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Methyl farnesoate induced ovarian vitellogenin syntehsis in freshwater rice field crab *Oziothelphusa senex senex* Fabricious

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Abstract

Reproduction in crustaceans is under the control of an array of hormones, internal and external factors including the terpenoid hormone methyl farneosate (MF). In the present study an attempt was made to induce vitellogenesis in crab *Oziotelphusa senex senex* by injecting MF on every alternative day up to 28 days at a concentration of 10^{-8} moles/crab. The concurrent control group received vehicle on par with MF injected group and eyestalk ablated group as positive control were maintained. The crabs were sacrificed on 7^{th}, 14^{th}, 21^{st} and 28^{th} day of experiment to check the stage of reproduction and to measure the ovarian vitellogenin levels. Significant ($p<0.001$) increase was observed in ovarian vitellogenin levels in MF injected and eye stalk ablation groups on 7^{th}, 14^{th}, 21^{st} and 28^{th}day of experiment, when compared to concurrent controls which are in immature stage throughout the experimentation. Also observed increase in the ovarian maturation and ovarian vitellogenin levels are significant in MF injected group than the eyestalk ablated group. Observed two reproductive cycles in the MF injected group of with a mean days of 11±2 in 28 days experiment. Whereas positive control group (ESX) is about to complete one reproductive cycle by the end of the experiment. The results of the present study clear indicate that MF induces vitellogenin synthesis there by ovarian maturation in crab by direct and/or indirect action.

Keywords: Crab, Ovarian maturation, Vitellogenin, Eyestalk ablation, Methyl farnesoate

1. Introduction

Crustaceans play an important role in producing high quality protein to meet the demands of ever growing human population in the world. There are many crustacean species culturing in and around sea coast in many countries. Out of all the problems facing by the crustacean aquaculture industry, getting quality seed is one of the major problems. In search of alternative at first the conventional method, the removal of eyestalks (ESX) unilaterally or bilaterally was introduced (1, 2) and found limitations as attaining inferior quality seed and damage in the brood stock due to loss of hemolymph by eyestalk cautering. In this scenario, the researchers are finding for new methods to adopt for crustacean aquaculture industry in specific hatchery industry. The best way of producing quality seed in hatchery industry is by manipulating the endocrine hormones without damaging other physiological processes of the brood stock. Many hormones in crustaceans are reported for their involvement in regulating reproduction.

Out of all hormones, some acts as reproduction inhibitors and others shows induction. The ovarian maturation inhibitory hormones named as vitellogenesis/gonad inhibiting hormone (VIH/GIH) and Mandibular organ inhibiting hormone (MOIH) (3-5) releases from eyestalk neuronal tissue along with other peptides hormones whose role on reproduction is still in its infant stage. Where as positive regulators of reproduction includes methyl farnesoate (MF) (6, 7), gonad stimulating hormone (GSH) (8-10), ecdysteroids (11-13), crustacean hyperglycaemic hormone (CHH) (14, 15), molt-inhibiting hormone (MIH)(16-18), red pigment concentrating hormone (RPCH) (19, 20) and other small molecules release from various sites of the body of a crustacean.

The major source of reproductive regulatory hormones in crustaceans is neurohemal tissue located in the eyestalks. The major hormones such as VIH/GIH, MOIH, MIH, CHH and RPCH synthesized and released from neurohemal X-organ sinus gland

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complex of eyestalks (21-23) and are collectively called as CHH-family peptide hormones. Among all eyestalk hormones except VIH/GIH and MOIH shows positive impact on ovarian maturation. The involvement of non-eyestalk hormones in reproduction is well defined in many crustaceans such as ecdysteroids in *Daphnia* (24) and *Metopograpsus messor* (25), amines in *O.senex senex* (26) and opioids such as leucine enkephaline in *O.senex senex* (27, 28). Besides all other hormones MF has pivotal role in regulating reproduction in crustaceans.

