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Relationship of claw form and exoskeleton condition to reproductive system size and methyl farnesoate in the male spider crab, *Libinia emarginata*

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Summary

Methyl farnesoate (MF) expression and reproductive system size were compared in five representative groups of male *L. emarginata* selected from a sample collected in November. The groups differed from each other with respect to carapace size (small, intermediate and large), relative propodus size (small and large claw forms), and condition of the exoskeleton (abraded and unabraded). Large males with large claws and abraded exoskeletons had reproductive system indices which were significantly larger than any other group. The mandibular organs of these crabs also had significantly higher rates of methyl farnesoate synthesis *in vitro*. Hemolymph titers of methyl farnesoate were also highest in this group, but were not significantly different from the group with small carapaces, small claws and unabraded exoskeletons. Methyl farnesoate titers were significantly lower in all other groups of unabraded animals with small or large claws. These results suggest that methyl farnesoate may play a role in morphogenesis and reproduction in male *L. emarginata*.

Keywords: morphogenesis, methyl farnesoate, male reproduction, Majidae

Introduction

Male spider crabs (Majidae) exhibit marked differences in relative claw size during post-larval development. The phenomenon of differential growth, known as allometry, is typically described by regression analysis of log-transformed variables. Regression lines differing in slope or intercept indicate changes in growth patterns which occur during development (Tessier, 1960). Using these criteria, Hartnoll (1963) described three forms of male spider crabs based on propodus and carapace sizes which he termed "immature', 'pre-pubescent' and 'mature'. Similar forms were observed in *Libinia emarginata* by Aldrich (1974). Although the differentiation of the claws in male spider crabs may correspond to changes in reproductive status, the relationship of relative claw size to gonadal development and mating behavior has not been described in detail.

Abbreviations:

MF = methyl farnesoate
LLA = large carapace, large claw, abraded exoskeleton

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LL = large carapace, large claw, unabraded exoskeleton
IL = intermediate carapace, large claw, unabraded exoskeleton
IS = intermediate carapace, small claw, unabraded exoskeleton
SS = small carapace, small claw, unabraded exoskeleton

The onset of gonadal maturation in spider crabs may vary, occurring either in the pre-pubescent or mature form, depending on the species (Hartnoll, 1963). However, the criteria by which mature and immature gonads are distinguished is not always clear. Small, 'immature' *L. emarginata* (19 mm carapace length) have sperm which are morphologically indistinguishable from sperm of large, 'mature' males (Hinsch, 1969). It is not known whether these small males are able to inseminate mature females. Morphological and behavioral polymorphism, in which small males as well as large males are fertile but have different mating strategies, has been reported for some decapod (Kuris et al., 1987) and isopod (Shuster, 1987) crustaceans.

Spider crabs are thought to become terminally anecdysic, or incapable of molting, following the transition from the pre-pubescent to mature form (Tessier, 1935, Hartnoll, 1963, Hinsch, 1972) Signs of anecdysis include calcification of the proximal limb following autotomy, an accumulation of epibiota on the carapace and the absence of exuviae for extended periods of time. In other spider crabs, the y-organs, which produce the molting hormone 20-hydroxyecdysone, appear to degenerate following the molt of puberty (Carlisle, 1957, Chaix et al., 1976).

A more detailed description of the male reproductive system during adult development may clarify the relationship between the claw forms, molt interval and reproduction. In addition to the gonads, the internal reproductive system of male *L. emarginata* consists of the vas deferens, androgenic gland and ejaculatory duct (Hinsch and Walker, 1974). A prominent accessory gland, which presumably secretes components of the seminal fluid, is attached to the distal end of the vas deferens (Andrews, 1883).

In insects, morphological and reproductive development have a common endocrine regulator. High titers of juvenile hormone maintain the expression of immature characteristics during larval stages. Reduced titers of the absence of juvenile hormone permit the expression of pupal or adult features, respectively. In adult insects, juvenile hormone stimulates reproductive processes in both females and males. Additionally, juvenile hormone has been implicated in the regulation of insect polymorphism. For a recent review of the functions of juvenile hormone, see Kumaran (1990).

