Reproductive Endocrinology of the Shrimp *Sicyonia ingentis:* Steroid, Peptide, and Terpenoid Hormones

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ABSTRACT

Female reproduction in penaeid shrimp is carefully regulated by several different endocrine factors. Their precise modes of action have not yet been fully elucidated. Three endocrine factors, each representing a different chemical class of hormones, have been investigated in the penaeid shrimp *Sicyonia ingentis* in our laboratory: ecdysteroids, vitellogenesis-inhibiting hormone (VIH), and methyl farnesoate (MF). Ecdysteroids (the steroid molting hormones of arthropods; predominantly 20-hydroxyecdysone), are initially present in low levels (<10 ng/mg) in shrimp embryos. As development of the embryos nears time of hatch, the ecdysteroid levels increase to approximately 150 ng/mg, indicating that they may be of embryonic origin and involved in embryonic development. An assay was developed for shrimp VIH, which presumably is a protein. Delay of onset of the next reproductive cycle was observed following injection of sinus gland extracts into shrimp that had previously had their eyestalks removed. A photoaffinity analog was synthesized for the putative shrimp reproductive hormone MF—a terpenoid. This analog, farnesyl diazomethyl ketone (FDK), was used to demonstrate the presence of specific binding proteins for MF in shrimp hemolymph.

Introduction

The control of female reproduction in penaeid (members of the superfamily Penaeoidea) shrimp is highly complex. It appears that a number of environmental signals can influence different hormonal factors which in turn regulate various aspects of the reproductive process. The understanding of this regulation is an area of intense research (*see* reviews by Adiyodi 1985; Charniaux-Cotton and Payen 1988). In addition to the basic biological studies in comparative endocrinology, there is great interest in the applied aspects of this research.

Penaeid shrimp comprise one of the most economically important marine products both domestically and worldwide (Rosenberry 1990). While natural fisheries for shrimp have declined, there has been a concomitant surge in aquatic culture of penaeids. One of the major problems preventing optimization of the commercial culture of shrimp is control of female reproduction.

We examined three areas of shrimp reproduction. First is the role of the arthropod molting hormones, ecdysteroids, in ovarian and embryonic development. Although there have been a number of studies on the role of ecdysteroids in crustacean molting (Chang 1989), relatively little is known about the action of these steroid molting hormones on ovarian and embryonic development.

Second, we investigated the activity of the vitellogenesis-inhibiting hormone (VIH). The initial observations that provided a basis for the existence of the VIH were made by Panouse (1943, 1944). He observed that, depending upon the molt stage, removal of the eyestalks from the shrimp Palaemon serratus resulted in accelerated ovarian development and spawning. Similar observations have been made in a number of natantians (*see* Chang 1992).

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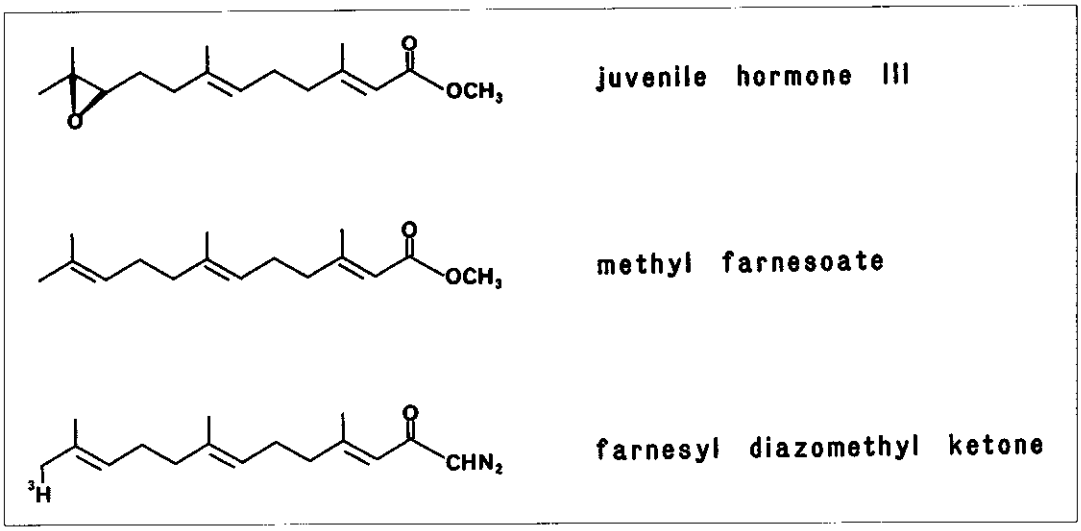

