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FEEDBACK REGULATION OF METHYL FARNESOATE SYNTHESIS BY MANDIBULAR ORGANS OF THE CRAYFISH

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ABSTRACT

The effect of different concentrations of methyl farnesoate (MF), a compound with juvenile hormone activity found in crustacea, was investigated on the regulation of MF synthesis by the crayfish mandibular organ (MO) *in vitro*. Nonvitellogenic animals have small ovaries with 0.12 gonad somatic index (GSI), MF in the blood is low at 0.30ng/ml and MF synthesis by MOs was low at 8.5ng/hr. Vitellogenic animals have 10 times larger ovaries, 3 times more MF in the blood and 2 times more MF synthesis by MOs than nonvitellogenic animals. In controls the right and left paired glands (MOs) have the same MF synthesis rates. A 1ng/ml of MF added to the media increased MF synthesis by 70% over controls in MOs from nonvitellogenic animals. This concentration did not alter MF synthesis in MOs from vitellogenic animals. A 20ng/ml concentration of MF added to the media of MOs from vitellogenic animals significantly decreased MF synthesis by 21%. A 20ng/ml MF in the media did not alter the MF synthesis by MOs from nonvitellogenic animals. These results indicate that in nonvitellogenic animals, MF synthesis by MOs is increased by low levels of MF while in MOs from vitellogenic animals at higher MF concentrations MF synthesis is decreased *in vitro*. These data suggest that MF levels in the hemolymph may play in regulating MF synthesis *in vitro* both in a positive direction in early vitellogenic and a negative direction in later vitellogenic stages, possibly by feedback. Such feedback control may also function *in vivo*.

KEYWORDS: Crayfish, Methyl Farnesoate, *In Vivo* Feedback, Mandibular Organ

INTRODUCTION

The mandibular organ (MO) of crustacea is a small ductless gland associated with the mandibular tendon which produces methyl farnesoate (MF)[1]. MF is the unepoxidated form of the insect juvenile hormone, JH III. In insects, juvenile hormone plays several regulatory roles both as gonadotropins and morphogens[2]. In crustacea, MF is thought to play a role in morphogenesis [3, 4], reproduction[5,6,7], larval development[8], osmoregulation[9] and molting[7,10,11] in a manner similar to insect JH.

Knowledge is accumulating about the regulation of MF synthesis by the MO. Synthesis of MF is inhibited by a peptide, mandibular organ inhibiting hormone (MOIH), secreted from the sinus gland X-organ complex in the eyestalks[11,12,13,14,15,16,17]. Eyestalk ablation increases biosynthesis of MF and is accompanied by a three fold increase in MO protein content in crayfish *Procambarus clarkii*[17]. *Carcinus maenas* hemolymph levels of MF increase in response to osmotic stress[9]. In *Libinia emarginata*, variations of MF concentration in the haemolynph and its synthesis by the MO depends on developmental stage, gender, and sexual maturity. Juvenile and non-reproductive females have the lowest levels of MF in the haemolymph compared to reproductive animals[1,6] In females the production of MF by MOs increases during vitellogenesis, then decreases to previtellogenic level when oocytes mature[1]. In *Oziotelphusa senex senex* the *in vivo*

MF levels have been correlated with ovarian maturation and molting [7]. In addition to this, MF levels may also vary, depending on color, behavior, and stress[10]. In *Libinia emarginata* degradative activity of the MF varies seasonally and according to the reproductive state of the animal[18]. The degradation of MF is attributable to specific MF esterase(s)[18,19]. The half-life of MF was estimated to be about 30 minutes with the hepatopancreas(HP) being most active in deesterification.

In addition to the factors mentioned above regulating MF, there may be others. These may include uptake and sequestration by cells and tissues, metabolic alteration, and excretion. All of these suggest possible additional regulation of this compound by various mechanisms.

In the present study we investigate the effect of MF as a possible regulatory molecule. We test two different concentrations of MF on the regulation of MF synthesis by MOs *in vitro* from crayfish of two different stages of ovarian development. The data suggest one positive and one negative feedback regulation of MF synthesis by MOs in culture.

MATERIALS AND METHODS

Animals and Maintenance

Red swamp crayfish *Procambarus clarkii* were obtained from Dr. Robert Romaire at the Louisiana State University. Females were separated from males and they were acclimatized and maintained in recirculating freshwater at 18°C in a living stream tank equipped with a biofilter and were feed with commercial shrimp pellets (Rangen Inc., Buhl, Idaho-83316, USA) *ad libitum*.

