J. Fish. Sci. Tech. 1(2), 269~275, 1998

Effects of Eyestalk Ablation on the Embryogenesis of Spider Crab, *Libinia emarginata*

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(Received April 1998, Accepted December 1998)

Spider crabs, *Libinia emarginata*, were eyestalk-ablated unilaterally and bilaterally to manipulate endogenous methyl farnesoate (MF) to increase during the embryogenesis. Endogenous MF were measured weekly over the embryogenesis of the crab, using HPLC with the aids of GC/MS and MS database (CAS 010485-70-8) for the identification of the hormone.

Initial MF titers both in the hemolymph and embryos of intact control were at bottom levels and the hormone concentrations kept unchanged ($p<0.05$), reflecting physiological unnecessaries of the hormone in the embryogenesis. Eyestalk ablation significantly stimulated the crabs to increase endogenous MF in both tissues ($p<0.01$). In the response of the embryos to the increased MF, no growth stimulations were observed, at least, in the first part of embryogenesis. The increased mortalities and immature sheddings of embryos resulted from the crabs under the influence of elevated MF in both tissues, instead, suggesting that the elevated MF against the crab's requirement blocked the normal developmental process of the crab embryos.

These data can give crustacean endocrinologists some insights to understand the effects of the hormone on the crustacean reproduction studied previously in which JH analogs ambiguously affected the crustacean reproduction depending on the reproductive stages. The data also can give shrimp aquaculturists some implication of a possible generation of unfavorable shrimp seeds attributed to elevated egg MF originated from their eyestalk-ablated mother shrimp.

Key words: crab (*Libinia emarginata*), embryogenesis, eyestalk ablation, methyl farnesoate

Introduction

The process of eyestalk ablation has been used in almost every crustacean maturation/reproduction facility to stimulate the animals to develop their gonads both in research and commercial purposes (Bray and Lawrence, 1992). The relationship between eyestalk ablation and ensuing gonadal development was first discovered by Panouse (1943) in the shrimp *Palaemon serratus*. This and subsequent findings (Brown and Jones, 1949; Borst et al., 1987) suggested that reproduction in crustaceans is under inhibitory control by neurosecretory factors in the eyestalks. Eyestalk ablation causes hypertrophy and changes in the ultrastructure of mandibular organ (MO) (Hinsch, 1977; LeRoux, 1983), further suggesting that MO is inhibited by one of eyestalk factors, known as MO-inhibiting hormone (Laufer et al., 1987a). MO synthesizes methyl farnesoate (MF), a crustacean analog to insect juvenile hormone (JH) (Laufer et al., 1987a).

Juvenile hormone, a family of epoxidated sesquiterpenoids, has been captivated by insect endocrinolgists because of its diverse endocrine functions since the first form of the family, JH I (methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate), was isolated by Roller et al. (1967) from the silk moth, *Hyalophora cecropia*. First identified and named for its regulation of metamorphosis, the JH I together with other forms of JHs found lately in insects and crustaceans (Fig. 1) is now known to have a wide variety of other regulatory roles including development and reproduction (Engelmann, 1983; Watson et al., 1985; Laufer et al., 1993; Riddiford,

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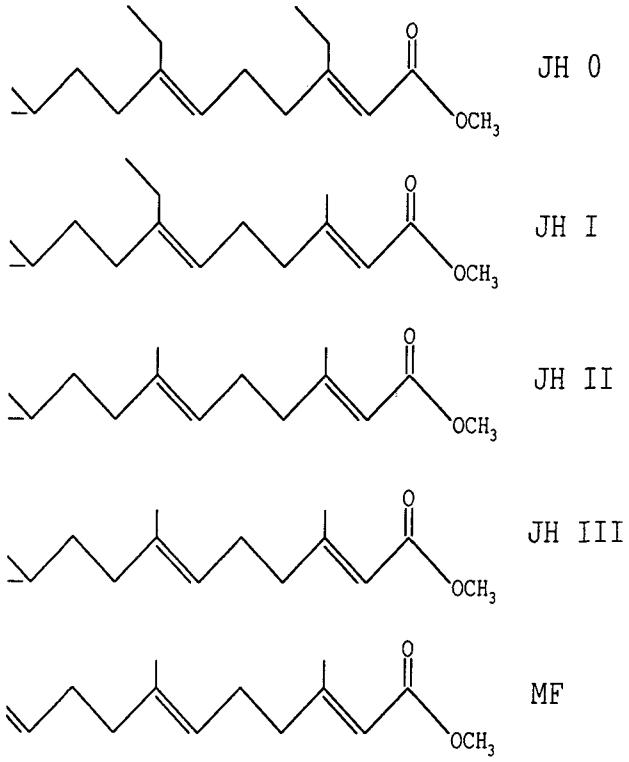

Fig. 1. structures of juvenile hormone (JH) family found in insects and crustaceans. JH 0 to III are found in insect and methyl farnesoate, an unepoxidated form of JH III, is a crustacean JH.