Methyl farnesoate (MF) is a member of the juvenile hormone family which is synthesized by the mandibular organs of crustaceans (Laufer et al., 1987). Experimental evidence suggests that MF may function analagously to juvenile hormone in insects (Hinsch, 1980, Charmantier et al., 1987, Laufer et al., 1987). In this study, we investigate whether claw form and exoskeleton condition reflect differences in reproductive system development and methyl farnesoate expression in male *L. emarginata.*

Materials and Methods

Animal collection and maintenance

Libinia emarginata were collected by the staff of the New England Utilities Environmental Laboratory, Waterford, CT in November, 1989 using a 30 ft trawl net with 1 inch mesh. The crabs were held in 500 gallon recirculating tanks equipped with a biological filter at 11 C. Artificial seawater was prepared at a salinity of 1.02 specific gravity. Crabs were fed with frozen squid twice a week.

Selection of experimental groups

Measurements were made to the nearest mm using vernier calipers. Carapace length was measured from the orbit to the median posterior margin of the carapace. Propodus length was measured from the proximal lateral condyle to the distal tip (Kuris et al., 1987). The propodus to carapace ratio (R) was calculated as an index of relative claw size. Logarithmic transformation of the propodus and carapace lengths were used to increase homoscedasticity and normality (Tessier, 1960). The claw forms were assigned as in Aldrich (1974). Randomness of residuals was confirmed for the two linear regression functions.

Five groups of seven individuals from a sample of 118 *L. emarginata* were selected on the basis of carapace size, claw from, and appearance of the exoskeleton. These parameters were defined within as narrow ranges as possible to minimize variation within the groups and to contrast the reproductive and hormonal differences among animals with different body sizes, claw forms, and conditions of the exoskeleton. Animals less than 35 mm in carapace length were not included in this study for practical reasons.

Reproductive system study

Body weight was measured to ±.01 g. The testes, vas deferens and accesory glands were dissected and weighed separately to ±0.01 g (wet weight). The accessory gland was attached to the posterior vas deferens near the opening in the base of the fifth walking leg and appeared to be composed of cecae filled with a viscous, translucent fluid. The reproductive system index was calculated by adding the testes, vas deferens and accessory gland weights, dividing by body weight and multiplying by 100. Separate organ indices were calculated by a similar formula. Semen from the vas deferens was examined under a light microscope for the presence of spermatophores.

Hormonal study

Mandibular organs were dissected and cultured attached to the mandibular tendon in 400 ul of media for 2 hours with gentle agitation at 22–25°C. Culture medium was Pantin's saline containing 20 mM HEPES (pH 7.4), 3.8 mM dextrose and 0.2% BSA and [methyl-^{3}H]-methionine (ICN), (S.A. = 200 mCi/mmole, T.A. = 40 μCi/ml). Following incubation, the cells were fixed with ethanol, homogenized and extracted with 1 ml hexane. The activity in a 100 μl hexane aliquot was quantitated using liquid scintillation spectrometry. Methyl farnesoate constitutes 97% of the radioactive material present in the hexane extract (Li and Borst, 1991).

Hemolymph titers of MF were determined according to the method developed by Laufer et al., (1987) and modified by Tsukimura et al. (1989). Hemolymph samples (0.5–6.0 ml) were collected in new 15 ml Kimax culture tubes with teflon lined caps containing 2.5 volumes of ice cold acetonitrile and 1 volume of 4% NaCl. 25 ng of the cis-trans (non-biological) isomer of MF was added to each tube as an internal standard. Samples were extracted twice with 0.5 ml hexane. Aliquots of the hexane extract were analysed on a Waters HPLC with model 501 pumps and a 5μ Econosil silica column (Alltech). The running solvent was 1% diethyl ether in hexane flowing at 2.5 ml/min. A Lambda Max 481 UV detector was used to monitor the wavelength at 218 nm. Peak areas were calculated using a Waters 740 data module.

Statistical analysis

Linear regression analysis, Pearson correlation coefficients, and Duncan's multiple range test were calculated using Statistical Analysis System (SAS) software on an IBM mainframe computer.