Mandibular organ (MO) was first described by *Le Roux* (1968) and later Laufer *et al* (29) identified its secretory product, MF using gas chromatography/ mass spectroscopy. It is observed in all members of crustacean family like, crayfishes (30, 7), crab (31) and shrimps (32). MO is analogous to corpora allanta of insects and is derived ectodermally. MF is structurally similar to unepoxidated form of juvenile hormone III of insects secreted by corpora allanta and mediate in growth and metamorphosis of larvae. JH III in insects not only involved in metamorphosis it also play an important role in reproduction by stimulating the maturation of the ovaries, synthesis of yolk by fat bodies and alters the oocyte membrane for provision of the yolk. MF is the major gonadotropin hormone stimulating gonadal development and maturation of sexual behaviour (33) and has a half-life of about 1hr. The secreted MF is having low aqueous solubility and is transported by means of binding proteins named as MF binding proteins (MFBP) in the hemolymph to the site of action. MF is involved in the maturation of the ovaries and thus stimulates the reproduction by showing oocyte growth (34). Evidences clearly subject that the farnesoic acid (FA) is the main precursor of the MF and is then converted into MF by the action of enzyme farnesoic acid O-methyl transferase (FAOMeT). This enzyme catalyzes the methylation of the carboxyl acid group of FA converting to MF with S-adenosyl -1- methionine as a co-factor.

Various methods are adopted to measure induced reproduction in crustaceans. The parameters like ovarian index, oocyte diameter and ovarian histology are in practice to determine various stages of reproduction in crustaceans (5, 27). In recent days, isolation of ovarian vitellogenin and measuring its levels in ovary or in hemolymph became a direct indicator for reproduction in many crustacean species.

Despite the role of MF on reproduction in crustacean models, there is tranquil in research knowing the effectiveness of MF on maturation induction in crustaceans. Therefore the present investigation an attempt is made to know the role of MF, on ovarian vitellogenin synthesis in freshwater crab *Oziothelphusa senex senex* in comparison with ESX as positive control.

2. Materials and methods

2.1. Chemicals used

The test chemical MF was purchased from Echlon Biosciences, Salt Lake City, USA and purchased other chemicals from Merck, Mumbai, India and HiMedia Private Limited Laborateries, Mumbai, India used in the present study.

2.2. Procurement of animals and housing

Intact adult female freshwater crabs, *Oziotelphusa senex senex* Fabricius with a body weight of 27 ± 3 g and body size of 36 ± 3 mm were collected from the rice fields and irrigation canals in and around Tirupati and Renigunta (13^o 36' N, 79^o 21' E), Andhra Pradesh, India. Animals 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. They were acclimatized to the laboratory conditions (temperature 27 ± 1^oC; relative humidity 75% and a light period of 12 h) for 7 days. During their sojourn, the crabs were fed with sheep meat *ad libitum*. Feeding was stopped one day before the commencement of the experiment to avoid changes due to prandial activity.

2.3. Experimental design

A total of 115 female crabs were taken and divided in to four groups. Group 1 (5 crabs) and group 2 (20 crabs) were served as initial and concurrent controls. Group 3 consisting of 50 crabs were treated as eyestalk ablated (ESX) group. Fourth group received MF and consisted of 40 crabs. The volume of hemolymph and injected volume of MF is calculated according to Reddy (35) and Tamone and Chang (36). Initial control crabs were sacrificed on day one whereas concurrent controls are injected with absolute alcohol and treated them on par with MF injected animals. On the first day of experiment third group animals were subjected for eyestalk ablation and MF was injected in a concentration of 10^{-8} moles/crab at the base of third walking leg to the 4^{th} group in an every alternative day. The concurrent controls, ESX animals and MF injected animals were sacrificed on 7^{th}, 14^{th}, 21^{st} and 28^{th} day of experimentation. After scarification by ice unesthetization animals from all the groups were dissected for examine the ovarian developmental

stages and identified stages were recorded. Later on ovaries were isolated, cleaned and used for isolation of ovarian vitellogenin for all the animals of each group. No mortalities were observed during experimentation except in ESX group (30%).

