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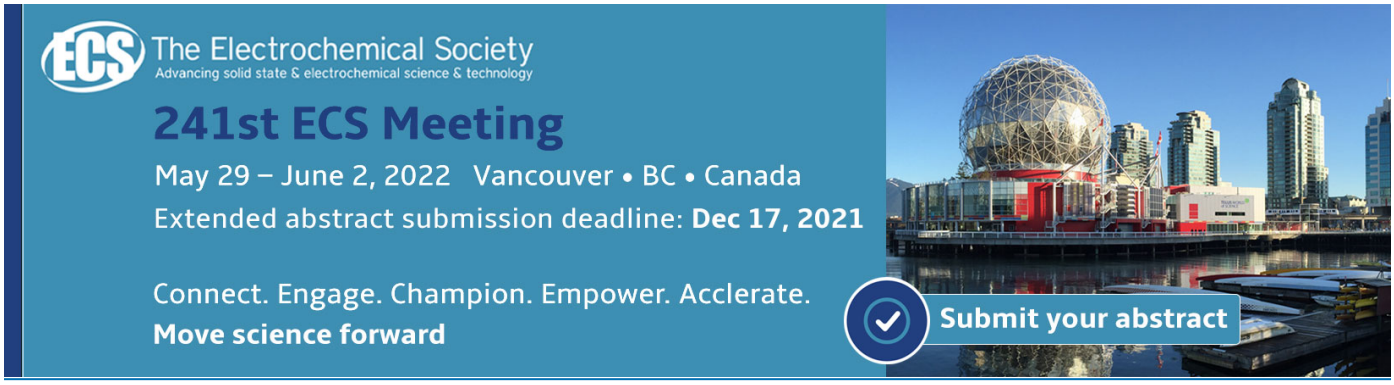

Dietary methyl farnesoate, a potential growth inducer in male crab *Oziothelphusa senex senex*

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Abstract. Insect juvenile hormone-like compound methyl farnesoate (MF), identified through 'reverse endocrinology' in crustaceans is a sesqui-terpenoid and plays crucial role in growth well proved by direct administration into the animals at laboratory conditions. However, these studies are not reached to the cultural ponds. Moreover, dietary supplementation of MF and its effects on growth in crustaceans is still at infancy. The present study tested MF (concentration of 10^{-9}, 10^{-8} and 10^{-7} moles/crab added to commercial shrimp pellet diet) in the growth of male crab *Oziothelphusa senex senex* (Oss) supplemented every alternative day for about 40 days. Along with experimental group control and eyestalk removed (ESX) groups are maintained. Dietary MF induced significant enhancement in the growth of male crab. The most effective group MF 10^{-8} moles/crab supplemented. The frequency of growth induction found in this study is MF 10^{-8}>10^{-9}>10^{-7} moles/crab ≤ ESX and molted percentage is 27.5%, 17.5%, 10%, 10% in each group, respectively. The dietary supplementation of MF effective in inducing growth in cultured crustaceans thereby increases the yield of crustacean protein.

1. Introduction

Crustacean aquaculture industry plays crucial position in producing quality protein in agriculture. The flavour, deliciousness and limited availability of crustacean protein made it as one of the precious proteinaceous food on the globe. The worldwide production of crustacean protein facing many problems/difficulties. Methods are in pipeline to produce quality protein by inducing growth in culture species. One of such common technique followed to induce growth is traditional eyestalk ablation (ESX), where one-sided (unilateral) or two-sided (bilateral) eyestalk ablation tests were conducted [1]. Consequently, ablation of eyestalk triggers ecdysteroid secretion from Y-organ thereby induces precocious molting and tested in many decapods [2-4]. Although, ESX induces molting effectively in Aquaculture species, but it has its own limitations. ESX endorses with mortality owing loss of large amount of hemolymph and causes mortality. A few alternatives are in search alongside ESX and one of the best methods to improve crustacean protein identified in many studies using growth regulating hormone(s) manipulation. A process where exogenous molecules/chemicals exposed to the test animal for its growth enhancement called endocrine manipulation. Crustacean growth has been manipulated using a list of internal and external endocrine modulators [5-7].