Figure 1
Chemical structures of juvenile hormone III, methyl farnesoate, and tritiated farnesyl diazomethyl ketone.

Finally, we examined the presence of binding proteins for the putative gonadotropin methyl farnesoate (MF). Based upon the similarities between crustaceans and insects in terms of the endocrine regulation of molting, it was hypothesized that an analog to the insect juvenile hormone (JH; Fig. 1) may be present in crustaceans. Recent work indicates that a sesquiterpenoid other than JH may be the modulator of crustacean development. The related compound, methyl farnesoate (Fig. 1), was isolated from the hemolymph of the crab *Libinia emarginata* (Laufer et al. 1987). Further evidence for an endocrine role of MF in crustaceans would be the demonstration of a hemolymph binding protein, similar to that found in insects (*see* Goodman and Chang 1985).

Materials and Methods

Experimental Animals

Sicyonia ingentis (ca. 24 g wet weight) were collected off Southern California and maintained in seawater tanks (15 ± 1° C) under an ambient photoperiod at the Bodega Marine Laboratory.

Embryonic Ecdysteroids

To measure the circulating levels of ecdysteroids, 25 µL samples of hemolymph were removed and extracted with 75% methanol at various times during the course of the molt cycle. The extracts were centrifuged (5000 × g, 10 min) and the pellets washed with 75% methanol. The supernatants were combined and analyzed by radioimmunoassay (RIA) according to the method of Chang and O'Connor (1979).

Concentrations of ecdysteroids in whole animal extracts of *S. ingentis* from spawning to early nauplii were also determined by RIA. At least 100 embryos or larvae were collected on a filter disc at various times and vacuum dried for one min to remove excess water. They were homogenized in a Dounce homogenizer, extracted with methanol, and centrifuged (5,000 × g, 10 min). The supernatants were dried and then solubilized in water. The extracts were loaded onto C_{18} Sep-Pak (Waters Assoc.) cartridges and the ecdysteroids were eluted with 60% methanol and then assayed by RIA.

Vitellogenesis-Inhibiting Hormone

Females with well-developed ovaries were held in a tank with running seawater until they initiated prespawning swimming behavior. They were removed to a smaller tank for spawning and then placed into a separate holding tank following spawning. Bilateral eyestalk ablations were performed within 24 hours with the use of iris scissors. Shrimp were allowed to recover for 24 hours before receiving their first injection.

The injections consisted of 1.0 sinus gland equivalent or, for the controls, an equivalent amount of non-sinus gland neural tissue. The source of both tissues were eyestalks of reproductively quiescent females obtained during February and March (winter animals). The glands or control tissue were homogenized with a Dounce homogenizer in 4 µL of distilled water per gland or equivalent. The homogenate was stored at –75° C until needed. Prior to injection, the homogenate was thawed, spun (5000 × g, 5 min) and the supernatant was removed. A 10 µL syringe (Hamilton) with a 28 gauge needle was used to inject 4 µL of the supernatant. The animal was immobilized and the needle was inserted from

one side of the ventral midline of the fourth abdominal segment to the first segment where the sample was injected. The needle was withdrawn after 10 seconds to minimize sample leakage.

Injections of sinus gland or control tissue extracts were administered every 48 hours until 30 animals in each group had received four injections. The females were monitored daily and the subsequent time to spawn was noted.

