Insect Biochem. Molec. Biol. Vol. 23, No. 1, pp. 171–174, 1993
Printed in Great Britain.

0965-1748/93 $6.00 + 0.00

Ecdysteroids and Juvenoids in Two Male Morphotypes of *Libinia emarginata*

H. LAUFER,* G. WAINWRIGHT,† N. J. YOUNG,† A. SAGI,* J. S. B. AHL,*‡ H. H. REES†

Two forms of the large-clawed male spider crab, *Libinia emarginata*, from the same molt stage, were examined to determine possible roles of ecdysteroids and juvenoids (methyl farnesoate) during maturation of the reproductive system and mating behavior. These forms are distinguished by the condition of the epicuticle covering the exoskeleton, abraded (worn away) and unabraded (intact), and mating behavior, active and inactive, respectively. Methyl farnesoate (MF) levels in the blood are high during active reproduction (abraded males)—mating, mate guarding and male competition; but, when the animals are in reproductive diapause (unabraded males), these levels are low. Ecdysteroids are found primarily in the testes of both male types. However, the amounts detected are highest in the non-reproductive unabraded males, and lowest in the actively mating abraded ones. It appears that ecdysteroids may function early in maturation in unabraded animals by making the gonad competent, while MF may be functioning in mature males with developed reproductive systems and exhibiting mating behavior.

Ecdysteroids Methyl farnesoate Mating behaviour Reproduction Reproductive diapause Juvenoids

INTRODUCTION

Reproductive maturation in crustaceans is a complex process that includes maturation of the reproductive system and initiation of mating behavior. In the male spider crab, *Libinia emarginata*, this process extends beyond the initial production of sperm, the onset of which occurs in very small males (19 mm carapace length) (Homola *et al.*, 1991). Non-reproductive individuals continue to grow until they reach a final "maturational" molt (Hartnoll, 1963). Male spider crabs that have undergone the "maturational" molt have developed large claws which are used to compete for females, and to maneuver their mates into position for insemination. There are two morphotypes of these large-claw males, abraded and unabraded, which are distinguished by the condition of the epicuticle covering the exoskeleton, and by their mating behavior. The abraded morph is the primary reproductive type that aggressively competes for a female, then will carry and guard her until oviposition (Sagi *et al.*, 1991a). Abraded males have been anecdysic for at least a year as evidenced by the presence of epibionts on the exoskeleton, and the epicuticle is worn away (Homola *et al.*, 1991). The unabraded morph has recently molted as evidenced by the pubescent nature of the epicuticle (Homola *et al.*, 1991), and does not display reproductive behavior (Sagi *et al.*, 1991a).

The hormonal control of reproduction in crustaceans is known to involve neurosecretions from the sinus gland that regulate the ovarian cycle of females (see Charniaux-Cotton and Payen, 1983). However, there is also evidence that vitellogenesis is stimulated by low blood levels of ecdysteroids, (Charniaux-Cotton, 1985), and elevated titers of methyl farnesoate (MF) (Laufer *et al.*, 1986, 1987), which in insects is a precursor to the juvenoid hormones that regulate development and reproduction.

Ecdysteroids are produced by the Y organs, which degenerate after the "pubertal" molt ("maturational" molt of Hartnoll, 1963) in spider crabs (Chaix *et al.*, 1976). So in female crabs, the source of ecdysteroids during reproduction may be the ovary, since these hormones are present in this tissue throughout vitellogenesis (Chaix and DeReggi, 1982; Laufer *et al.*, 1988). However in males, this final molt may be better termed a "differentiational" molt because the claws become greatly enlarged. Sperm are present in the reproductive systems of all males greater than 19 mm carapace length (Homola *et al.*, 1991), and small-clawed crabs are also capable of mating. Thus, these large-clawed crabs clearly have not undergone a "pubertal" or a "maturational" molt.

What is known about the endocrine basis for male reproduction in crustaceans is limited to differentiation and maintenance of the gonad which is controlled by secretions from the androgenic gland (Charniaux-Cotton and Payen, 1983). Recently we have found that

*Department of Molecular and Cell Biology, The University of Connecticut, Storrs, CT 06269 and the Marine Biological Laboratory, Woods Hole, Mass., U.S.A.
†Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, England.
‡College of Basic Studies, University of Hartford, CT 06117, U.S.A.

MF levels are high in reproductively active male *L. emarginata* (Homola *et al.*, 1991; Sagi *et al.*, 1991a), but low in non-reproductive animals, suggesting that this compound may be involved in reproductive maturation. Although ecdysteroids have been reported to be present in the testes of the crab *Pachygrapsus crassipes* (Chang *et al.*, 1976), their role in male reproduction remains to be elucidated.