Crustacean growth is under the control of a range of endocrine hormones. Methyl farnesoate (MF) and farnesoic acid (FA) are secretory products of mandibular organ (MO) and ecdysteroids the products of Y-organ regulate crustacean growth positively [8, 9]. In contrary, molt-inhibiting hormone (MIH) and mandibular organ inhibiting hormone (MOIH) the secretory products of eyestalk regulate negatively.

Crustacean MF (methyl-(2E,6E,10E)-3,7,11-trimethyldodecatri- 2,6,10-eneoate) structural analogue to insect JHIII (methyl 9-(3,3-dimethyloxiranyl)-3,7-dimethyl-2,6-nonadienoate) and contrary by not holding an epoxide moiety at the terminal end. The spider crab *Libinia emarginata* (*L. emarginata*) is a first source to isolate and characterize MF [10]. Since then, MF characterized in more than 35 crustaceans. Presence of MF was confirmed in crabs, shrimps, prawns, lobsters, and crayfishes. MOIH, a product of eyestalk X-organ sinus gland complex regulates the synthesis and secretion of MF in crustacean MO [11]. MF released into hemolymph from MOs, binds with a protective lipoprotein called MF-binding protein (MFBP) transports to target and regulates growth positively [12, 13]. Moreover, MF also reflected with positive regulation of reproduction in crustaceans [7, 14].

Science advances should come into reality to get product. Available literature well represented the direct effect of MF on growth induction in crustaceans. Our lab has taken initiative to study the MF induced molt in crab *Oss* and other crustacean species [15]. However, these earlier reports constrained only to the laboratory, but not reached/practiced in the culture ponds on culturing crustacean species for growth induction. Few reports are focused to induce molt by supplementing the MF through diet in the laboratory and these are not successful at the field level. Yield (growth) enhancers of crustaceans at the field level has great potentiality to produce high amounts of protein. At this juncture, the present study focused on feed supplementation of MF induced growth in male crab *Oziothelphusa senex senex* at semi-field experiment with natural pond environment.

2. Materials and Methods

2.1. Collection and maintenance of crabs

Intact male freshwater crab *Oziothelphusa senex senex* obtained from rice fields located in and around Kadapa (14° 28' 39N, 78° 49' 25E), A.P, India. To maintain crabs semi-controlled small natural pond (sand with enough water) environment was created at Yogi Vemana University, Kadapa. Initially about a week time crabs were adapted to the pond conditions. Shrimp feed available commercially was used to feed the crabs in every alternative day *ad libitum* with recycled water.

2.2. Body weight of crabs

The average body weight of male crabs used in the present study is 25 ± 3 g. Weights of the male crabs were measured on day one and during the experimentation process using Shimadzu (AY220) electronic balance.

2.3. Eyestalk removal (ESX)

To deprive the eyestalk growth inhibitory principle in selected male crabs, bilateral eyestalk ablation has been done by cautering at the base without prior ligation. This group of crabs are maintained as positive controls.

2.4. MF dosage

Methyl farnesoate (MF) the test chemical of the present study purchased from Echlon Biosciences, Salt Lake City, USA. The other chemicals used are purchased from HiMedia Private Limited Laboratories, Mumbai, India and Merck, Mumbai, India. MF stock was prepared in 95% ethanol and crab ringer solution prepared with 6.5g NaCl, 0.42g KCl, 0.25g $CaCl_2$ and 0.2g of sodium bicarbonate used for diluting MF stock to attain ethanol final concentration as 10%. The final concentration of MF

10^{-9}, 10^{-8} and 10^{-7} moles prepared by adding each to the 100g of commercially purchased shrimp pellets. This MF concentration calculated according to Tamone and Chang [16], and Reddy et al. [15].