Methyl Farnesoate Photoaffinity Analog

Chemicals—The unlabeled and tritium-labeled hormones farnesyl diazomethyl ketone (FDK) and methyl farnesoate (MF) were synthesized as described by Ujváry and Prestwich (1990) and purified by silica gel chromatography. The [^{3}H]-FDK had a specific activity of 6.6 Ci/mmol.

Dissection and tissue preparation—Dissected tissues were homogenized (Dounce) in TM buffer (10 mM Tris HCl, 5 mM $MgCl_2$, pH 6.9) and centrifuged for 20 min at 10,000 × g (4° C) to remove cellular debris. Each supernatant was diluted with TM buffer to give a final protein concentration between 0.5 and 2.5 mg/mL.

Photoaffinity labeling—Glass plates with depression-wells (ca. 250 µL volume) were coated with 1% polyethylene glycol (PEG) MW 20,000 and then rinsed with water. Each experiment consisted of competed and uncompeted samples for each tissue. Thus, for each tissue, two wells were loaded with 100 µL of a pH 8.3 buffer (20 mM Tris HCl). Four µL of either ethanol or the competitor (MF) in ethanol (1.6×10^{-2} M) were added and the solutions were gently mixed on an orbital shaker for 20 minutes. The plates were then cooled on an ice pack while 100 µL of tissue homogenate was added to each well. The chilled samples were mixed for 30 minutes, and then 2.5 µL of a 5×10^{-5} M solution of [^{3}H]-FDK in ethanol was added to each well. Mixing continued for 45 minutes at 4° C, and then the wells in the chilled plate were irradiated from ca. 3 to 4 cm above with an 8-watt, 254-nm germicidal lamp for times ranging from 15 seconds to 4 minutes

Electrophoresis and autoradiography—A 70 µL aliquot of each well was transferred into a 0.5 mL plastic tube containing 60 µL of 2× SDS-sample

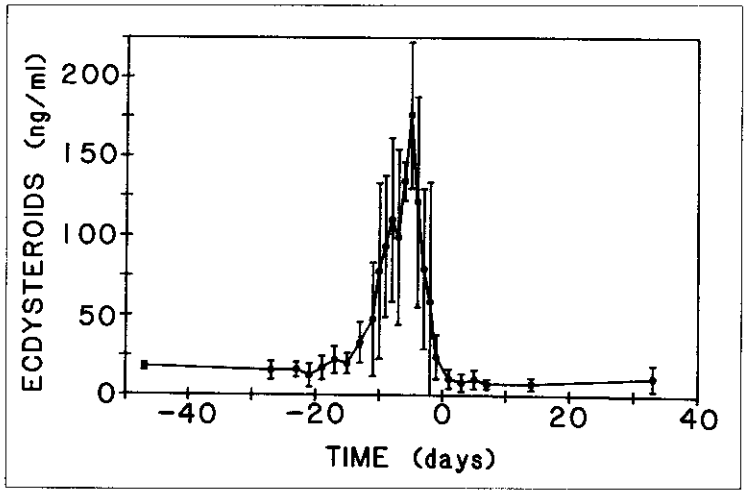

Figure 2
Ecdysteroid titers (mean ± S.D.) of *Sicyonia ingentis* at various times before (negative days) and after molt (positive days). Molt occurred on day 0. Hemolymph (25 µL) was removed and extracted with 75% methanol and analyzed by radioimmunoassay (n = 3 to 16).