In this paper we report on the status of ecdysteroids and juvenoids in two large-claw forms of the male spider crab, *L. emarginata*, from the same molt stage to determine their possible roles during maturation of the reproductive system and mating behavior.

MATERIALS AND METHODS

Animals

Libinia emarginata were collected by the staff of the New England Utilities Environmental Laboratory, Waterford, Conn. in April of 1991; and by the Department of Marine Resources of the Marine Biological Laboratory, Woods Hole, Mass. in July 1991.

The morphotypes were identified on the basis of log propodus (claw) to log carapace length ratios, and the appearance of the epicuticle covering the exoskeleton (Homola *et al.*, 1991). Reproductive behavior was assessed by observing male competition in the holding tanks, and during a 15 min period when individual males were isolated with a receptive female (Sagi *et al.*, 1991a).

Extraction, purification and analysis of free ecdysteroids

Hemolymph (2 ml) samples were taken from the base of the walking legs, then transferred to 15 ml culture tubes with caps. Testes, vas deferens and accessory glands were dissected out separately, weighed to the nearest 0.01 g, then transferred to culture tubes and capped. Both the hemolymph and tissue samples were stored at −20°C until assayed. Before ecdysteroid extraction, the tissue samples were lyophilized and reweighed.

Total free ecdysteroids were extracted and purified as described previously by a combination of silicic acid column chromatography and C_{18} Sep-Pak fractionation (Rees and Isaac, 1985; Mercer *et al.*, 1987; Young *et al.*, 1991). The samples were then analyzed by RIA using H-22 antiserum (generously provided by Dr L. I. Gilbert; Warren and Gilbert, 1986) and expressed as ecdysone equivalents.

Analysis of the reproductive system

The reproductive system index was calculated by adding the weights of the testes, vas deferens and accessory glands, dividing by body weight, then multiplying by 100. Semen from the vas deferens was examined under a light microscope for the presence of spermatophores.

Methyl farnesoate hemolymph titers

Hemolymph samples (2 ml) were taken from the base of the walking legs, extracted with hexane then analyzed using HPLC according to Homola *et al.* (1991). *Cis–trans* MF was used as an internal standard and *trans–trans* MF was determined by reading O.D. at 215 μm.

Data analysis

Differences between mean values were assessed using a *t*-test (Sokal and Rolf, 1973).

RESULTS

Ecdysteroids were present primarily in the hemolymph and the testes of each morphotype (Fig. 1). The highest concentration of ecdysteroids per dry weight was in the testes of the unabraded males (132.15 ± 25.87 ng/g), but their hemolymph contained the lowest concentration (0.88 ± 0.36 ng/ml). The abraded males had less than one fifth (26.92 ± 12.4 ng/g) the concentration of ecdysteroids in their testes as the unabraded males, which was significant ($P \leqslant 0.05$). However, their hemolymph contained more than twice (2.28 ± 0.77 ng/ml) the amount detected in the unabraded animals.

The abraded males actively competed for, mated with and carried the receptive females. Their reproductive indices (Table 1) (2.45 ± 0.46% of the body weight vs 1.07 ± 0.85%), and their hemolymph titers of MF (Table 1) (53.2 ± 11.4 ng/ml vs 23.6 ± 10.3 ng/ml), were significantly greater ($P \leqslant 0.05$) than those of the unabraded males. The unabraded males made little or no attempt to compete for, carry or mate with receptive females, even when isolated with them.

Examination of the semen from the vas deferens revealed that both abraded and unabraded large-claw males contained spermatophores.

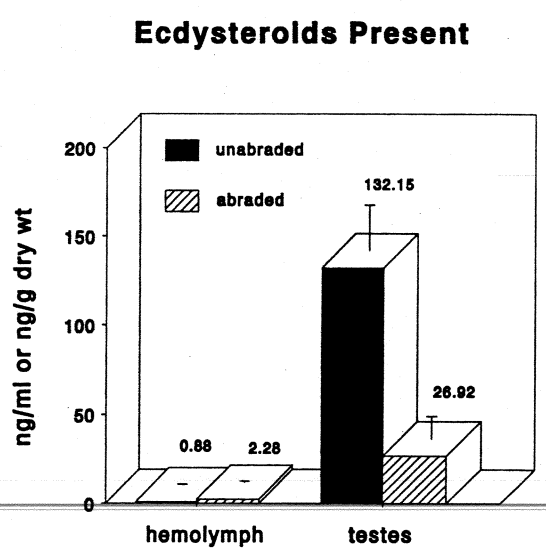

FIGURE 1. Ecdysteroids present in the hemolymph (ng/ml) and testes (ng/g dry wt) of unabraded (solid bars) and abraded (cross-hatched bars) male *L. emarginata* as detected by RIA. The value above bar is the mean from 3, 6, 2 and 5 animals, respectively.