2.5. Experimentation

About 40 clearly labelled male crabs in each group were maintained in five small ponds. The first two ponds were maintained with control (1) and ESX (2) crabs, respectively and are fed with normal pellet diet. Pond 3, 4 and 5 crabs maintained by feeding every alternative day with 10^{-9}, 10^{-8} and 10^{-7} moles of MF/each 100 mg pellet diet, respectively.

2.6. Molt stage determination in crab

Decapod crustacean growth cycle determined by its morphological change. For the crab *Oss* molt staging (premolt; D) is determined as per Hosamani et al. [17]. Premolt is sub-divided into early (D_0–D_1) and late (D_2–D_4) premolt in this crab. During experimentation selected animals from each group were placed for ice anesthetization and determined the molt stages.

Mastigobranch of maxilliped 3 setal development determines the molt stages in crab. Some of the control crabs sacrificed on day 0 to determine the molt stage. The remaining were sacrificed on day10, 20, 30 and 40 along with all other groups and recorded the molt stages. Using Olympus phase contrast microscope (Model BX41TF, Japan) setal changes were determined and molt frequency was recorded.

3. Result

All the crabs of control group were in intermolt (C_4; 100%) stage throughout the experimentation. In the experimental animals of each group, molting incidence (frequency) was determined on day 10, 20, 30 and 40. Only ESX group showed mortality during experimentation. The positive control (ESX) group initiated the molt cycle on day 10, but most of them were found in intermolt (C_4; 60%), few were in early premolt (D_1; 30%) stage. The percentage of mortality in this group is 30.77 on day 10. Few crabs were found in intermolt (C_4; 10%) and majority in early premolt (D_1; 90%) in group supplemented with 10^{-9} moles MF/crab. In 10^{-8} moles MF/crab received group were observed in premolt D_1 (70%) and D_2 (30%) stage on day 10. Whereas intermolt (C_4; 40%) and premolt (D_1; 60%) were found on day 10 in 10^{-7} moles MF/crab supplemented group (Figure 1).

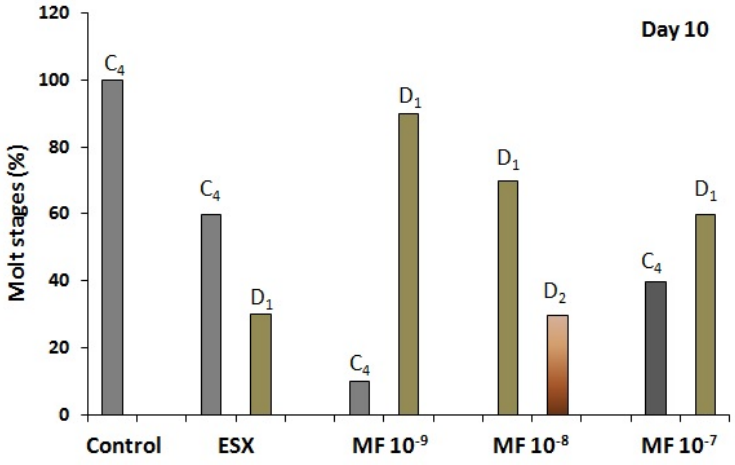

Figure 1. Effect of methyl farnesoate (MF) pellet diet on percentage of molt sub-stages at day 10 in male crab *Oziothelphusa senex senex*.

C_4: Intermolt; D_1 and D_2 premolt sub-stages; ESX: eyestalk ablated.

On day 20, besides 20% mortality, 80% crabs were found with premolt (D_1; 40% and D_2; 40%) in ESX group. Late (D_3; 50%) and middle (D_2; 50%) premolt stages were observed in 10^{-9} moles MF/crab supplemented group on day 20. Crabs received 10^{-8} moles MF/crab on day 20 recorded with 80% premolt (D_2; 30% and D_3; 50%) stages and 20% molted. Male crabs in 100% premolt (D_1; 50%, D_2; 30% and D_3; 20%) stages were recorded in 10^{-7} moles MF/crab on day 20 (Figure 2).