2.4. Isolation of ovarian vitellogenin

Ovarian vitellogenin was isolated using the protocol described by Tsukimura *et al* (37). In brief, 10% (W/V) ovarian tissue homogenates were prepared in homogenization buffer (pH 7.8) containing 0.1 M sodium chloride (NaCl), 0.05 M Tris, 1 mM ethylenetriamine tetra acetic acid (ETTA) and 0.1% tween-20 with 10 mg/ml phenylmethylsulfonyl fluoride (PMSF), using an ice cold glass-glass homogenizer. The homogenate was centrifuged at 4000 X g for 5 min. at 4ºC. Resultant supernatant was collected and again centrifuged at 20,000 X g for 20 min. at 4ºC. To the supernatant, ammonium sulfate (SAS) was added to produce a 25% SAS solution. After mixing the contents for 1 h at 4ºC, the homogenate was centrifuged at 20,000 X g for 10 min. at 4ºC. The supernatant was collected and SAS was added to produce 40, 50 and 60% SAS solution sequentially. The pellets of 60% SAS solution was suspended in homogenization buffer and dialyzed three times against homogenization buffer at 4ºC for 12 h each. The purified ovarian vitellogenin was stored at -20ºC until further use.

2.5. Estimation of protein content

The ovarian vitellogenin content in each purified sample was estimated by the method of Bradford (38). To 0.1 ml of isolated vitellogenin sample 0.9 ml of 0.1M phosphate buffer (pH concentrations of Bovine serum albumin and used to determine the protein concentration in the purified samples. The protein content was expressed as mg/g tissue. 7.5) was added followed by 5.0 ml of Bradford reagent. After mixing the contents, the absorbance of the colour was measured at 595 nm against reagent blank in Hitachi Model U-2001 UV-VIS spectrophotometer. A standard curve was prepared against graded.

2.6. Preparation of antisera

Polyclonal antisera were raised in New Zealand white rabbits by injecting the equal volumes of purified protein (700 µg) and Freund's complete (in the first week) and incomplete (2^{nd}, 3^{rd} and 4^{th} weeks) adjuvant. After one week of the last injection blood was collected by ear puncture. The blood was kept at room temperature for 1 hour and then at 4°C overnight. The blood was centrifuged at 1000 X g, 4°C for 20 min. and the titer of anti-sera was determined using double immunodiffusion according to the method described by Ouchterlony (1958). Collected anti-sera were stored at -20ºC until further use.

2.7. Measurment of vitellogenin levels by ELISA

The isolated vitellogenin was estimated by Enzyme Linked Immune Sorbent Assay (ELISA). The well of certified 96-well microtiter plate were coated with 20 µl of concentrated sample diluted in 1:10 ratio with coating buffer (carbonate buffer: 1.59 g sodium carbonate, 2.93 g sodium bicarbonate in 1000 ml distilled water, Diethyl dithio carbomate 56 mg per 25 ml buffer). The wells coated with buffer alone were treated as blank. The covered plate was incubated in humid chamber at 37°C for 2 h. After incubation contents were discarded and the plate was filled with 200 µl of diluted (1:1000) primary antibody (antibodies raised in rabbits against *O. senex ssenex* vitellogenin isolated from vitellogenic stage III ovary) in 0.1 M Phospahte Buffer Saline containing 0.05% Tween 20, pH 7.2 (PBST) with 2% polyvinyl pyrrolidine, 0.2% ovalbumin (PBST-PO). Incubated for 2 h at 37°C followed by discarding, the plate was filled with 200 µl of horseradish peroxidase (HRP) conjugated anti IgG antibody (1:1000 dilution with PBST-PO; purchased from Genei, Bangalore) and incubated for 1 h at 37°C. Each step is followed by successive washing of plate with PBST for 3 times. Finally the plate was added with 200 µl Tetra Methyl Benzidene (TMB in 0.015% hydrogen peroxide) and incubated for 1 h at 37°C. The reaction was stopped by adding 50 µl of 1.0 M phosphoric acid per well. Absorbance of each well was measured with ELISA reader (Bio-Rad, Imark, USA) at 450 nm. All standards and samples measurement were performed in duplicate.

