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Effect of Methyl farnesoate in stimulation of moulting in the female crab *Oziothelphusa senex senex* (Fabricius, 1798)

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Short communication

Abstract

In crustaceans, eyestalk ablation (ESA) is commonly used in aquaculture to stimulate moulting, and methyl farnesoate (MF) effects crustacean moulting. The influence of methyl farnesoate in the regulation of moult was investigated by administration of MF with three tested concentrations of 10^{-9}moles/crab; 10^{-8}moles/crab and 10^{-7} moles/crab. Highest moult induction frequency was obtained at MF 10^{-8}moles/crab injected group as evidenced on day 7th, 14th, 21st and 28th of the experiment. The moult induction frequency of MF from the above experiment in crabs is summarized as 10^{-8} moles/crab (33.33%) > 10^{-7} moles/crab (17.5%) > 10^{-9} moles/crab (16.6%) > ESX (16.6%) respectively. Injection of MF into inter-moult crabs increased moulting percentage, moulting stimulation, growth acceleration and adaptability. Hierarchial cluster and dendogram analysis quantify the close relation among the experimental groups.

Keywords: *Moulting, methyl farnesoate, crustacean, edible crab, Oziothelphusa senex senex*

Introduction

Growth is a major challenge in crustacean aquaculture and is essentially linked to moulting cycles. Moulting and breeding are seasonal events in crustaceans. Most of the crabs program their moult and breeding in antagonistic fashion; only the inter-moult females engage in breeding during the reproductive seasons (July to September) and undergo moulting during non-breeding (March to June/July) seasons (Kappalli *et al.,* 2012; Sarika *et al.,* 2014). Moulting is regulated by steroidal moulting hormones, ecdysteroids produced by the Y-organ (Nakatsuji *et al.,* 2009) and sesquiterpenoid methyl farnesoate (MF) (Laufer *et al.,* 1998). Several moult regulatory hormones and other factors, plays a major role in the regulation of moulting in crustaceans (Diwan, 2005). Bilateral eyestalk ablation had a significant effect on moulting in the crab *Oziothelphusa senex senex* (Neelima *et al.,* 2016).

The eyestalk ganglia contain the X-organ/sinus gland (XO-SG) complex, which produces a moult-inhibiting hormone (MIH). As a member of the crustacean hyperglycemic hormone (CHH) subfamily II, the MIH peptide hormone acts on the Y-organ, suppressing 20-Hydroxyecdysone (20E) synthesis (Covi *et al.,* 2009; Nakatsuji *et al.,* 2009; Mykles, 2011; Nagaraju, 2011). As the result of ESA, inhibitory factors, including MIH, are removed,

increasing 20E production and up-regulating its physiological response. Although ESA is an effective strategy for inducing a moult, it resulted in high mortality

Methyl farnesoate (MF) is a sesquiterpenoid compound found in decapod crustaceans and is structurally similar to the juvenile hormone (JH) of insects. However, MF differs from juvenile hormone (JH III) in containing an epoxide moiety at the terminal end. Crustaceans appear to lack epoxidase and S-adenosyl-methionine-dependent methyl transferase, which convert farnesoic acid (FA) to JH III (Hui *et al.,* 2010). Therefore, crustaceans lack JH III, and MF is the end product of sesquiterpenoid biosynthesis. In insects, JH III is the major hormone related to metamorphosis, gonad maturation, and may play a role in moulting (Belles *et al.,* 2005; Tsubota *et al.,* 2010). The major production site for MF is the mandibular organ (MO) and its biosynthesis is negatively controlled by the mandibular organ-inhibitin g hormone (MOIH), secreted from the XO-SG complex at the terminal end of the eyestalk (Nagaraju, 2007). The MOIH peptide hormone suppresses the production of MF.

Previous studies suggest that MF treatment results in a physiological response similar to that to 20E in decapod crustaceans. Several studies showed that MF stimulates moult acceleration in addition it causes reproduction in several crustacean species. However, the effects varied among species, and no consensus has yet been reached.

The main goal of this study was to compare with three tested concentrations–as low, medium and high dosages effects of MF on the growth of the crab *O. senex senex*. We evaluated moult frequency, after the injection of 1.8, 2.0 and 2.2ng/ml MF for 1 month on alternate days of the experiment.

Material and methods

Collection and maintenance of crabs

Procurement of animals and intact adult intermoult freshwater crabs, *O.senexsenex* with a body weight of 27 ± 3 g and body size of 36 ± 3 mm width were collected from rice fields and irrigation canals in and around Tirupati and Renigunta (13° 36′ N, 79° 21′ E), Andhra Pradesh, India. Animals were housed in 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°C; 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.