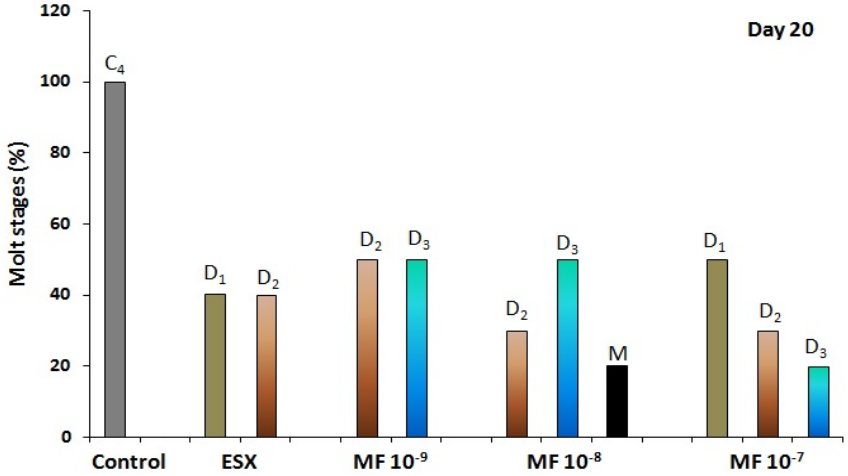

Figure 2. Effect of methyl farnesoate (MF) pellet diet on percentage of molt sub-stages at day 20 in male crab *Oziothelphusa senex senex*.
C_4: Intermolt; D_1, D_2 and D_3 premolt sub-stages; M: molted; ESX: eyestalk ablated.

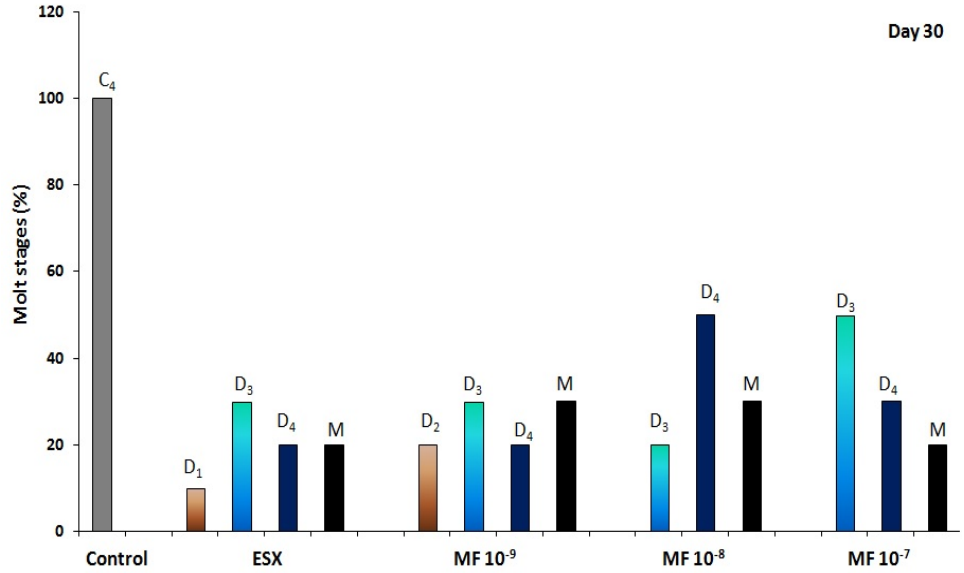

Figure 3. Effect of methyl farnesoate (MF) pellet diet on percentage of molt sub-stages at day 30 in male crab *Oziothelphusa senex senex*.
C_4: Intermolt; D_1, D_2, D_3 and D_4 premolt sub-stages; M: molted; ESX: eyestalk ablated.