2.8. Statistical analysis

By using the statistical programme GraphPad. Prism. version 5.0.3.477, the data obtained were analyzed statistically and data were processed with Two- way ANOVA followed by student's t-Test. The 'p' value <0.05 was considered as significant. The graphs were drawn by using programme Origin Pro version 8.

3. Results

Throughout the experiment no mortality was observed both in concurrent control and MF injected groups. There was 30% mortality recorded in ESX group. On the sacrification days i.e., 7^{th}, 14^{th}, 21^{st} and 28^{th} day of experiment, the ovarian vitellogenin

stages was identified by seeing the morphology (colour) of ovary.

The macroscopic description of ovary at different stages of ovarian maturation is presented in Table 1. In 28 days of experiment observed two reproductive cycles by MF injection whereas only one cycle in the positive control (ESX) group.

In the present study, found that all the concurrent controls sacrificed during experimentation are in immature stage (5 crabs on each day). On 7th, 14th, 21st and 28th day (number of crabs i.e., n=5 in each stage) observed that the ovary of MF received animals are in vitellogenic stage II and III. Where as in ESX group 7th to 28th day of experimentation observed progression in the ovarian maturation i.e. vitellogenic stage I (7th day n=5; 14th day n=5; 21st day n=5), II (14th day n=5; 21st day n=5; 28th day n=5) and III (28th day n=5). Ovulation was observed only in the MF injected group between 11-15 days (n=18) and 26-28 days (n=5) of experimentation. Crabs ovulated between 11-15 days were continued with MF injections after removing eggs from brood sac for the second reproductive cycle.

The vitellogenin from ovaries of crabs sacrificed on 7th, 14th, 21st and 28th day of experiment was isolated and the isolated samples were tested for protein estimation for loading in to the ELISA plate. For all the isolated vitellogenin samples starting from control, concurrent control, ESX and MF injected groups were subjected to ELISA to find out the concentration of vitellogenin by using vitellogenin polyclonal antisera raised against crab *O. senex senex* vitellogenin in rabbit.

On 7th day of experiment the mean values of levels of ovarian vitellogenin in MF injected (vitellogenin stage II, 0.514 ± 0.029 and vitellogenic stage III, 0.844 ± 0.035) and ESX (vitellogenic stage I, 0.243 ± 0.04) groups were significantly ($p<0.001$) increased when compared to concurrent control crabs (immature, 0.094 ± 0.007) (Figure 1).

Similarly on 14th day of experiment significant increase ($p<0.001$) in the mean values of ovarian vitellogenin levels were notified in both MF injected (vitellogenin stage II, 0.626 ± 0.031 and vitellogenic stage III, 0.858 ± 0.042) and ESX (vitellogenin stage I, 0.298 ± 0.009 and vitellogenic stage II, 0.478 ± 0.012) crabs over the concurrent control group (immature, 0.098 ± 0.005) (Figure 2).

The MF injected (vitellogenin stage II, 0.672 ± 0.02 and vitellogenic stage III, 0.981 ± 0.036) and ESX (vitellogenin stage I, 0.343 ± 0.018 and vitellogenic stage II, 0.644 ± 0.022) crabs were shown significant increase ($p<0.001$) in the mean values of ovarian vitellogenin levels on 21st day of experiment when compared to the concurrent control crabs (immature, 0.120 ± 0.014) (Figure 3).

The same trend has been observed in the mean values of levels of ovarian vitellogenin on 28th day of experimentation in MF injected (vitellogenin stage II, 0.828 ± 0.024 and vitellogenic stage III, 0.990 ± 0.035) and ESX (vitellogenin stage II, 0.692 ± 0.014 and vitellogenic stage III, 0.861 ± 0.022) groups when compared to concurrent control crabs (immature, 0.217 ± 0.016) (Figure 4) and found significance at the 'p' value <0.001.