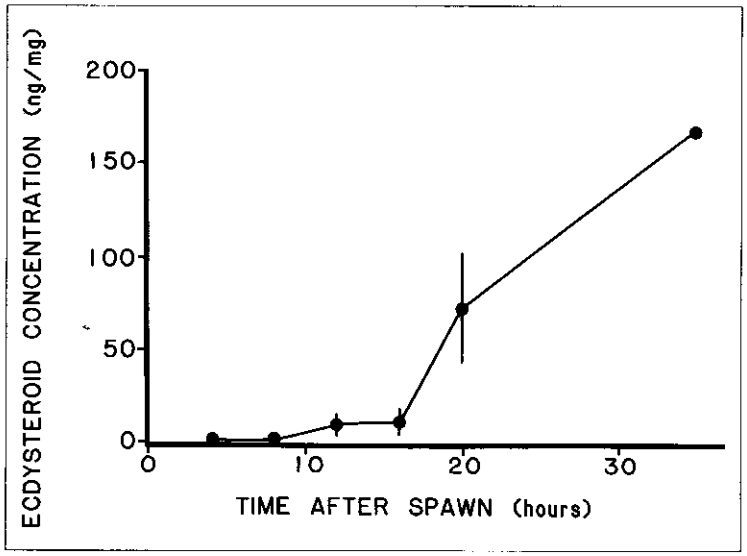

Figure 3
Concentrations (mean ± S.D.) of ecdysteroids in whole animal extracts of *Sicyonia ingentis* from spawning (= fertilization; 0 h) to early nauplii. Hatching occurred at approximately 20 hours. At least 100 embryos or larvae were collected on a filter disc, homogenized, and assayed by radioimmunoassay as described in Materials and Methods section. Sample numbers at 4, 8, 12, 16, 20, and 35 hours were 7, 4, 4, 3, 2, and 1, respectively.

buffer, mixed, and boiled 4 minutes. Samples (45 μL) were loaded onto a 0.75 × 150 × 150 mm denaturing polyacrylamide gel (SDS-PAGE, 12% acrylamide), stacked at 13 mA per gel, and separated at 10° C at 18 mA per gel using a Tris-glycine pH 8.3 running buffer. Gels were stained (4 h) with Coomassie Blue R250 and then destained (95% ethanol:H_2O:acetic acid, 50:40:10).

The destained gel was rinsed for 5 minutes with glacial acetic acid and then impregnated with diphenyloxazole (15% PPO in glacial acetic acid) for 25 minutes. The acetic acid was poured off and the gel was treated with 50% PEG 2000 at 50–75° C for 30 to 40 minutes. The miniaturized gels (Mohamed et al. 1989) were dried and exposed to pre-flashed Kodak XAR-5 x-ray film for 5–15 days at –75° C.

Results and Discussion

Embryonic Ecdysteroids

The concentration profile of ecdysteroids in the hemolymph of Sicyonia ingentis is similar to those of most other crustaceans that have been examined (Fig. 2; *see* Chang 1989). There are low levels during postmolt and intermolt with a dramatic increase just prior to ecdysis (premolt). This peak falls back to basal levels just prior to ecdysis. Approximately similar values were obtained in the shrimp *Palaemon serratus* (Baldaia et al. 1984; Van Wormhoudt et al. 1986).

Since ecdysteroids play such an important role in larval, juvenile, and adult molting (Chang 1989), we assayed extracts of developing embryos to determine if these steroid hormones were also involved in embryonic development. Figure 3 shows the total RIA activity of extracts of embryos at various times after spawning (fertilization). Negligible levels of ecdysteroids were present in the embryos at spawning, but the concentrations increased significantly as embryonic development proceeded. These data imply that there is relatively little maternal investment of ecdysteroids in the eggs and that the increasing levels of hormone observed in the extracts is likely due to endogenous synthesis.

These data are in apparent contrast to observations that our laboratory has made in the crab *Cancer anthonyi* (Okazaki and Chang 1991). These crab embryos have relatively high levels of ecdysteroids at the time of spawn (ca. 9 ng/mg wet weight) that decrease during embryonic development. This implies an embryonic utilization of the hormones during development. These differences may be due to either the dramatically different rates of development (*S. ingentis* hatches after ca. 30 hours, *C. anthonyi* after ca. 35 days) or to differences in the chemical forms of the molting hormones. For example, conjugated metabolites of ecdysteroids have a much lower affinity for the ecdysteroid antiserum and hence, if present, would give the overall appearance of less total ecdysteroid RIA activity. However, we favor the former explanation (differential rates of development).