ESX crabs were 66.66% in premolt (D2; 11.11%, D3; 33.33% and D4; 22.22%) stage, 22.22% molted and 11.11% died on day 30 of experimentation. In case of 10-9 moles MF/crab supplemented

group found with 70% premolt (D2; 20%, D3; 30% and D4; 20%) stages and 30% molted on day 30. About 70% were in premolt (D3; 20% and D4; 50%) stages and 30% molted on day 30 in group supplemented with 10-8 moles MF/crab. Whereas observed 80% premolt (D3; 50% and D4; 30%) and 20% molted on day 30 of experiment in 10-7 moles MF/ crab supplemented group (Figure 3).

Premolt (D_3; 37.5% and D_4; 37.5%) 75% and molted 25% were recorded in ESX group on day 40. There are 40% molted and 60% premolt (D_3; 30% and D_4; 30%) on day 40 in the 10^{-9} moles MF/crab supplemented group. In crabs supplemented with 10^{-8} moles MF/crab found 40% premolt (D_3) and 60% molted on day 40 of experiment. About 20% molted and 80% premolt (D_3; 30% and D_4; 50%) on day 40 in 10^{-7} moles MF/crab received group (Figure 4).

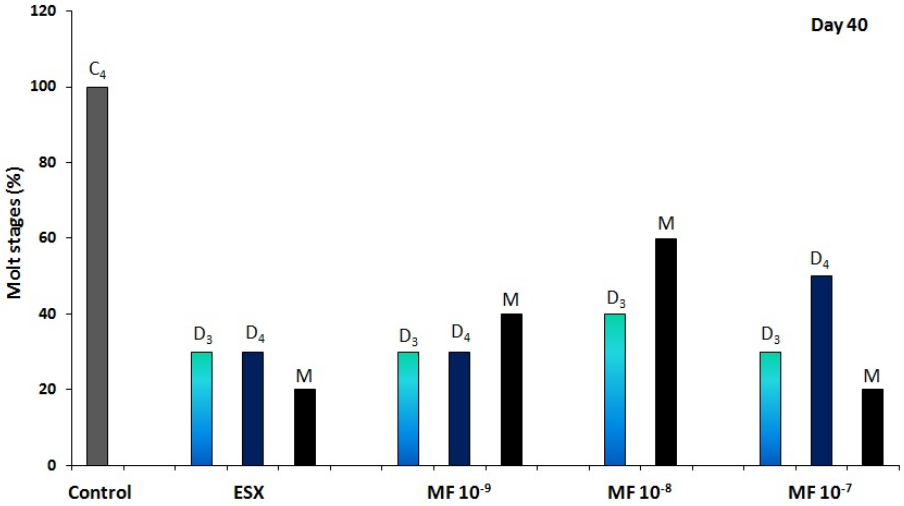

Figure 4. Effect of methyl farnesoate (MF) pellet diet on percentage of molt sub-stages at day 40 in male crab *Oziothelphusa senex senex*.
C_4: Intermolt; D_3 and D_4 premolt sub-stages; M: molted; ESX: eyestalk ablated.

The results of the present study clearly indicate that the frequency of molt as 10^{-8} moles/crab > 10^{-9} moles/crab > 10^{-7} moles/crab ≤ ESX. The highest molted group in the present study is MF 10^{-8} moles/crab with a percentage of 27.5. Whereas 17.5, 10 and 10 percent molt were recorded in 10^{-9} moles MF/crab, ESX and 10^{-7} moles MF/crab groups respectively (Table 1).

Table 1. The overall percentage of molt in eyestalk ablated (ESX) and methyl farnesoate (MF) supplemented male crabs.