Results

Description of collection and experimental groups

Forty-nine percent of 118 *L. emarginata* males collected were small claw forms. The carapace sizes of these crabs ranged from 36 to 76 mm and the propodus to carapace ratios were between 0.47 and 0.83. The remainder of the crabs were classified as large claw forms; carapace sizes ranged from 40 to 80 mm and propodus to carapace ratios were between 0.68 and 1.20.

The experimental groups chosen for the reproductive and hormonal study were as follows: (1) small carapace (36–45 mm), small claw (R = 0.47–0.57), unabraded (SS), (2) intermediate carapace (52–58 mm), small claw (R = 0.63–0.69), unabraded (IS), (3) intermediate carapace (51–59 mm), large claw (R = 0.96–1.17), unabraded (IL), (4) large carapace (62–79 mm), large claw (R = 0.90–1.05), unabraded (LL), and (5) large carapace (62–82 mm), large claw (R = 1.00–1.20), abraded (LLA) (Fig. 1). The carapace sizes of groups IS and IL were not significantly different, nor were those of groups LL and LLA, all others were significantly different. Each group was significantly different with respect to propodus to carapace ratio.

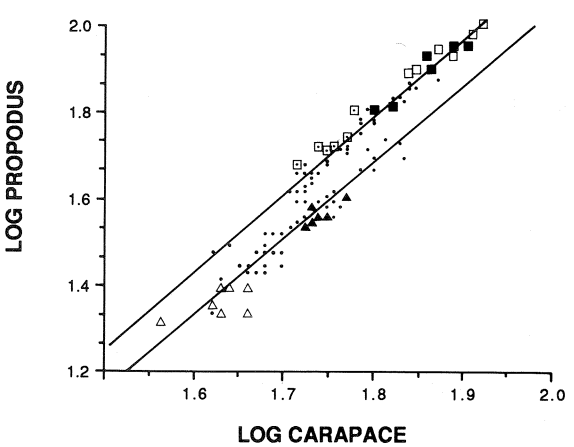

Fig. 1. Experimental groups of crabs selected for further study. Each group consists of 7 animals. Group LLA = large carapace, large claw, abraded exoskeleton (□). Group LL = large carapace, large claw, unabraded exoskeleton (■). Group IL = intermediate carapace, large claw, unabraded exoskeleton (⊡) Group IS = intermediate carapace, small claw, unabraded exoskeleton (▲). Group SS = small carapace, small claw, unabraded exoskeleton (△) (●) symbols represent crabs collected but not chosen for further study. Solid lines represent the regression lines of claw forms.