Recently, data were presented that implicate a role for ecdysteroids in the mediation of embryogenesis in the shrimp *Palaemon serratus* (Spindler et al. 1987). In that species, although much lower concentrations were measured (peaks of about 80 ng/g), a similar hormone pattern was observed of low levels of ecdysteroids at egg extrusion followed by increasing levels near hatch.

Vitellogenesis-Inhibiting Hormone

S. ingentis is a useful species for the assay of VIH because it undergoes several cycles of reproduction without intervening molt cycles in the summer months. Following a spawn, shrimp were injected with extracts of sinus glands obtained from winter (nonreproductive) female shrimp. A significant inhibition ($P<0.01$) of ovarian development and spawning resulted. This effect was not observed in

Table 1
Effect of shrimp sinus gland extracts on spawning duration.

Extract	Spawning Duration (days ± S.D.)	*N*
nonsinus gland neural tissue	16.31 ± 1.31	16
sinus glands	19.93 ± 2.08[a]	15

[a] $P < 0.01$ (Student's *t*-test).

control shrimp that were injected with nonsinus gland neural tissue (Table 1) or summer shrimp that were injected with sinus gland extracts obtained from summer donor females (Chang and Hertz, unpubl. data).

We have observed differences in the peptide profiles from sinus glands of winter (quiescent) and summer (active) shrimp following separation using high-performance liquid chromatography (Chang and Hertz, unpubl. data). We have purified these shrimp sinus gland peptides and are currently assaying them for VIH activity.

There are reports that provide some chemical information on VIH. Most of these previous experiments utilized a heterologous assay system. Bomirski et al. (1981) extracted a factor with a molecular weight of approximately 2000 from eyestalks of the crab *Cancer magister*. Their assay consisted of measuring ovarian growth in the shrimp *Crangon crangon*. A 5000 dalton factor was extracted from eyestalks of the spiny lobster *Panulirus argus*. It was assayed by measuring ovarian growth in the fiddler crab *Uca pugilator* (Quackenbush and Herrnkind 1983). A 7500 dalton peptide was isolated from sinus glands of the American lobster *Homarus americanus* and assayed in vivo in the shrimp *Palaemonetes varians* (Soyez et al. 1987). The assay consisted of measuring oocyte diameters. Antisera were also raised against this lobster VIH which cross-reacted with sinus gland extracts from a number of different decapods (Meusy et al. 1987). A 3300 dalton factor was characterized from eyestalks of *Penaeus setiferus* as assayed in vitro using fiddler crab ovaries. This assay utilized precipitation of radiolabeled leucine by antibodies that had been generated against fiddler crab vitellogenin (Quackenbush and Keeley 1988).

Farnesyl Diazomethyl Ketone

Using the radiolabeled photoaffinity analog of methyl farnesoate, [^{3}H]-FDK, we examined a number of different tissues from several different species for specific hormone binding. We examined both the cytosol and membrane fractions of these tissues. Although we were unable to demonstrate cellular binding proteins for [^{3}H]-FDK from any tissue, we consistently observed specific binding in the hemolymph. Figure 4 shows the effects of increasing amounts of unlabeled MF in the presence of a constant amount of [^{3}H]-FDK. The radiolabeled analog forms a covalent bond with a binding protein of ca. 36,000 daltons and is effectively competed with a 250-fold excess of the unlabeled hormone. In addition,