Measurement of body weights

Animals were acclimatized to laboratory conditions and their body weights were measured on the initial day of the experiment and during the experiment before sacrificing, using an electronic balance (Shimadzu AY220).

Eyestalk ablation (ESX)

Bilateral eyestalk ablation was conducted in female crabs. Eyestalk ablation (ESX) deprives the crab from eyestalk hormones as they regulate the major physiological processes like moulting in crustaceans.

Chemicals used

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

Dosage of MF

MF was dissolved in 95% ethanol and diluted with crab ringer solution (6.5g NaCl, 0.42g KCl, 0.25g $CaCl_2$ and 0.2g of sodium bicarbonate) so that the final concentration of ethanol was made into 10%. To calculate dosage of MF, the weight of the crabs was measured and the volume of haemolymph calculated according to body weight of individual crabs. The volume of haemolymph and injected volume of MF was calculated according to Reddy *et al.* (2004). Injections were given as low, medium and high dosages on alternate days to the experimental groups and were sacrificed on first, seventh, fourteenth, twenty first and twenty eighth day of the experiment.

Determination of MF optimum concentration on moult induction

To study the induced moult cycle of the crab *O. senex senex*, about 184 crabs of each sex with a weight of 30 ± 2 g were divided into five groups containing controls (n=4), concurrent controls: (n=20) and 4 experimental groups, each consisting of 40 crabs. The crabs in the first group were sacrificed on day '0' of the experiment. The crabs in second group which served as concurrent controls were treated as experimental samples, not givenMF injection. The crabs in the 3rd group were ESX. Eyestalks were removed and wounds were cauterized to minimize hemolymph loss, as described by Salma *et al.* (2012). Group 4,5 and 6 were administered MF 10^{-9} moles/crab, MF 10^{-8}moles/crab, MF 10^{-7} moles/crab respectively. The specified number of crabs in group 2, 3, 4, 5 and 6 were sacrificed to determine the moult stages on 7th, 14th, 21st and 28th day of the

experiment. Inter-moult and pre-moult stages were determined based on the setal development in the mastigobranch of the 3rd maxillepede.

Determination of moulting stage

Moult stages were determined based on morphological changes in the decapods during the moulting cycle. In the sub-stage D_0, the underlying epidermis was separated from the exoskeleton and it is termed as apolysis. In sub-stage D the retraction of epidermal layer from old cuticle and loss of external setae was observed with appearance of rudiments of setal grooves from the old epidermal layer in mastigobranch for development of new setae. The sub-stage D_2, in this substage, the new pigment layer was synthesized. In sub-stage D_3, the newly formed integument became completely detached from the old exoskeleton. In sub-stage D_4, the inner newly formed integument was dark brown or black in colour and remained detached from old exoskeleton. For determining moulting stages, live crabs were checked daily and the selected animals were placed on ice.

Hierarchial clustering and Dendogram

Moult stages were selected into groups and quantified between four experimental groups *i.e.* ESX, MF 10^{-9}, MF10^{-8}, MF 10^{-7} were dissected on day 7th, 14th, 21st and 28th of the experiment. Hierarchial Cluster and Dendogram (Wards method) were analysed using software JMP 14.0.1(SAS Institute Inc., 2016).

Results

Similar to males, females also exhibited significant induction of moulting on the 7th, 14th, 21st and 28th days of the experiment when compared to controls and concurrent controls. The ESX group on day 7 of the experiment were found in inter-moult stage C_4 (100%). Crabs with MF 10^{-9}moles/crab were in early pre-moult D_1 (99.99%). The animals that received MF 10^{-8}moles/crab were in pre-moult stages D_1 (55.55%) and D_2 (44.44%). Whereas moult stages C_4 (66.66%) and D_1 (33.33%) were observed in crabs that received 10-7 moles/crab of MF on the7th day of the experiment (Fig. 1a).

On the 14th day of experiment ESX crabs had entered into the early pre-moult D_1 stage (66.66%) and the middle pre-moult D_2 (33.33%) stage. Female crabs that were injected with MF 10^{-9}moles/crab were found in pre-moult D_1 (44.44%) and D_2 (55.55%) stages. Crabs injected MF 10^{-8}moles/crab was observed in pre-moult stages D_2 (55.55%), D_3 (33.33%) and few had moulted (11.11%). Pre-moult stages D_1 (44.44%) and D_2