S. No.	Treatment group	Number of crabs molted (n=40)	Molt percentage
1	ESX	4	10
2	MF 10^{-9} mole/crab	7	17.5
3	MF10^{-8} mole/crab	11	27.5
4	MF10^{-7} mole/crab	4	10

4. Discussion

Molting, a process of increasing the size of crustacean body under the control of several internal and external factors. Major growth controlling factors are endocrine hormones like MIH, MOIH, ecdysteroids, MF etc. Elimination of growth inhibitory principles promotes molting process. Removal

of eyestalk eliminates MIH and MOIH and induces molting process. Moreover, external administration of growth promoters like MF, ecdysteroids etc., induces molt cycle [17].

The regulation of molting by removal of eyestalk is well defined in many studies. Several crustacean species like *Aphanomyces astacus* [18], *Procambarus clarkii* [19], *L. emarginata* [20], *Macrobrachium rosenbergii* [21] and *Scylla serrate* (*S. serrata*) [22] were tested for molt induction by ESX. These studies agree with the present study where molt induction has been done through ESX in crab *Oss*. ESX has its own limitations including loss of hemolymph, elimination of other physiological elements and is reported in *Panulirus argus* [23]. Though ESX inducing molting, but about 17.5% crabs were died in the present experiment. It is suggested that ESX is not a preferable method for growth induction in cultured crustaceans.

The minimum effective dietary supplementation dose of MF on molt induction in male crab *Oss* at semi-controlled pond conditions was determined in the present study. The dietary MF levels tested are 10^{-7}, 10^{-8} and 10^{-9} moles/crab and are shown significant effect in inducing molt cycle in male crabs. Out of three concentrations tested minimum effective feed supplementary dose identified in the study is 10^{-8} moles MF/crab. Similar study was done with female *Oss* in this lab and the results are identical one to other [24], but it is found that the MF 10^{-8} moles/crab is most effective in case of males than females. This might be due to the synchronous effects of MF on both molting and reproduction especially in female crustaceans [9]. Many studies are focused to test the molt induction role of MF in several crustacean species. Injection of MF induced molt in male and female crab *Oss* were reported from our laboratory [4]. It is found that the MF administration reduced the duration of molt cycle to 14 days. Similarly, crustaceans like *Cancer magister* [16] , *Homarus americanus* [25], *L. emarginata* [20], *Cherax quadrcarinatus* [26], *Metapenaeus ensis* [27], *Scylla olivacea* [3], *Chionectes opolio* [28], *Litopenaeus vannamei* [29], *Portunus trituberculatus* [30], *S. serrate* [31], *Neocaridina denticulate* [32] and *Travancoriana schirnerae* [33] were tested and reported with MF induced growth at laboratory conditions.

A few studies proved the molt induction capacities of MF in cultured crustaceans. Though the high molt frequency observed by MF injection, it is not materialized at the field level. The present investigation clearly indicates that 10^{-8} moles MF/crab feed supplementation in male crabs increased molt cycle frequency. In crustaceans it is found that MF may induce molt directly or indirectly be inducing molting hormone (ecdysteroids) from Y-organs or by both. It is also clear from the present study that MF (27.5%) induced molt is much effective than ESX (10%). Moreover, the duration of molt cycle is significantly reduced in MF (10^{-8} moles/crab; 12-14 days) supplemented group than ESX (25-28 days). This study is the first report showing the growth induction of MF through feed supplementation and promotes the usage of MF at the farm level.

In conclusion, this study provides a base to induce molt cycle of a cultured crustacea through MF containing pelted feed supplementation. This application of MF may be effective to reduce molt cycle duration and increases the molt frequency in crustaceans other than *Oss*. This ultimately decreases the duration of crop with increased amount of crustacean protein, which improves economic status of farmers and country. Nevertheless, some more field studies are needed to confirm the field level application effectively.

Acknowledgements
The author thankful to University Grants Commission, New Delhi, India for granting financial support in the form of research grant [F.No. 41-582/2012 (SR)] to Dr. P. Ramachandra Reddy.

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