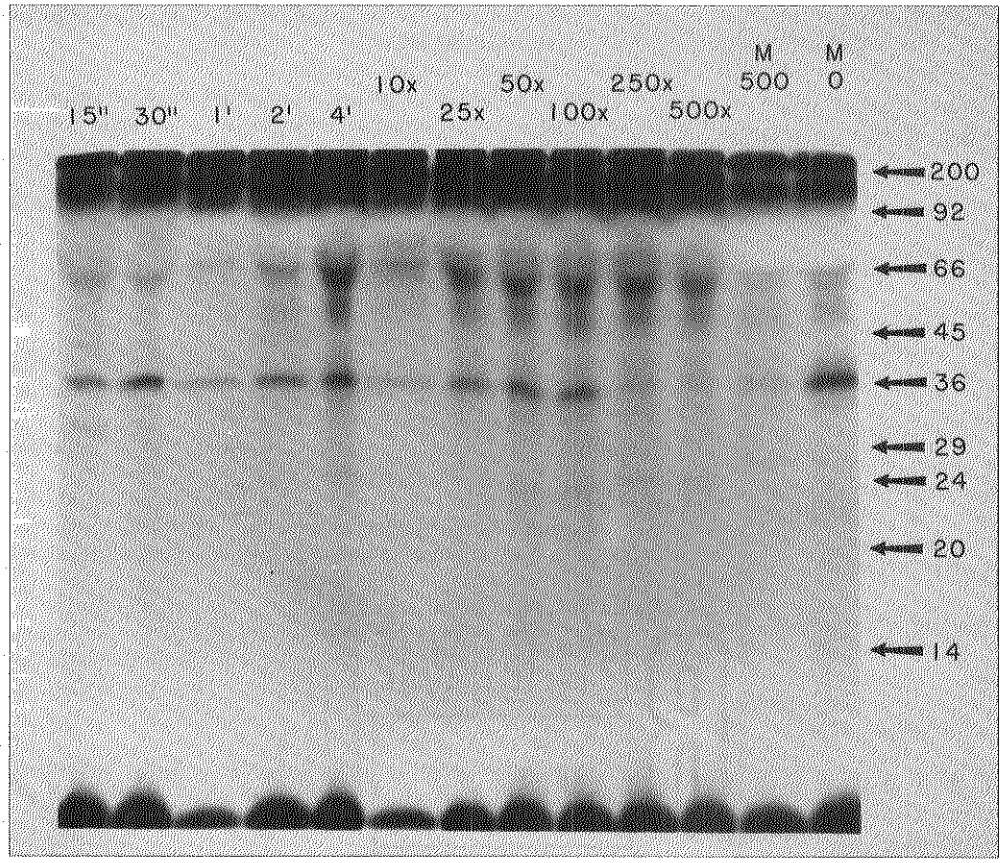

Figure 4
Comparison of photolysis times (15 s, 30 s, 1 min, 2 min, and 4 min), and the quantity (fold excess) of unlabeled methyl farnesoate (MF) used to displace the labeling of female shrimp hemolymph with [^{3}H]-farnesyl diazomethyl ketone (photolysis time was 4 min). The band at ca. 36,000 daltons appears to be specifically labeled. The lanes marked M500 and M0 represent hemolymph from male shrimp labeled with [^{3}H]-FDK with and without a 500-fold excess of unlabeled MF, respectively. Molecular weight markers ($\times 10^{-3}$) are given on the right of the autoradiograph. No labeling is observed at zero photolysis time.

the effects of increasing the length of time of U.V. irradiation is demonstrated. A high degree of attachment of [^{3}H]-FDK to the hemolymph binding protein is observed after only 15 sec (Fig. 4). The 36,000 dalton protein appears to be present in both female and male shrimp (Fig. 4).

We have also recently utilized [^{3}H]-FDK to characterize an analogous binding protein for MF in the hemolymph of adult female American lobsters (Prestwich et al. 1990). The lobster MF binding protein has an approximate molecular weight of 42,000.

Although a definitive hormonal role for MF has not yet been established, these data suggest that MF has a specific binding protein that may prevent its rapid degradation and may facilitate its cellular action. The role of the crustacean MF binding protein may be analogous to the insect juvenile hormone binding protein.

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