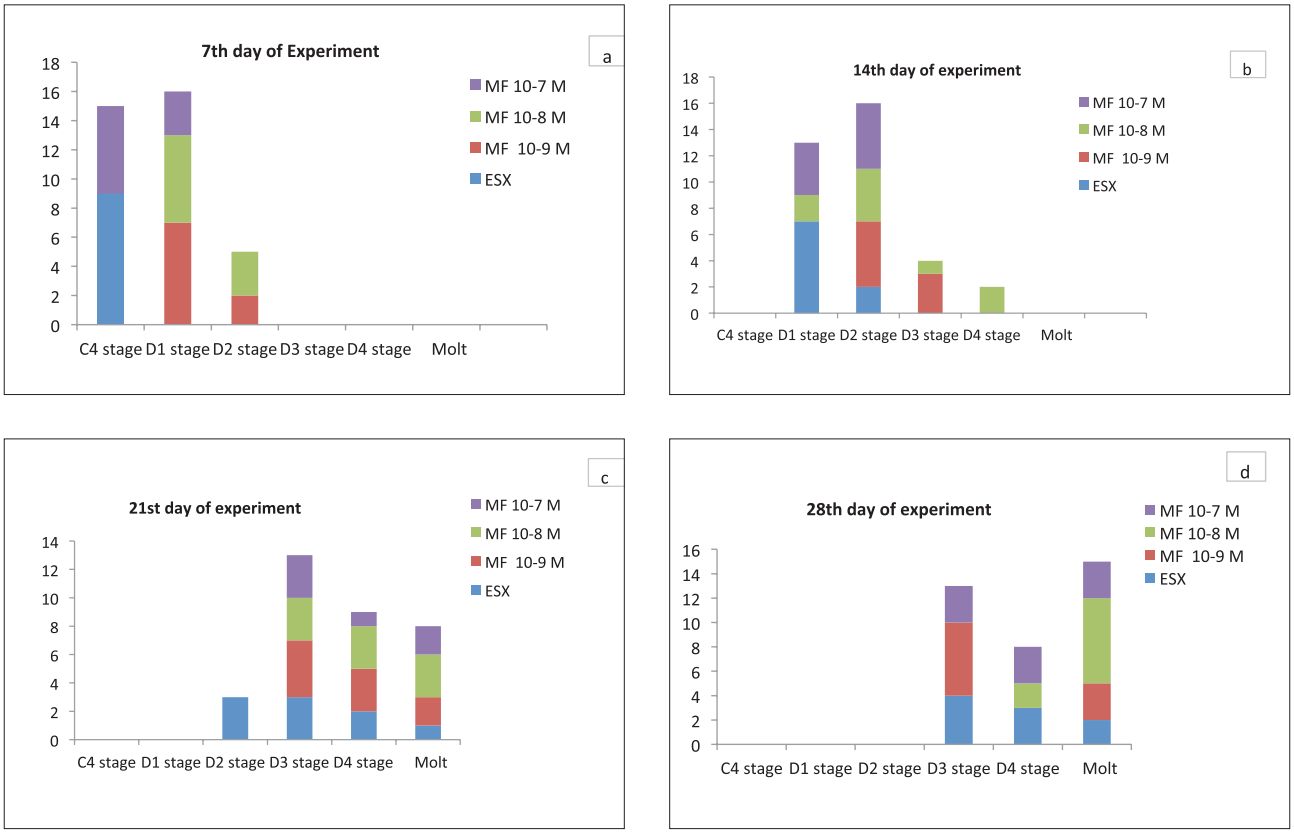

Fig. 1. Different premolt substages and molted in female crab during 28 days of experiment

(55.55%) were observed in crabs that received10^{-7} moles/crab of MF on the 14th day of the experiment (Fig. 1b).

Those crabs administered ESX entered into middle pre-moult stage D_2 (33.33%) and D_3 (33.33%), late pre-moult D_4 (22.22%) and moulted (11.11%) on day 21 of the experiment. Groups given MF 10^{-9}moles/crab were found in pre-moult stage D_2 (33.33%), D_3 (33.33%), D_4 (22.22%) and moulted (11.11%) on 21st day of experiment. Animals that received MF 10^{-8}moles/crab were observed in pre-moult D_3 (44.44%) and D_4 (33.33%) and 22.22% moulted. On day 21 of the experiment pre-moult stages D_2 (55.55%), D_3 (22.22%), D_4 (11.11%), and 11.11% of moulted (11.11%) crabs were observed among crabs that received10^{-7} moles/crab of MF (Fig. 1c).

On 28th day crabs with ESX were found in pre-moult D_3 (44.44%) and D_4 (33.33%), and 22.2% moulted. Crabs given MF10^{-9}moles/crab were in pre-moult D_3 (33.33%) and D_4 (44.44%) stages, and some moulted (22.22%) on the 28th day of the experiment. MF 10^{-8} moles/crab group were in pre-moult D_3 (55.55%) and moulted (44.44%). The pre-moult stages D_3 (44.44%), D_4 (33.33%) and moulted (22.22%) crabs were observed in MF 10^{-7} moles/crab injected group on 28th day of experiment (Fig. 1d).