TABLE 1. Reproductive indices and hemolymph titres in the large-clawed male morphotypes of *Libinia emarginata*

Morphotype	*n*	Reproductive system index [%body wt (±SE)]	Methyl farnesoate [ng/ml (±SE)]
Unabraded	7	1.07 (±0.12)	23.6 (±10.3)
Abraded	8	2.45 (±0.03)	53.2 (±11.4)

DISCUSSION

While the role of ecdysteroids and juvenoids during maturation of the reproductive system and mating behavior in Crustacea is still tentative, we note certain trends in two forms of the large-claw male spider crab *L. emarginata*. Abraded males have high blood levels of MF, large reproductive systems and display mating behavior. However, ecdysteroids are present in relatively small amounts in the blood and in the testes. The unabraded males show a reversal in these trends. Their MF and ecdysteroid blood levels are low, and they do not display reproductive behavior. Although their reproductive systems are less developed and smaller than those of the abraded males, there are comparatively large amounts of free ecdysteroids present in the testes.

Ecdysteroids have been shown to enhance cell proliferation in the primary culture of lobster testes (Brody and Chang, 1989), and stimulate DNA synthesis in testes of the freshwater prawn *Macrobrachium rosenbergii* (Sagi *et al.*, 1991b) possibly during early phases of spermatocyte proliferation. This has also been demonstrated in insect species, such as *Rhodnius prolixus* (Dumser and Davey, 1974), and many others (see Hagedorn, 1983). That ecdysteroid levels in testicular tissue from unabraded males are greater than those of the abraded males, suggests that these steroids may function by stimulating spermatogenesis or growth of the gonad.

Even though ecdysteroids are found in, and have an effect on testes in crustaceans, it is not clear exactly where they are being produced in spider crabs, since it is assumed that the Y-organs have disintegrated prior to the differentiational molt.

The lack of mating behavior, small reproductive system, and low MF blood titers in the unabraded male suggest that this crab may be in a state of reproductive diapause. In insects, this state commonly occurs in newly eclosed adults, and is due to a juvenile hormone (JH) deficiency (DeWilde, 1983; Denlinger, 1985). JH has been found to have a negative effect on spermatogenesis (Dumser and Davey, 1974), so ecdysteroids may work to make the gonad competent in the absence of juvenoids during the period of reproductive diapause. It has even been suggested that ecdysteroids may regulate juvenoid production by promoting the release of an allatostatin (Tobe, 1980), which inhibits the corpora allata that produce the juvenoids in insects.

In conclusion, during development of the reproductive system, ecdysteroids may function early in maturation by stimulating spermatogenesis, while MF may be functioning during later stages, and may somehow stimulate or promote the development of mating behavior. However, further studies are needed to complete our understanding of the roles of ecdysteroids and juvenoids in crustacean reproduction.

REFERENCES

Brody M. D. and Chang E. (1989) Development and utilization of crustacean long-term primary cell cultures: ecdysteroid effects *in vitro*. *Invert. Reprod. Dev.* **16,** 141–147.

Chaix J. C. and DeReggi M. (1982) Ecdysteroid levels during ovarian development and embryongenesis in the spider crab *Acanthonyx lunulatus*. *Gen. comp. Endocr.* **47,** 7–14.

Chaix J. C., Trilles J.P. and Vernet G. (1976) Degenerescence de l'organe Y chez les males puberes d'*Acanthonyx lunulatus* (Crustacea Decapoda Oxyrhyncha). *C.r. hebd, Séanc. Acad. Sci., Paris, Ser. D* **283,** 523–525.

Chang E. S., Sage B. A. and O'Connor J. D. (1976) The qualitative and quantitative determinations of ecdysones in tissues of the crab, *Pachygrapsis crassipes*, following molt induction. *Gen. comp. Endocr.* **30,** 21–33

Charniaux-Cotton H. (1985) Vitellogenesis and its control in malacostracan Crustacea. *Am. Zool.* **25,** 197–206.

