14	0.99± 0.12	1.33± 0.05	1.21± 0.06	1.1± 0.05	1.24± 0.08	1.35± 0.07	64.66± 0.58	75.27± 1.00	297.7±1.48	65.49±1.27	64.14±0.55
1×10^{-7}	0.92±0.05	0.94± 0.02	2.51± 0.13	3.15± 0.15	0.96± 0.04	1.33± 0.04	1.25± 0.01	1.15± 0.04	1.06± 0.05	1.18± 0.04	64.06± 0.26	70.66± 0.04	219.56± 1.05	289.37± 1.95	65.66±1.62
1×10^{-8}	0.92± 0.05	0.93± 0.03	1.21± 0.16	2.71± 0.25	3.58± 0.17	1.33± 0.04	1.3± 0.02	1.19± 0.02	1.08± 0.02	1.09± 0.01	64.49± 0.89	68.81± 0.05	162.63± 1.48	299.73±1.34	300.94±1.37

Table 2. Effect of various doses of serotonin injection on lipid level in ovary and hepatopancreas of the mole crab *E.emeritus*

Treatments		Total lipid mg/g				
		0 day	**1st day**	**6th day**	**12th day**	**18th day**
Ovary	**Control**	17.63± 0.32	17.78± 0.29	19.54± 0.32	23.70± 0.24	27.05± 0.75
	$1x10^{-6}$	17.54± 0.21	19.17± 0.18	26.92± 0.21	17.59± 0.40	17.7± 0.53
	$1x10^{-7}$	17.61± 0.34	18.26± 0.17	23.81± 0.36	26.30± 0.27	17.77± 0.14
	$1x10^{-8}$	17.64± 0.46	17.96± 0.56	20.86± 1.00	26.34± 0.89	26.96± 0.13
Hepato pancreas	**Control**	14.4± 0.04	14.4± 0.31	16.51± 0.62	18.59± 0.93	22.59± 0.42
	$1x10^{-6}$	14.39± 0.12	15.81± 0.45	22.07± 0.62	14.89± 0.21	14.59± 0.22
	$1x10^{-7}$	14.4± 0.32	15.16± 0.15	19.07± 0.60	21.55± 0.43	14.79± 0.29
	$1x10^{-8}$	14.35± 0.06	14.88± 0.88	17.07± 0.24	21.74± 0.30	21.81± 0.43

Table 3. Effect of various doses of serotonin injection on triglycerides, fatty acids and lipase activity in ovary and hepatopancreas of the mole crab *E.emeritus*

Treatments		Triglycerides mg/g					Free fatty acids mg/g					Lipase activity **µmole/ mg**				
		0 day	**1st day**	**6th day**	**12th day**	**18th day**	**0 day**	**1st day**	**6th day**	**12th day**	**18th day**	**0 day**	**1st day**	**6th day**	**12th day**	**18th day**
Ovary	**Control**	3.24± 0.17	3.25± 0.36	3.86± 0.14	5.56± 0.23	6.71± 0.47	5.75± 0.03	5.74± 0.03	5.20± 0.06	4.91± 0.12	4.32± 0.08	7.37± 0.22	7.38± 0.25	6.79± 0.14	4.37± 0.32	3.71± 0.20
	$1x10^{-6}$	3.25± 0.14	3.59± 0.19	6.41± 0.32	3.70± 0.50	3.95± 0.28	5.77± 0.04	5.55± 0.03	4.61± 0.07	5.30± 0.07	5.68± 0.06	7.32± 0.16	6.97± 0.14	3.65± 0.28	7.08± 0.23	7.35± 0.07
	$1x10^{-7}$	3.21± 0.06	3.45± 0.25	5.19± 0.15	6.01± 0.10	3.81± 0.31	5.72± 0.03	5.66± 0.04	4.93± 0.07	4.33± 0.10	5.42± 0.06	7.33± 0.06	7.06± 0.15	4.35± 0.32	4.06± 0.32	6.63± 0.35
	$1x10^{-8}$	3.23± 0.08	3.29± 0.24	4.00± 0.12	6.19± 0.17	6.29± 0.43	5.73± 0.03	5.69± 0.05	5.14± 0.06	4.49± 0.09	4.93± 0.06	7.31± 0.16	7.21± 0.13	6.17± 0.31	3.82± 0.49	4.26± 0.22
Hepato pancreas	**Control**	2.80± 0.12	2.89± 0.11	4.12± 0.17	5.04± 0.24	6.01± 0.12	4.80± 0.05	4.82± 0.07	4.36± 0.15	3.83± 0.15	3.64± 0.34	4.38± 0.18	4.39± 0.07	3.96± 0.18	3.61± 0.23	3.20± 0.22
	$1x10^{-6}$	2.73± 0.23	3.15± 0.17	6.04± 0.26	3.07± 0.17	2.89± 0.28	4.87± 0.04	4.58± 0.07	3.29± 0.16	4.51± 0.11	4.77± 0.14	4.34± 0.24	4.36± 0.1	3.35± 0.07	4.09± 0.19	4.50± 0.11
	$1x10^{-7}$	2.73± 0.16	3.03± 0.15	5.02± 0.13	5.76± 0.11	3.19± 0.31	4.80± 0.02	4.66± 0.05	3.78± 0.18	3.52± 0.05	4.29± 0.11	4.38± 0.17	4.26± 0.16	3.78± 0.19	3.70± 0.18	3.94± 0.16
	$1x10^{-8}$	2.74± 0.13	2.95± 0.18	4.35± 0.19	5.92± 0.14	5.84± 0.16	4.77± 0.04	4.61± 0.02	4.20± 0.07	3.75± 0.21	3.76± 0.12	4.38± 0.3	4.29± 0.12	3.74± 0.11	3.39± 0.15	3.19± 0.31