The moult induction frequency of MF was 10^{-8}moles/crab > 10^{-7}moles/crab >10^{-9}moles/crab. High moult percentage were recorded as 17.5% in MF 10^{-8} moles/crab and low (7.5%) in MF 10^{-9} moles/crab. It is clear from the results of these experiments that effective concentration of MF for inducing moult in crab *O. senex senex* is 10^{-8} moles/crab and is fixed asan MF concentration for further experiments.

Dendogram and cluster quantify the close relation among the groups based on analysis on day wise experiment. On 7th day of the experiment in group A MF10^{-9} moles/crab and MF 10^{-8}/crab was high in premolt D_1 stage and ESX was observed in inter-moult C_4stage. This clearly show the close relation among MF 10^{-9} and 10^{-8}. On 14th day in group B ESX were in early pre-moult stage $D_1$and MF 10^{-9}, 10^{-8}, 10^{-7}were observed to be in middle pre-moult stage (D_2). Hence there is close relation between dosages of MF 10^{-9}, 10^{-8}, 10^{-7}.On 21st day ESX showed early and middle pre-moult D_2 and D_3 stages, and MF 10^{-8} and 10^{-7} were in late pre-moult D_3, D_4 and some animals entered into moult stages. In group C it clearly indicated the close relation among the groups MF 10^{-8} and MF 10^{-7}. On 28th day of experiment close relation was observed between MF 10^{-8} and MF 10^{-7} by entering into moult stage. Similarly, ESX and MF 10^{-9}had close relation. Hierarchical cluster clearly indicates that MF 10^{-8} has high moult percentage and close relation with the dosage of MF 10^{-7}.

Discussion

Moulting is a complex process consisting of cyclic physiological and biochemical changes in the structure of the integument (Anger, 2001) which is controlled by endocrine hormones (Molina and Cadena, 2001). Though many of these studies are focused on induction of moulting, the effective moult inducing factors are not yet implemented at the level of aquaculture. The present study was conducted in search of effective moult inducing factors in the crab *O. senex senex*.

The role of MF and the minimum effective concentration of MF in inducing moult were determined in the present study in the crab *O. senex senex*. Out of three concentrations of MF tested, MF 10^{-8} moles/crab injection was found to be effective moult inducer. MF stimulated moulting in *Cherax quadricarinatus* (Abdu *et al.,* 2001), *Carcinus magister* (Tamone and Chang (1993), *Penaeus vannamei* (Hui *et al.,* 2010), *Liagore emarginata* (Rotllant *et al.,* 2000), *Scylla serrata* (Girish *et al.,* 2015), *Portunus trituberculatus* (Xie *et al.,* 2015), *Neocaridina denticulate* (Sin *et al.,* 2015), *Metapenaeus ensis* (Gunawardene *et al.,* 2002), *Homarus americanus* (Homola and Chang, 1995) and *Penaeus monodon* (Suneetha *et al.,* 2010).

Administration of MF also caused moult acceleration in crayfish *C. quadricarinatus* (Abdu *et al.,* 2001) and *Procambarus clarkii* (Laufer *et al.,* 2005), and in crab *O. senex senex* (Reddy *et al.,* 2004). However, moulting of adult crayfish was induced by the administration of JH III (Rodriguez *et al.,* 2002). It indicates that MF modulates ecdysteroid activity in crustaceans. MF may involve directly or indirectly in moulting by stimulating the secretion of ecdysteroids from Y-organ. Another hypothesis is MF could delay moulting in larval crustaceans (Borst *et al.,* 1987). Evidences also support the idea that MF regulates the increase of growth in crustaceans.

The role of bilateral eyestalk ablation in the regulation of moult was tested and had a significant effect on moulting in the crab *O. senex senex* (Neelima *et al.,* 2016). Reduced inter-moult periods and shortened moult cycle lengths *i.e.*, 21 days were reported. Crabs usually moult only once or twice per year. Eyestalk ablation induced moulting has been reported in fiddler crabs (Hopkins 1982), shrimps (Vijayan *et al.,* 1997), crayfish (Majid, 2008) and fresh water prawns *Macrobrachium rosenebergii* (Okimura and Aida, 2001). Stella *et al.*, (2000) had drawn the importance of bilateral eyestalk ablation on moulting of *Chasmagnathus granulata* and reported that ESX is a strong inducer of moulting.

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