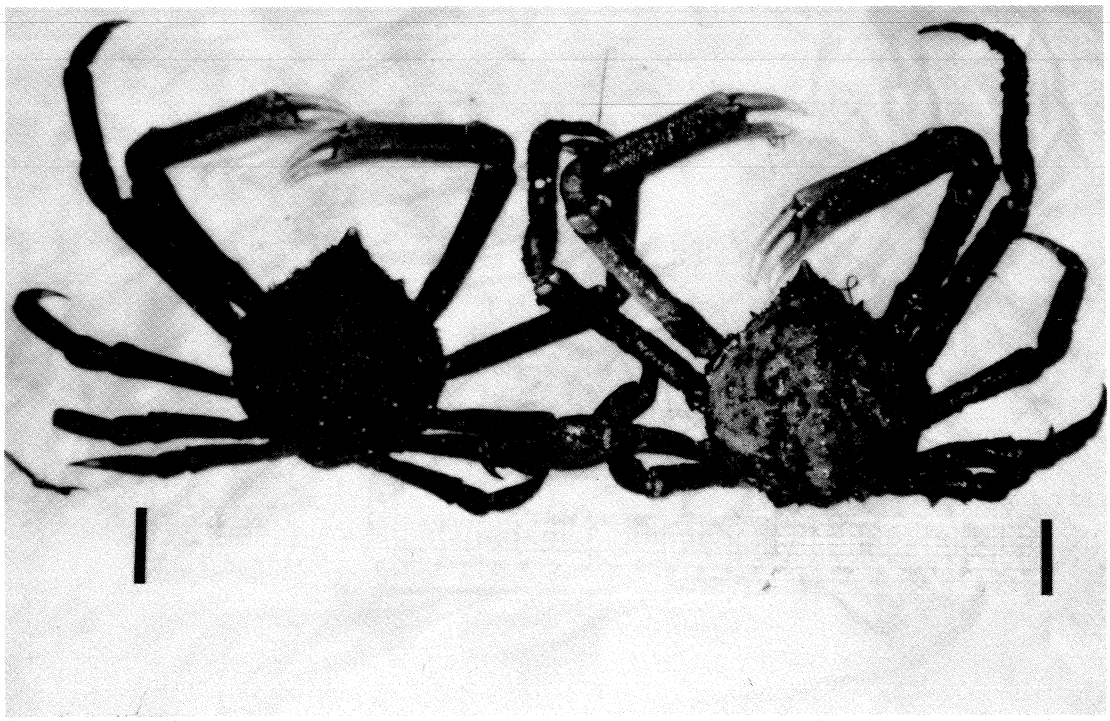

Fig. 2. Different exoskeletal conditions of male *L. emarginata*. Both crabs are classified as large claw forms. The crab on the left is an example of the unabraded condition and has a more flexible exoskeleton covered with a scaly epicuticle. The crab on the right is an example of the abraded condition and has a heavily calcified exoskeleton. The scales have been removed from exposed parts of the exoskeleton and remain in the recessed portions of the carapace. Note the presence of epibiota on the abraded specimen. Distance between bars equals one foot.

Approximately 12% of the large claw form crabs had worn, heavily calcified exoskeletons which were conspicuously colonized by epibiota such as barnacles, sponges and algae. These animals were termed abraded. All others of both forms had exoskeletons which were more flexible and covered with pubescent epicuticle; these were termed unabraded (Fig. 2).

Reproductive system

All 35 males examined, from carapace size 36 to 82 mm had sperm within spermatophores present in the vas deferens.

The components of the reproductive system were highly correlated with respect to size (Fig 3). Pearson's coefficient of correlation (R^2) ranged from 0.94 to 0.97 among the testes, van deferens and accessory gland weights from individual crabs. Therefore, the reproductive system was considered as a unit when making statistical comparisons among groups.

The average reproductive system index of the abraded, large claw crabs in group LLA was an order of magnitude larger than that of any other group ($x = 2.39 \pm 0.43$). The large claw crabs with

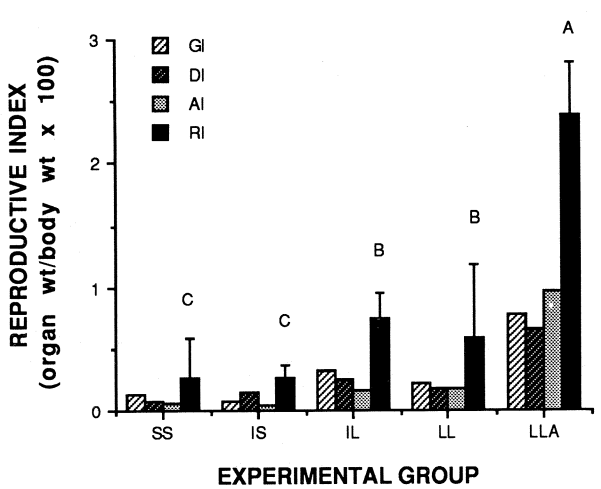

Fig. 3. Relative weights of the testes (GI), vas deferens (DI), accessory glands (AI) and total reproductive systems (RI) were determined by dividing organ weight by body weight and multiplying by 100. The bars represent the mean organ index for $N = 7$ observations. Standard deviations and statistical analysis are given for total reproductive system index only. The bars labelled with different letters are significantly different according to Duncan's multiple range test ($P < 0.0001$). SS = small carapace, small claw, unabraded. IS = intermediate carapace, small claw, unabraded. IL = intermediate carapace, large claw, unabraded. LL = large carapace, large claw, unabraded. LLA = large carapace, large claw, abraded.

unabraded exoskeletons in groups LL and IL had reproductive indices that were significantly lower than that of the LLA group. The indices of the intermediate sized crabs (group IL) were greater ($x = 0.74 \pm 0.22$), but not significantly different from that of the larger crabs of the LL group ($x = 0.58 \pm 0.22$). The average reproductive indices of the small claw crabs were significantly smaller than those of the other groups. The IS group ($x = 0.27 \pm 0.32$) was not significantly different from the SS group ($x = 0.27 \pm 0.09$) with respect to reproductive system index.