On 12th day of the experiment, the ovary showed immature oocytes, reduction in the size of the oocyte and absence of yolk material indicating that the crab has spawned having immature ovary. Whereas on 18th day observation of spawned ovaries indicated that they were in early pre-vitellogenic stage of development (Figure 1). The results clearly indicated that the serotonin had influenced crabs to reach the vitellogenic stage in 14 days advance. The lipid content did not show much variation in the ovary of control and serotonin injected crabs on 0 day of the experiment. On 6th day of exposure the lipid content in the ovary of the crabs injected with $1x10^{-6}$mol/crab of serotonin amounted to 26.92±0.21mg/g and decreased on the subsequent 12th and 18th days of treatment (Table 2). On the 18th day of experiment the triglyceride content of control and serotonin injected crabs at $1x10^{-8}$ mol/crab was 6.01±0.12 mg glycerol/g and 5.84±0.16 mg glycerol/g respectively. The lipase activity in the hepatopancreas of the control group decreased from 4.38±0.18 µmole/mg on 0 day to 3.20±0.22 µmole/ mg on 18th day of the experiment. The lipid content and triglyceride levels significantly increased and whereas free fatty acids and lipase activity decreased in the ovary, hepatopancreas and hemolymph of the crab.

DISCUSSION

Neuropeptides synthesized and stored in the XOSG complex play an important role in regulating physiological processes in crustaceans (Kuo and Yang, 1999). It has been reported that biogenic amines indirectly influence metabolism and reproduction in decapod crustaceans by modulating the release of neurohormones including CHH and GIH from the eyestalks (Luschen *et al*., 1993). Physiological processes in crustaceans are regulated by neuropeptide synthesized and stored in X-organ sinus gland complexes (Kuo and Yang, 1999). Biogenic amines indirectly influence metabolism and reproduction in decapod crustaceans by modulating the release of neurohormones including CHH and GIH from the eyestalks (Luschen *et al*., 1993). The hepatosomatic index decreased while the gonadosomatic index and the lipid content of the ovary increased, this suggested that the hepatic lipids are transported to the developing ovaries of *E.emeritus* which corroborates with earlier studies in Chinese mitten crab *Eriocheir sinensis* (Cheng *et al*., 1998). Similar results were observed in other crustaceans (Allen, 1972; Castille and Lawrence, 1989). Among growth indices, Gonadosomatic index (GSI) has been considered a tool to evaluate maturation and breeding potential of different organisms. An increase in GSI might reflect an increase in ovarian growth and oocyte development. A significant increase observed in the GSI of serotonin treated *E. emeritus* over concurrent controls may be attributed to the development of greater number of oocytes in response to serotonin injection. Serotonin has been reported to cause a dose dependant increase in ovarian development in *U. pugilator* and *P. clarkia* (Richardson *et al.*, 1991; Kulkarni *et al*., 1992; Sarojini *et al.*, 1995) and not to act directly on the target organ of the neurohormone in *U. pugilator* (Rao and Fingerman, 1970). The increase in oocyte diameter caused by serotonin injection may be due to the accumulation of yolk granules occupying the whole of the oocyte. This suggests that serotonin stimulated gonadal maturation and spawning by interaction with the release of neurohormones and the GIH has intense control over ovaries and hepatopancreas as suggested by Vaca and Alfaro (2000). Hepatopancreas is the metabolic centre for body reserves in crustaceans (Ravid *et al.*, 1999). The vitellogenin, which is responsible for ovarian maturation, is first synthesized in the hepatopancreas and transported to the ovary during vitellogenesis (Wilder *et al*., 1999). Synthesis of yolk precursor proteins by the hepatopancreas was reported in many decapods crustaceans during ovarian development (Quackenbush, 1989). Injection of serotonin caused a significant decrease in HIS compared to control in *E. emeritus*. Allen *et al.* (2001) observed a decrease in the size of the hepatopancreas in the alvinocarid shrimp, *Rimicaris exoculata* during advanced female reproductive stages.

