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Accumulation of Ovarian Ecdysteroids in Synchronization with Gonadal Development in the Giant Freshwater Prawn, *Macrobrachium rosenbergii*

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ABSTRACT—Changes in ovarian ecdysteroids in association with molting and gonadal developemnt were investigated by high performance liquid chromatography and radioimmunoassay in *Macrobrachium rosenbergii*. The reproductive molt cycle was synchronized with vitellogenesis, during which gonadosomatic index (GSI) increased from basal values of 0.41 to 8.73 immediately prior to molting. Throughout the common molt cycle, oocytes remained in a previtellogenic state and increases in GSI were not observed. During stages C_0-D_0, ovarian ecdysteroid concentrations were approximately 1–2 ng/g tissue. At premolt stage D_3, concentrations during the reproductive molt cycle (22.9 ng/g) reached values 2-fold greater than those during the common molt cycle (11.4 ng/g). Following reproductive molting, peak ecdysteroid concentrations and high GSI values were maintained until spawning. This indicates that ecdysteroids were accumulated in maturing ovaries in conjunction with the uptake of yolk substances. In contrast, following common molting, ecdysteroid concentrations returned immediately to basal levels of 1.0 ng/g, suggesting that ecdysteroids detected in ovaries during the common molt cycle were due to the presence of circulating hemolymph ecdysteroids. During both molt cycles, major ecdysteroid groups were 20-hydroxyecdysone and immunoreactive high-polarity products, with lesser amounts of ecdysone, inokosterone, and immunoreactive low-polarity products.

INTRODUCTION

Work in insect endocrinology in the 1950's firmly established the identity of 20-hydroxyecdysone as the molting hormone. However, with findings that ecdysteroids are present in adult insects (in which molting has ceased) and are generally confined to the ovaries prior to egg-laying [1], a role for ecdysteroids in reproduction became an area of focus [2]. In Crustacea, in which a number of species continue to molt in synchrony with ovulation and spawning throughout reproductive adulthood, ecdysteroid hormones have long been considered to play an additional simultaneous role in the control of ovarian maturation [3, 4].

In insects, ecdysteroids are in general synthesized in the follicle cell epithelium [2, 5, 6] and accumulated in the ovarian follicles [7]. Evidence exists that ecdysteroids trigger meiotic reinitiation in the locust *Locusta migratoria* [8, 9], the cricket, *Gryllus bimaculatus* [10], the cockroach, *Periplaneta americana* [10], and the shrimp, *Palaemon serratus* [11, 12] and stimulate vitellogenesis in the fleshfly, *Sarcophaga bullata* [13], and the isopod crustacean, *Porcellio dilatatus* [14]. In *P. serratus*, Lanot and Clédon [15] have revealed that the process of oocyte development is closely linked with the molt cycle, with germinal vesicle breakdown (GVBD) occurring at specific molt stages; this is correlated to increases in ovarian ecdysteroids, and is followed by exuviation [12, 15].

We have previously investigated ecdysteroid levels during embryogenesis in another palaemonid species, *Macrobrachium rosenbergii*, revealing that ecdysteroids are present in newly-laid eggs and post-vitellogenic ovaries [16]. Thus, we considered the possibility that in this species as well, oocyte development progresses with the molt cycle in association with the accumulation of ecdysteroids in ovaries, prior to oviposition. Ecdysteroid

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determinations and histological studies were undertaken on *M. rosenbergii* at every stage of both the reproductive molt cycle (mating and spawning following ecdysis), and the common molt cycle (ecdysis only). As ovarian ecdysteroid levels have also been investigated in the crabs, *Acanthonyx lunulatus* [17], and *Carcinus maenas* [18, 19], the amphipod, *Orchestia gammarellus* [20], and in *P. serratus* [15], the results of the present investigation are compared to previous reports.

MATERIALS AND METHODS

Sampling

Macrobrachium rosenbergii (body weight 12–36 g) were obtained from a commercial prawn culture farm in Osaka, Japan, and were maintained at 28°C (molt cycle approximately 28 days). Prior to sampling of ovaries, animals were staged according to molt stage criterion developed by Han [21] for application to *Macrobrachium nipponese*. Individuals were sacrificed at each molt stage, corresponding to both the reproductive molt cycle (visible development of the ovaries; enlarged and orange in appearance as viewed through the carapace) and common molt cycle (no visible ovarian development); body weight was determined, ovaries were quickly excised, and gonadosomatic index (GSI = gonad weight × 100/body weight) was determined. A small portion of ovarian tissue was taken for histological investigations, and the remaining tissue was immediately frozen at −80°C and stored at −30°C until further analysis.

Histology

Histological studies were employed to confirm the extent of vitellogenesis in ovaries as a function of molt stage. Ovarian tissue was fixed for 24–48 hrs in Bouin's solution and subsequently dehydrated through several steps of increasing alcohol concentration. Tissues were then embedded in paraffin, sectioned to 5–7 μm, and stained with hematoxylin-eosin. Ovarian maturational stages were classified in reference to those of Fauvel [22] for *M. rosenbergii* and those of Han [21] for *M. nipponese*.