Methyl Farnesoate synthesis and titers

Mandibular organs from the large, abraded males of the large claw form (group LLA) synthesized significantly more MF *in vitro* than any other group ($x = 645{,}000 \pm 500{,}000$ dpm/gl/hr). The unabraded, large sized, large claw males (group LL) group had the next highest average synthetic rate ($x = 290{,}000 \pm 170{,}000$). The unabraded, intermediate sized crabs were identical with respect to mandibular organ activity regardless of relative claw size. Group IL averaged $155{,}000 \pm 58{,}000$ dpm/gl/hr and Group IS averaged $155{,}000 \pm 33{,}000$ dpm/gl/hr. The unabraded, small sized, small claw crabs (group SS) had the lowest average rate of synthesis ($x = 50{,}000 \pm 40{,}000$ dpm/gl/hr) (Fig. 4). MF synthesis rates of groups SS, IS, IL and LL were not significantly different. The synthetic activity of the mandibular organs *in vitro* was moderately correlated with body weight ($R^2 = 0.68$) and carapace size ($R^2 = 0.64$).

MF titers were the highest in the LLA group ($x = 38.8 \pm 49.1$ ng/ml) and were significantly different from all other groups except those of the SS group ($x = 16.4 \pm 7.4$ ng/ml). The lowest titers were observed in the IL group ($x = 2.9 \pm 3.1$ ng/ml) but they were not significantly different from those of the IS group ($x = 9.9 \pm 5.6$ ng/ml) or LL group ($x = 5.2 \pm 3.3$ ng/ml) (Fig. 5).

Discussion

The different claw forms of male *L. emarginata* presumably serve a reproductive function in courtship and/or territorial behavior. It is not known whether these forms represent obligatory developmental stages which terminate in a single reproductive form, or if the different forms possess alternative mating strategies such as those described for *Macrobrachium rosebergii* (Kuris et al., 1987). However, we can conclude from the results of this experiment that maximal reproductive system size is concommitant not with the large claw form but with the

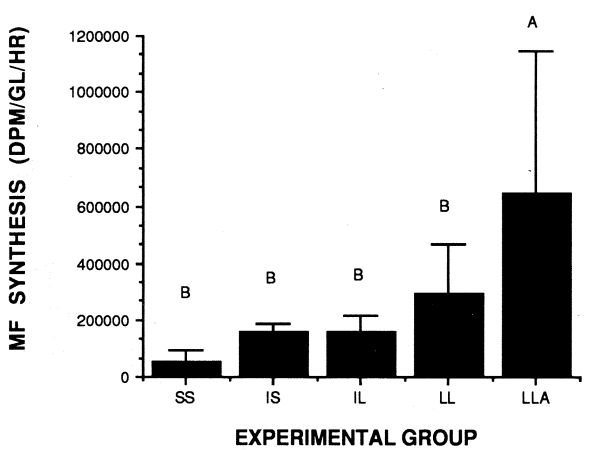

Fig. 4. The synthetic activity of the mandibular organ *in vitro* was determined by culturing the gland in the presence of ^3H methionine, extracting the MF and determining incorporation using liquid scintillation spectrometry. The mean synthetic activity expressed in DPM/hour/gland is plotted for each group. Error bars represent the standard deviation. Different letters indicate that the values are significantly different according to Duncan's multiple range test ($P < 0.001$). SS = small carapace, small claw, unabraded. IS = intermediate carapace, small claw, unabraded. IL = intermediate carapace, large claw, unabraded. LL = large carapace, large claw, unabraded. LLA = large carapace, large claw abraded.