1994, 1996). Crustacean JH, methyl farnesoate, was first identified by Laufer et al. (1987a) from spider crab *Libinia emarginata*. This unepoxidated form of JH III is also known to regulate many crucial physiological processes such as development, morphology and reproduction in crustaceans (Laufer et al., 1987a, b; Borst et al., 1987; Laufer et al., 1993).

In spite of a sheer number of studies which explain pivotal roles of JH in the physiological processes, some of this hormonal roles in reprodction are still in ambiguity. For example, injection of JH I into the amphipod *Orchestia gammarellus* inhibited vitellogenesis (Charniaux-Cotton, 1974). Treatment of female *Libinia emarginata* with JH analogues inhibited ovarian development and had deteriorative effects on oocyte morphology (Hinsch, 1981). These negative effects of JH analogues on crustacean reproduction were also found in the studies of Payen and Costlow (1977) and Templeton and Laufer (1983) when the crustaceans were totally exposed to the juvenile hormone analogs (JHs). In the previous study, we found that MF stimulated vitellogenesis of female *L. emarginata*, but the time regime the elevated MF positively affected the process was within a narrow window just around the time when marked gonado-somatic index (GSI) increase was initiated (Jo et al., 1999). The increased MF beyond the window inhibited normal growth of the oocytes in vitellogenesis, suggesting that the oocytes respond to the hormone stage-specifically.

Eyestalk ablation has been routinely used for sexual maturation in captivity. However, the negative effects of eyestalk ablation on the shrimp seed production have also been considered (Bray and Lawrence, 1992). Recently, shrimp seeds from eyestalk-ablated spawners are more liable to be affected by diseases than those from wild spawners (personal communication). Crustaceans treated with exogenous MF timely bring precocious gonadal growth in captivity. Elevated MF caused by eyestalk ablation also introduces precocious maturation. However, the effects of the eyestalk ablation-induced MF elevation after the hormone is no longer required remain unresolved.

In an attempt to understand the MF influence on embryogenesis, we determined modes of MF titers in the hemolymph and embryos from the commencement of embryogenesis of female spider crabs. Eyestalks of the crabs were also ablated to study how the ablations stimulate MF production and how the manipulated MF affects the embryo growth.

Materials and Methods

1. *Crabs*

In the spawning season of spider crabs, *Libinia emarginata*, about 120 healthy female crabs obtained from the coast of Connecticut, Connecticut, USA, were selectively collected into the indoor rectangular tanks. Of them 107 animals with just spawned embryos in their abdomens were finally selected: 10 for initial sacrifice, 30 for intact control, 32 for unilateral eyestalk ablations (UEA), and 35 for bilateral eyestalk ablations (BEA).

2. *Eyestalk ablations and culture of crabs*

Stalked eyes were unilaterally and bilaterally ablated and cauterized as was in our previous study (Jo et al., 1999). Animal room was controlled by temperature (20 ± 1℃) and photoperiod (14L:10D). Rearing seawater was artificially prepared and managed in terms of salinity, nitrogen compounds, and pH throughout the animal culture. Twenty

two crabs died: throughout the experiment : 3 from control, 7 from UEA, and 12 from BEA.

3. *Purification of MF*

Ten crabs were sacrificed as an intial control. Thereafter 24 animals, 8 from each group, were weekly sacrificed for the purification and quantification of MF. The protocols used for MF purification and quantification in the hemolymph and embryos were just the same as described by Jo et al. (1999). These protocol was modifiedly prepared from that for hemolymph MF (Homola et al., 1991; Sagi 1993; Ahl et al., 1996). In brief, the preserved samples in acetonitrile and NaCl were first centrifuged at about 900 g for 15 min with the addition of 1 mℓ hexane and 15 μℓ *cis-trans* MF (10 ng/10 mℓ hexane), and only two thirds of the supernatant were collected. For the second extraction 0.5 mℓ of hexane was added and then two thirds of hexane phase were collected after centrifugation. The pooled MF hexane solution from the first and second extractions was concentrated into 400 μℓ for duplicate HPLC run with the addition of 1% ethyl ether solution. For the purification of embryo MF, the vortexed samples were extracted twice in the more amount of internal standard, 20 μℓ MF isomer (10 ng *cis-trans* MF/10 μℓ hexane) and longer centrifuge at about 900 g for 20 min.