Lipids provide energy for almost all endergonic process and are of importance in maintaining the structure and physiological integrity of cellular and subcellular structures in crustaceans and are also required during reproductive cycles. The ovarian lipid concentration increased steadily from 0 to 6th day of the experiment and decreased to the lowest levels after spawning at 12th and 18thday of the experiment in the mole crabs injected with serotonin at $1x10^{-6}$ mol/crab. Total lipid has been reported to be involved in ovarian maturation in *P.hydrodromous* and *P. clarkia* by stimulating the release of GSH (Sarojini *et al*., 1995; Ragunathan *et al.*, 1999). During ovarian maturation, body lipid reserves, particularly hepatopancreatic lipids are transported to the ovaries resulting in increased ovarian lipid levels compared to those in hepatopancreas (Castille and Lawrence, 1989; Harrison, 1990). Similar mechanism has occurred in *E. emeritus* such as increased lipid content of ovary, hepatopancreas and hemolymph after serotonin administration. Other crustaceans like *U. pugilator* (Richardson *et al.,* 1991) and *P.clarkii* (Kulkarni *et al.,* 1992) obviously support the results of the present study. Triglyceride is one of the main lipid components, responsible for an increase in the quantity of ovarian total lipid during maturation (Ravid *et al.,* 1999).

This study reveals a result that serotonin treatment might have enhanced ovarian maturation as a result of increase in the hepatopancreatic and ovarian lipids constituted by triglycerides and free fatty acids of decrease in lipase activity. Several other authors also have documented mobilization of lipid reserves from the hepatopancreas to the gonads for the buildup of gametes in other crustaceans viz. *Penaeus kerathurus* (Mourente and Rodriguez, 1991), *M .rosenbergii* (Lee *et al*., 1997), *P. vannamei* (Palacios *et al*., 2000), *Litopenaeus vannamei* (Wouters *et al*., 2001), *Aristeus antennatus* (Rosa and Nunes, 2003) and *M. idella idella* (Dinakaran and Soundarapandian, 2009). There was significant decrease in the hepatopancreatic and ovarian lipase activity in serotonin injected crabs. In the promotion of reproductive growth with the elevation of GSH, the crabs prefer to reduce their degradation of lipid. Lipid requirement for gamatogenesis is well known (Cuzin *et al*., 1999). Decrease in lipase activity with serotonin treatment clearly suggests that higher rate of accumulation of lipid in the form of yolk globules during ovarian maturation. These histological findings evidence that serotonin influence the number and size of oocytes in the ovary of *E.emeritus,* and reveals that serotonin might have induced gonadal maturation in association with increased hepatopancreatic and ovarian energy reserves as a result of stimulation of GSH and inhibition of GIH.

Conclusion

The results clearly indicate that serotonin is involved in the induction of ovarian maturation in the mole crab *E.emeritus.* Based on the experimental data it is suggested that selective use of serotonin would be advantageous to induce ovarian growth and spawning in crustaceans.

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Conflict of Interest

There is no conflict of interest.

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