Table 1. The macroscopic observation of ovary in crab *Oziothelphusa Senex senex* at different stages of ovarian development.

Ovarian Stage	Macroscopic description
a. Immature	Ovaries are thin strand like and translucent to opaque white; recovering spent ovary in few crabs.
b. Mature	
Vitellogenesis I	Ovaries are thickened and dark yellow; oocytes are arranged compactly.
Vitellogenesis II	Ovaries further thickened and orange in colour; oocytes are clearly visible in the ovary.
Vitellogenesis III	Ovaries swollen and large dark brown or bright orange oocytes are clearly visible in the ovary.

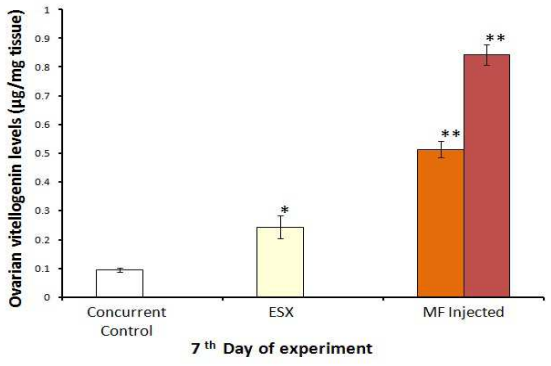

Figure 1. Effect of methyl farnesoate injection and eyestalk ablation on ovarian vitellogenin levels on 7th day of experiment in the crab *Oziothelphusa senex senex.*

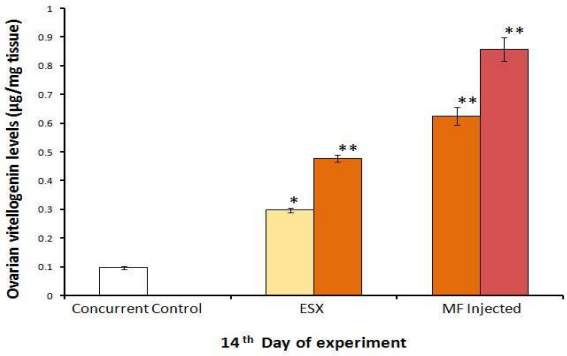

Figure 2. Effect of methyl farnesoate injection and eyestalk ablation on ovarian vitellogenin levels on 14th day of experiment in the crab *Oziothelphusa senex senex.* Values are mean ± SEM of five crabs. The significance was considered at (*)p<0.001 and (**)p<0.0001.

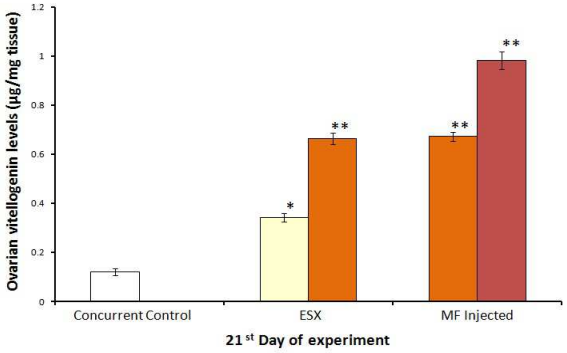

Figure 3. Effect of methyl farnesoate injection and eyestalk ablation on ovarian vitellogenin levels on 21st day of experiment in the crab *Oziothelphusa senex senex.* Values are mean ± SEM of five crabs. The significance was considered at (*)p<0.001 and (**)p<0.0001.