Results

1. *Purification and quantification of MF*

Mass spectra of two known MF isomers (*cis-trans* and *trans-trans* MF) and biologically active MF from sample were compared (Fig. 2). The ion fragments were further compared with those from MS database for MF (CAS 010485-70-8). All MF spectra from different sources had common ions at *m/z* of 69, 108, 114, 121, 147, 149, 175, 207, and 250. When an ion at *m/z* 69 was designated as base peak, the peak with the highest abundancy was ion at *m/z* 121 in the spectra analyzed while it was the ion at *m/z* 114 in the MS database. Molecular ion was observed at *m/z* 250, indicating the molecular weight of the hormone.

2. *MF concentrations in the hemolymph and embryos*

The mean MF concentrations (± SE) in the hemolymph and embryos were shown in the three-grouped crabs of intact control, UEA, and BEA

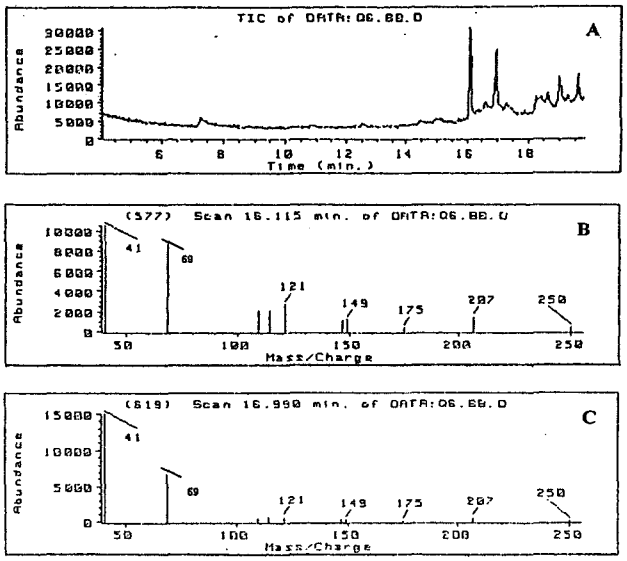

Fig. 2. GC chromatograms for *cis-trans* (internal standard) and *trans-trans* (sample) methyl farnesoate, where the peaks at 16.2 and 16.9 minutes represent cis-trans and *trans-trans* MF, respectively (A). Injected is 5 μg of extracted methyl farnesoate (400 μg) in which 15 μg (ng/mℓ MF) of internal standard is included in the total of 1 g ovary sample. Mass spectra of *cis-trans* (B) and *trans-trans* (C) methyl farnesoate. Both spectra have common ions at *m/z* 69 (base ion), 108, 114, 114, 121, 147, 175, 207, and 250 (molecular ion).

(Figs. 3 and 4). The eyestalk-ablated animals had significantly higher MF levels ($p<0.01$) compared with intact animals. In comparison between two eyestalk-ablated groups (UEA and BEA), more significant MO releases of MF into hemolymph were observed in BEA than in UEA ($p<0.01$). In the embryo MF, BEA levels in the first two weeks were higher than UEA ($p<0.05$), while they equiled UEA in the last week.

3. *MF concentration of intact control*

MF titers in the hemolymph and embryos were represented during the embryogenesis of the control crabs (Fig. 5). Hemolymph MF concentrations in mean (± SE) remained low but showed a decreasing trend as the time proceeded from initial 0.43 ng/mℓ to 0.36 ng/mℓ by the 3rd week. There were no two consecutive concentrations which were characterized by significant changes in the hemolymph MF. The embryo MF concentrations, however, fluctuated ($p\leq0.05$) from the maximum, 0.45 ng/g by the first week to minimum, 0.28 ng/g by the last week. Although the concentrations varied

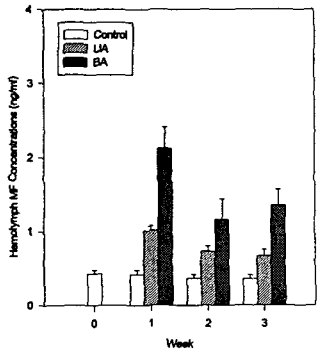

Fig. 3. Methyl farnesoate titers in the hemolymph of control, UEA, and BEA during the embryogenesis of spider crab, *L. emarginata*, where UEA and BEA mean unilateral and bilateral eyestalk-ablated crabs, respectively.