Selection of Groups

Animals were categorize into two groups at the time of dissection on the basis of color and development of the ovary. One group was established having white-yellowish, small previtellogenic oocytes and small ovaries and the other group had brown larger vitellogenic oocytes, and larger ovaries.

Gonadosomatic Indices

Ovarian indices were determined to indicate the reproductive condition of the animals. The gonadosomatic index (GSI) was calculated by determining the goand weight as a percentage of the total live weight of the animals as was done by others [20].

Hemolymph Titers of MF

The MF concentration in the hemolymph was determined according to the method developed by Laufer *et al.*,[1]. Hemolymph samples (1ml) from each animal collected in new 15ml Kimex culture tubes with Teflon lined caps containing 5ml of ice cold acetonitrile and 2ml 4% NaCl. Ten ng of cis-trans (non-biological) isomer of MF was added to each tube as an internal standard. Samples were extracted twice with 0.5ml hexane. Aliquots of the hexane extract were analyzed on a Waters HPLC system with model 510 pumps and Milipore model1386 UV absorbance detector set at 215nm using a 5µm silica particle column,(20 cm length) (Rainin, Inc., catalog no.86-100-C5). The running solvent was 1% diethyl ether in hexane flowing at 1.5ml/min, at a pressure of 1800psi. The chromatorgrams were analyzed with maxima software. The all-trans MF present in a sample was estimated by comparing the peak area with the internal standard cis-trans isomer peak.

Mandibular Organ Culture

Controls

Paired MOs were dissected out and incubated as described by Laufer *et al.*,[1], in 400µl medium consisting of a freshwater crustacean saline[21], containing 1mci/25ml [methyl-3H] methionine for four hours. The experiments were conducted on 10 and 13 pairs of mandibular organs from animals with white and brown ovaries respectively to establish basal synthesis rates in culture and to determine wheather glands from the right and left sides of the animals synthesized MF symmetrically.

Exp-I (1ng/ml MF Added to the Culture Media)

Paired MOs were dissected out and incubated, either in 1ngMF/ml while its partner was incubated with no MF in 400 µl of medium as in the untreated group described above. This experiment was conducted on 22 and 11 pairs of mandibular organs from animals with white and brown ovaries respectively.

Exp. II (20ng/ml MF Added to the Culture Media)

Paired MOs were dissected out and incubated either in 20ng/ml MF or the other gland with no MF in 400 µl medium for four hours. The experiment was conducted on 18 and 14 pairs of mandibular organs from animals with white and brown ovaries respectively.

Following the incubation, the cells were fixed with ethanol, homogenized and extracted with 1ml hexane. The activity in a 100 µl hexane aliquot was quantitated using liquid scintillation spectrometry. The rate of MF synthesis (ng/hour) was determined from DPM.

RESULTS

Nonvitellogenic animals have ovaries that appear to be white to yellowish color and that are relatively small, with a GSI = 0.12. The MF concentration in the hemolymph of the animals was low at 0. 30 ng/ml (Table 1). Vitellogenic animals have ovaries that appear to be brown in color and about 10 times larger than the nonvitellogenic ovaries with GSI = 1.16. The MF concentration in the blood of vitellogenic animals was about 3 time higher 0.93 ng/ml (Table 1) than in the nonvitellogenic animals.

MF synthesis by the MOs: It was determined that untreated right and left MOs from an individual organism have nearly identical MF synthetic activity (Table 2).

While the rate of MF synthesis by MOs in vitellogenic animals was about 2 time higher than nonvitellogenic animals (Table -2). The MF synthesis by left and right glands from nonvitellogenic and vitellogenic animals was 8.64ng/hr, 8.38ng/hr and 17.14ng/hr, 16.96ng/hr respectively (Table 2).

Experiment I (1 ng/ml MF Added to the Culture Media)

In MOs from nonvitellogenic animals, the MF synthesis (ng/hr) by untreated left and treated (1ng/ml) right glands were 12.9 and 19.8 respectively (Table 2). This increase of MF in the media was statistically significant ($p \leq 0.05$) by Student's *t*-test.

In vitellogenic animals whose basal level of MF synthesis was higher than that from nonvitellogenic animals the MF synthesis (ng/ml) by MOs were decreased (Table 3) when 1ng/ml MF was added to the culture media. This is not a

significant change.

Experiment II (20ng/ml MF Added to the Culture Media)

In MOs from vitellogenic animals, the MF synthesis (ng/ml) by untreated left and treated (20ng/ml) right glands were 22.2 and 18.9 respectively (Table 2). This result was statistically significant at $p \leq 0.05$ level by the Student's *t*-test.

In MOs from nonvitellogenic animals the MF synthesis (ng/hr) by glands that were treated with 20ng/ml MF decreased by 1.1ng, a change which was not significant.