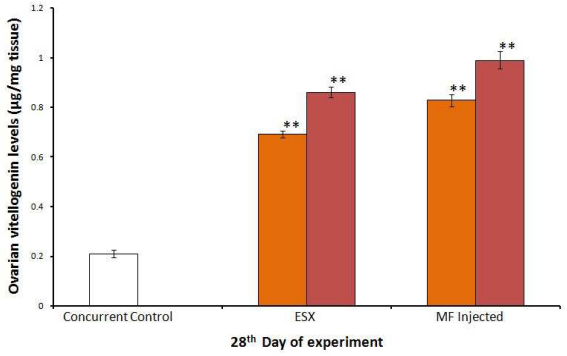

Figure 4. Effect of methyl farnesoate injection and eyestalk ablation on ovarian vitellogenin levels on 28th day of experiment in the crab *Oziothelphusa senex senex.*Values are mean ± SEM of five crabs. The significance was considered at (*)p<0.001 and (**)p<0.0001.

4. Discussion

Methyl farnesoate a naturally occurring crustacean terpenoid hormone has great potential to induce reproduction in crustaceans. Though many studies revealed the role of MF on crustacean reproduction, but still information is lacking on mechanistic behaviour of MF on reproduction and the other functions of MF is not clear. Studies are confined to know the induction of reproduction by studying different parameters. In recent days measuring vitellogenin levels in the ovary or/and in hehemolyph or vitellogenin synthesizing tissue became fascinated to study reproduction. In the present study though we are not proposing the mechanism of MF induced reproduction, we are adding MF role on vitellogenin levels in ovary of crab to the literature.

Significant increase ($p<0.001$) in the ovarian vitellogenin levels in MF injected crabs *O. senex senex* was observed along with the positive control group ESX over the concurrent controls on 7th, 14th, 21st and 28th day of experiment. Reddy *et al* (39) studied ovarian vitellogenin levels in natural and induced (EXS) reproductive cycle in the crab *O. senex senex.* In the same crab significant increase in the ovarian vitellogenin levels was reported by the injection of 17α-OHP (40). The positive role of MF on reproduction was reported in many crustaceans such as *Procabarus clarkii* (*P. clarkii*) (41), *Charybdis feriaturs* (42) and *Macrobrachium rosenbergii* (43). The reports are also available in combinational effect of MF with other vertebrate type steroids on reproduction in crustaceans. Rodriguez *et al* (44) injected MF and 17β-estradiol and found reproduction induction in *P. clarkii*. In the same report also mentioned that MF in combination with 17α-OHP inhibits ovarian maturation. The increased ovarian vitellogenin levels in crab *O. senex senex* by MF in the present study are in support of the publication evidences and suggesting the role of MF in the induction of vitellogenesis in crab.

Observed two reproductive cycles with a mean period of 13 ± 2 days in a span of 28 days experiment by the injection of MF in crab *O. senex senex.* The ESX group a positive control for this study have about to complete the reproductive cycle in 28 days of experiment. It is clear from the results of the present study that the administration of MF is effective in reducing the length of reproductive cycle in crab. The MF injections are more effective to increase the vitellogenin levels in ovary of crab than the ESX crabs, indicating that MF has an intrinsic role in promoting reproduction with an effective manner for producing quality seed. The possible

action of MF on ovarian maturation in crustaceans has reported and found that MF can induce ecdysteroid biosynthesis from Y-organs thereby reproduction (36). On the other hand the evidences also available on direct effect of MF/farnesoic acid (precursor for MF synthesis) on vitellogenin synthesis (42). However the MF induces reproduction in crustaceans either in direct way or in indirect way. But still there is controversy in MF mediated reproduction.

The present study clearly says that MF induces ovarian vitellogenin levels in crab *O. senex senex*. The level of induction is almost more than two times than the positive control i.e., ESX. Besides this it is also clear that MF strongly induce vitellogenesis from its synthesizing site directly or indirectly. In the indirect way it may induce the ecdysteroid biosynthesis thereby vitellogenesis. The high levels of vitellogenin accumulated in the oocytes by the action of MF leads to produce healthy and high quality seed. However the possible mechanism of MF induced reproduction in crab *O. senex senex* is in search.

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Conflict of interest Statement

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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