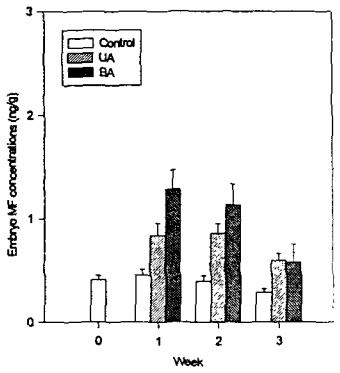

Fig. 4. Methyl farnesoate titers in embryos of control, UEA and BEA during the embryogenesis of spider crabs.

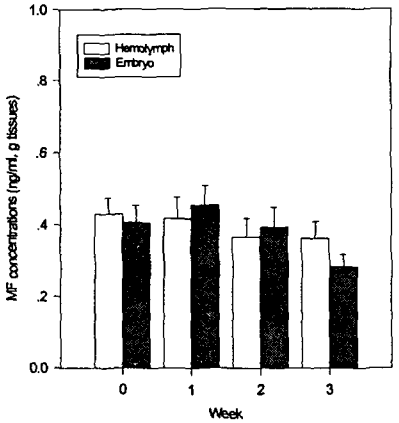

Fig. 5. Changes of MF titerss in relation to embryogenesis progress in the intact control of spider crabs. MF titers from the two tissues, hemolymph and embryos, were compared.

significantly it also remained low for the three weeks.

4. MF concentrations in UEA and BEA

UEA and BEA significantly stimulated MO release of MF into the hemolymph and embryos ($p<0.01$) compared to intact control (Figs. 6 and 7). In the stimulatory effects of the two eyestalk ablations, BA had higher effects than UEA both in the hemolymph and embryos except for just a case on embryo MF by the last week, where the MF concentrations in BA and UEA were competing. Although, in general, BEA had higher MF concentration than UEA, it was also characterized by higher SE. The average SE of BEA was 0.2230 while that of UEA was 0.0811 (Data, not shown).

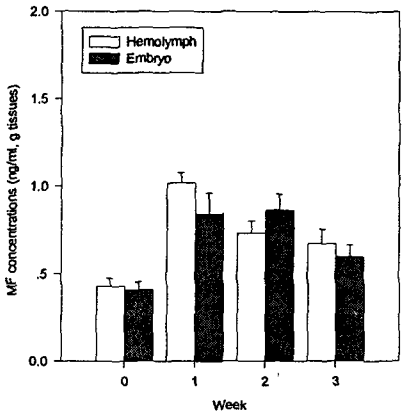

Fig. 6. MF concentrations of UEA during embryogenesis of spider crabs. MF titers from hemolymph and embryos were weekly compared.

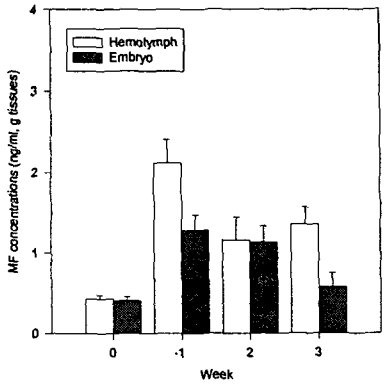

Fig. 7. MF concentrations of BA in the embryogenesis of spider crabs. The two MF titers in the hemolymph and embryos were weekly compared in the process.

5. Effects of elevated MF on embryogenesis

In control, normal embryonic development proceeded although delayed progress was observed, particularly in the late developmental stages (Data, not shown). In the comparison of the embryonic development and the modes of MF levels in control, no relativeness was noted. When MF concentrations were elevated during the same period of embryonic development, the MF blocked normal embryonic development in concentration-dependent manner. The abnormal development was represented by detaching of immature embryos (Table 1).

Discussion

In the present study, embryos of intact control failed to reach to maturity in captivity. However, normal embryonic development continued at least for 10 days although, thereafter, delayed developmental progress was noted. In the 10 days of normal embryonic development MF titers were low and remained unchanged (Figs. 3 and 4), reflecting that the control crabs do not require the elevated MF for the process. UEA and BEA significantly stimulated the crabs to increase their MF titers both in the hemolymph and embryos (Figs. 3 and 4). The MF elevations in the eyestalk-ablated crabs continued over the experiment and in some case, hemolymph MF elevation was much significant than embryo MF (Figs. 5 and 6). The elevated MF by eyestalk ablation caused immature shedding and high mortalities (Tables 1 and 2).