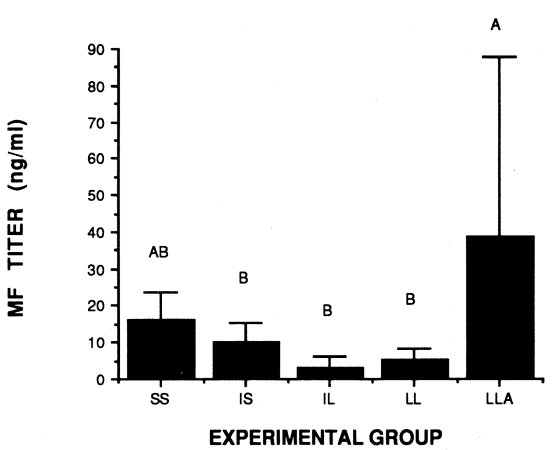

Fig. 5. MF titers were measured by separating hemolymph extract on normal phase HPLC. An internal standard was used to quantify MF by relative peak areas. The bars represent titers expressed in ng/ml ± standard deviation. Different letters indicate that the values are significantly different according to Duncan's multiple range test ($P < 0.037$). SS = small carapace, small claw, unabraded. IS = intermediate carapace, small claw, unabraded. IL = intermediate carapace, large claw, unabraded.IL = large carapace, large claw, unabraded. LLA = large carapace, large claw, abraded.

abraded exoskeleton. Exoskeleton condition may be useful in future experiments as a developmental marker for staging animals collected from the wild.

In addition to large reproductive systems, abraded males have increased mandibular organ activity, both in terms of methyl farnesoate synthesis *in vitro* and hemolymph titers *in vivo*. MF synthetic rates may be related to mandibular organ size, which in turn may be correlated with the body size of the donor. However, the average carapace size of the abraded LLA males was not significantly different from that of the unabraded group LL; therefore the differences in synthetic activities of these groups are independent of body size. In the future, it will be informative to measure the size of the mandibular organ as well as synthetic activity.

The coincidence of large reproductive system size and high MF titers in abraded crabs is consistent with the hypothesis that MF has a stimulatory role in crustacean reproduction. In insects, juvenile hormone regulates vitellogenesis in females by stimulating vitellogenin synthesis by the fat body and subsequent uptake of yolk proteins by the oocyte (Englemann, 1983). In males, juvenile hormone has a positive effect on reproductive accessory gland function. Ablation of the corpora allata, which synthesize juvenile hormone, causes atrophy of the male accessory glands in many species (Koeppe, 1985). Juvenile hormone has also been shown to stimulate RNA and protein synthesis in *Drosophila* accessory glands *in vitro* (Yamamoto et al., 1988).

To further investigate the role of MF in reproduction, it will be necessary to manipulate the titer of MF in order to establish a causal relationship between the hormone and the reproductive system. Preliminary experiments of this nature have been performed in female *L. emarginata* by Hinsch (1980). Implantation of mandibular organs from large males was reported to stimulate vitellogenesis in non-reproductive, juvenile females indicating that a positive relationship between MF and ovarian maturation may exist.

Future experiments concerning the function of MF in spider crab reproduction must also address the issue of anecdysis. Our data demonstrate that abraded crabs have undergone an important change in the regulation of methyl farnesoate levels and the reproductive system. In addition, these males may have experienced degeneration of the y-organs, as has been indicated for other members of the spider crab family (Carlisle, 1957, Chaix et al., 1976). Thus, the abraded crab may exhibit differences in expression of other hormones such as ecdysteroids, molt-inhibiting hormone and hypergylcemic hormone as well as MF.

If MF is indeed a crustacean gonadotrophin, then MF must have a different function in the small claw form since these animals had high titers of MF and small reproductive systems. Such is the case with juvenile hormone in insects. In larval stages, high titers of juvenile hormone maintain the expression of the immature phenotype but do not stimulate reproductive maturation. Experiments by Charmantier et al., (1987) demonstrated that exogenous juvenile hormone may cause the formation of intermediate stages in larval lobsters.

In insects, transitional molts require reduced titers of juvenile hormone to permit the development of adult structures. Indeed, the groups intermediate to the SS and LLA groups had significantly less MF in the hemolymph. This may indicate that MF is a morphogen which regulates the transition from the small claw to the large claw form. Longitudinal studies of titers from individuals during the molt cycle and subsequent development of male forms will provide a better understanding of role of MF in morphogenesis of *L. emarginata.*

In conclusion, our data suggest that methyl farnesoate may regulate not only the differentiation of the claws, but also the state of the reproductive system in different types of male *L. emarginata.*

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