In the control, the rate of MF synthesis from untreated pairs of MOs approached 100% (Table 3). In subsequent trials the contralateral gland was used to establish the experimental synthetic activity between control and experimental glands. The low MF (1ng/ml) treatment yielded an increase of MF synthesis which was 171% compared to 100% of controls (table-3) in nonvitellogenic animals, and the higher MF content of the media which was treated yielded an average of 81.3% of MF synthesis compared to the controls (Table 3) in vitellogenic animals. These results using Student's *t*-test were significant at the 0.05 level.

DISCUSSIONS

We have examined MOs at two stages of vitellogenesis from *Procambarus clarkii*, one pre-vitellogenic and the other early vitellogenic. The early vitellogenic population we examined had GSI =10x, MF concentration in the blood = 3x, and ovaries which were 10 times larger in comparison to nonvitellogenic ones. These facts are all in agreement with the earlier findings of Laufer *et al*.,[1,5], and Sagi *et al*., [6] .

It was demonstrated that several isoforms of MO-IH members of the crustacean hyperglycemic hormone (CHH) family inhibit MF synthesis by MOs [11,12, 13, 14,15,16,22]. In females the production of MF increases during vitellogenesis, then decreases to previtellogenic levels when oocytes mature[1]. MF degradation is attributable to specific MF esterase(s)[18]. The half life of MF was estimated to be about 30 minutes in the presence of hepatopancreas. Changes in the concentration of MF in hemolymph appears to be associated with vitellogenesis, in addition, decreased activity of MF is associated with seasonal changes.

Our results indicate that right and left MOs are equal in their MF synthesis rates in both stages of ovarian development. Tobe *et al*.,[23] found an asymmetry in MF synthesis in MOs in his earlier study.

The present results indicate that MF synthesis by the MO can be influenced *in vitro* by MF in the medium. This is different in animals in different states of vitellogenesis. These results indicate that in nonvitellogenic animals, with 0.3ngMF/ml in their hemolymph, MF synthesis by MOs are increased by 1ngMF/ml while in vitellogenic animals, with 0.9ngMF/ml in hemolymph MF synthesis by MOs is decreased by 21% with 20ngMF/ml. The possible explanation of these results is that the physiological role of MF in regulating MF synthesis by MOs may be by feedback regulation *in vitro*. To the best of our knowledge this is the first report of this kind of regulation in a crustacean.

The *in vitro* results suggest that possibly, the low MF level in the hemolymph during nonvitellogenic conditions trigger an increase of MF synthesis by the MOs by a positive feedback, while in animals with higher MF levels in the hemolymph during vitellogenic stages trigger a decrease of Mf synthesis by the MOs by a negative feedback. Such feedback mechanisms may be very important for regulating reproduction.

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APPENDICES

Table 1: Gonadosomatic Index and Mf Concentration in the Hemolymph of Red Swamp Crayfish *Procambarus Clarkii*

Ovary Color	N	GSI	N	MF in Hemolymph (ng/ml)
White (Std Err)	40	0.12 ±0.01	28	0.30 ±0.05
Brown (Std Err)	28	1.16* ±0.23	13	0.93* ±0.10

* = Significant at 0.05 level

Table 2: Mf Synthesis of MOs of Animals with Two Different Ovarian Stages Treated With Mf in Culture Mf Synthesis (Ng/Hr)

Ovary Color	Untreated			MF Treated					
				(1ng/ml)			(20ng/ml)		
	N	l	r	N	ul	tr	N	ul	tr
White (Std Err)	10	8.6 ±4.98	8.4 ±4.60	22	12.9 ±1.99	19.8* ±2.87	18	13.7 ±1.95	12.6 ±1.82
Brown (Std Err)	13	17.1 ±3.89	17.0 ±4.14	11	20.2 ±5.47	14.7 ±3.19	14	22.2 ±3.94	18.9* ±4.05

1= left gland, r=right gland, ul= untreated left, tr= treated right

*= Significant at 0.05 level

Table 3: % Mf Synthesis and %Change of Mf Synthesis by MOs Treated With 1ng/Ml or 20ng/Ml Mf in Culture

Ovary Color	Untreated		MF Treated					
			(1ng/ml)			(20ng/ml)		
	N	% (ng/hr)	N	% (ng/hr)	Change (% ng/hr, Treated VS Untreated)	N	% (ng/hr)	Change (%ng/hr, Treated VS Untreated)
White	10	101.19	22	171.5	+69.6*	18	94.8	-7.08
Brown	13	102.6	11	93.5	-9.1	14	81.3	-21.3*

*Significant at 0.05 level