Charniaux-Cotton H. and Payen G. (1983) Crustacean reproduction. In *Endocrinology of Selected Invertebrate Types* (Edited by Laufer H. and Downer R. G. H.), pp. 279–303. Liss, New York.

Denlinger D. L. (1985) Hormonal control of diapause. In *Comparative Insect Physiology, Biochemistry, and Pharmacology* (Edited by Kerkut G. A. and Gilbert L. I.), Vol. 8, pp. 353–412. Pergamon Press, Oxford.

DeWilde J. (1983) Endocrine aspects of diapause in the adult stage. In *Invertebrate Endocrinology. Vol. 1. Endocrinology of Insects* (Edited by Downer R. G. H. and Laufer H.), pp. 357–368. Liss, New York.

Dumser J. B. and Davey K. G. (1974) Endocrinological and other factors influencing testis development in *Rhodnius prolixus*. *Can. J. Zool.* **52,** 1011–1022.

Hagedorn H. H. (1983) The role of ecdysteroids in the adult insect. In *Endocrinology of Insects* (Edited by Downer R. G. H. and Laufer H.), pp. 271–304. Liss, New York.

Hartnoll R. G. (1963) The biology of Manx spider crabs. *Proc. Zool. Soc. Lond.* **141,** 423–496.

Homola E., Sagi A. and Laufer H. (1991) Relationship of claw form and exoskeleton condition to reproductive system size and methyl farnesoate in the male spider crab, *Libinia emarginata*. *Invert. Reprod. Dev.* **20,** 45–51.

Laufer H., Borst D. W., Foley T. A. and Landau M. (1988) Ecdysteroid titres in vitellogenic *Libinia emarginata*. *J. Insect Physiol.* **34,** 615–617.

Laufer H., Landau M., Borst D. and Homola E. (1986) The synthesis and regulation of methyl farnesoate, a new juvenile hormone for crustacean reproduction. In *Advances in Invertebrate Reproduction 4* (Edited by Porchet M., Andries J.-C. and Dhainaut A.), pp. 135–143. Elsevier Science, Amsterdam.

Laufer H., Borst D., Baker F. C., Carrasco C., Sinkus M., Reuter C. C., Tsai L. W. and Schooley D. A. (1987) Identification of a juvenile hormone-like compound in a crustacean. *Science* **235,** 202–205.

Mercer J. G., Munn A. E., Arme C. and Rees H. H. (1987) Analysis of ecdysteroids in different developmental stages of *Hymenolepis diminuta*. *Molec. Biochem. Parasitol.* **25,** 61–71.

Rees H. H. and Isaac R. E. (1985) Biosynthesis and metabolism of ecdysteroids and methods of isolation and identification of free and conjugated compounds. *Meth. Enzymol.* **111,** 377–411.

Sagi A., Ahl J., Danaee H. and Laufer H. (1991a) Methyl farnesoate and reproductive behavior in male morphotypes of the spider crab *Libinia emarginata*. *Am. Zool.* **31,** 87A.

Sagi A., Karp L., Milner Y., Cohen D., Kuris A. and Chang E. S. (1991b) Testicular thymidine incorporation in the prawn *Marcobrachium rosenbergii*: molt cycle variation and ecdysteroid effects *in vitro*. *J. exp. Zool.* **259,** 229–237.

Sokal R. R. and Rolf F. J. (1973) *Introduction to Biostatistics*, pp. 171–174. Freeman, San Francisco, Calif.

Tobe S. S. (1980) Regulation of corpora allata in adult female insects. In *Insect Biology in the Future* (Edited by Locke M. and Smith D. S.), pp. 345–367. Academic Press, New York.

Warren J. T. and Gilbert L. I. (1986) Ecdysone metabolism and distribution during the pupal-adult development of *Manduca sexta*. *Insect Biochem.* **16,** 65–82.

Young N. J., Webster S. G., Jones D. A. and Rees H. H. (1991) Profile of embryonic ecdysteroids in the decapod crustacean, *Macrobrachium rosenbergii*. *Invert. Reprod. Dev.* **3,** 201–212.

Acknowledgements—The research reported here was supported in part from the Sea Grant College Program and a Fellowship of the Lady Davis Trust to H.L.; a Fulbright Scholarship, and a Binational Agricultural Research Development Foundation Fellowship to A.S. We wish to thank the Environmental Control Laboratory of the Millstone Nuclear Power Station, Waterford, Conn. for generously providing living specimens of *Libinia emarginata*, Dr L. Renfro for help with their transport and Dr F. Mauri of the Biotechnology Center, University of Connecticut for providing technical expertise and assistance on HPLC.