Studies on endogenous MF effects on crustacean embryogenesis are lacking. In the studies of JH analog effects on crustacean reproduction, many JH analogs interfered with the normal egg development (Hinsch, 1981; Templeton and Laufer, 1983). However, there have been much more studies on this subject in insects from which an insight into MF role in crustacean embryogenesis can be given. In the early studies on JH roles in insect embryogenesis, Slama and Williams (1966) observed that a JH analog blocked embryonic development in *Pyrrhocoris apterus*. Later studies on the corpopus allatum synthesis of JH and on the effects of exogenous JH in the embryogenesis, however, confirmed that JH plays an important regulatory role in the embryogenesis, as do MF and JH in the vitellogenesis (Laufer et al., 1993; Homola and Chang, 1997; Riddiford, 1996; Wyatt and Davery, 1996). In the embryos of *Nauphoeta cinerea* the patterns of JH concentration were characterized by two conspicuous peaks at days 21 and 33 of embryonic development (Lanzrein, 1984). Similar observations were made in the insect embryogenesis (Lageux et al., 1979; Bergot et al., 1981; Burgin and Lanzrein, 1988). In their studies, JH concentrations were low or undetectable in the early part of embryogenesis but were elevated just before deposition of embryonic cuticles. Lowered concentrations of crustacean MF, a counterpart of insect JH, thus, appeared to be attributed to the physiological unrequirement of the hormone by the embryos for normal development at least in the early part of the embryogenesis of the crabs.

Eyestalk-ablated *L. emarginata* induced the crabs to increase MF concentration both in the

Table 2. Number of *L. emarginata* dead during the experiment

Animal Group	Week			
	0	1	2	3
Control	0 (30)	0 (22)〈8〉	1 (13)〈8〉	2 (3)〈8〉
UEA	0 (32)	3 (21)〈8〉	2 (11)〈8〉	2 (1)〈8〉
BEA	0 (35)	5 (22)〈8〉	3 (11)〈8〉	4 (0)〈7〉

( ) indicates number of animal survived
〈 〉 indicates number of animal sacrificed for MF and embryo studies.

Table 1. Accumulated number of *L. emarginata* with immature embryos detached off during the experiment

Animal Group	Days										
	0	2	4	6	8	10	12	14	16	18	20
Control	0 (30)	0 (30)	0 (30)	0 (30)	0 (22)	0 (21)	0 (21)	0 (13)	1 (12)	2 (12)	5 (11)
*UEA	0 (32)	0 (30)	0 (29)	0 (29)	0 (21)	1 (20)	3 (19)	7 (11)	11 (11)	–	–
*BEA	0 (35)	0 (32)	0 (31)	0 (30)	3 (22)	7 (21)	10 (20)	–	–	–	–

( ) means number of animal survived.
*UEA and BEA stand for unilateral eyestalk ablation and bilateral eyestalk ablation, respectively.

hemolymph and embryos (Figs. 6 and 7). The spider crabs with elevated MF by eyestalk ablations blocked progress of embryonic development by means of detaching dead embryos from day 8 in concentration-dependent manner (Figs. 5, 6 and 7 and Table 1). In 8 days of the experiment, no control crabs in which embryo MF concentrations were low showed detaching activities of dead embryos. Thus, although we could not make it sure when the deleterious effects of elevated MF on the embryo growth of the crabs started, it appeared that the lowered MF in the early stages of embryogenesis was critical to normal embryonic life.

Concerning the effects of JH on the embryo growth, the action mode of the hormone is also believed to be stage-specific. JH treatment of adult females and newly oviposited eggs of insects, *Hyalophora cecropia* and *Antheraea pernyyi*, for example, blocked embryonic development shortly after germ band formation (Riddiford and Williams, 1967; Riddiford, 1970). Hoffmann and Lagueux (19 85) did similar experiment in a number of other insects to get the same results. In their studies, higher JH esterase activities were observed in the early embryogenesis. This elevated enzyme might play a functional role in maintaining lowed JH concentrations early in the embryogenesis, necessary for normal embryo growth of the insects.

These data explain that a crustacean JH, believed to play a regulatory role in some period of embryogenesis, does not have biological activity for normal embryo growth, at least in the first half of the process in spider crabs. In case the hormone is endogenously elevated in the period, it seems to block the normal embryonic growth in concentration-dependent manner. In the sense of crustacean aquaculture, these results have some implications for crustacean aquaculturists who routinely ablate eyestalks for sexual maturation in captivity.

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