Ecdysteroid extraction and radioimmunoassay (RIA)

Extraction of ovarian tissue was performed as in the extraction of egg ecdysteroid [16], i.e., homogenization in methanol, and partitioning between chloroform/distilled water, and n-hexane/distilled water. For mature ovaries, 200–300 mg of tissue was utilized per individual. For immature ovaries, due to size and expected low ecdysteroid levels, same-stage samples (5–7 individuals per stage) were combined (amounting to as much as 400 mg), and subsequently extracted. All extracts (in water) were evaporated under reduced pressure, and the resultant residue was dissolved in Gel-PBS (0.02 M Phosphate buffer, pH 7.5, containing 0.1% gelatin, 0.14 M NaCl, and 0.1% sodium azide) for use in RIA.

RIA was performed as in Okumura *et al.* [23] and in Wilder *et al.* [16]. Antiserum (raised against the 2-, 3- and 22-monohemisuccinates of 20-hydroxyecdysone conjugated to human serum albumin) was kindly provided by Dr. Masao Nagata, The University of Tokyo. Briefly, samples (as above), or 20-hydroxyecdysone standards (2-fold serial dilution, 78–40000 pg/ml) in Gel-PBS, were taken in aliquots, and incubated with rabbit anti-20-hydroxyecdysone serum. This was followed by addition of tritium-labeled ecdysone (7000 cpm; New England Nuclear, Ltd.) and a second incubation of 24 hrs. Next, goat anti-rabbit IgG serum was added to aliquots, and then incubated for 24 hrs. Lastly, aliquots were centrifuged (3200 rpm, 30 min, 4°C), after which supernatant was discarded by aspiration. Upon addition of Liquiflour scintillator/Triton X-100 (2:1) to aliquots, ovarian ecdysteroid concentration as 20-hydroxyecdysone immunoreactive equivalents was determined.

High performance liquid chromatography (HPLC)

The total ecdysteroid profile for ovarian ecdysteroids corresponding to both molt cycles was obtained according to RIA as above; subsequently, individual extracts in Gel-PBS corresponding to the reproductive molt cycle were pooled stage-wise (5–6 per stage), and prepared for HPLC as described previously [16]. Low ecdysteroid concen-

trations and small GSI values of common-molt cycle ovaries did not permit HPLC analysis of all stages. The ecdysteroid extract of ovaries of stage D_3 only of the common molt cycle was prepared for and subjected to HPLC as a means of comparison to the ecdysteroids of the reproductive molt cycle.

Ecdysteroids were separated by reversed-phase HPLC (ODS-80TM, 4.6×250 mm, Tosoh Co., Ltd.) on two solvent phases consisting of methanol/acetonitrile/water. Solvent A (10:15:75) was run for 23 min, switching to a linear gradient reaching Solvent B (55:5:45) after 32 additional minutes. Fractions were collected every 30 sec, at a flow rate of 1.0 ml/min. Fractions were subsequently dried under reduced pressure, dissolved in Gel-PBS and subjected to RIA analysis.

Additionally, early-eluting immunoreactive fractions (high-polarity products; see Results) were further characterized for stage D_3 of the reproductive molt cycle by additional reversed-phase separation (on ODS-80TM column as above). Gradient elution (8%–40% CH_3CN in 20 mM Tris/$HClO_4$ buffer, pH 7.5 in 60 min) was performed as in Lafont *et al.* [24]. Fractions were collected at 30 sec intervals at a flow rate of 0.8 ml/min and subjected to RIA analysis as above. Reference compounds [^{3}H]-20-hydroxyecdysonoic acid, [^{3}H]-ecdysonoic acid, and [^{3}H]-20,26-dihydroxyecdysone were the generous gifts of Dr. René Lafont, Ecole Normale Supérieure.

Statistics

Statistical analysis was done by the Kruskal-Wallis test followed by Dunn's method.

RESULTS

Examination of molt stage and extent of ovarian development

Changes in GSI are shown in Fig. 1. The GSI during the common molt cycle remained fairly constant (0.37 ± 0.04 to 0.53 ± 0.10). During this entire cycle, oocytes remained in a previtellogenic stage, as described by Han [21]. In such oocytes, the cytoplasm was basophilic, and the nucleoli were clearly visible within the nucleus. Histological observation revealed that a disparity in the stage of oocyte development between the reproductive and common molt cycle was first apparent at molt stage C_1; the GSI corresponding to the reproductive molt cycle (3.14 ± 0.63) began to depart from that of the common molt cycle (0.48 ± 0.03) at this stage. Ovaries of individuals at stage C_1 of the reproductive molt cycle had progressed to the latter stages of secondary vitellogenesis, in which the oocytes were completely filled with yolk globules. However, a few individuals exhibited ovaries which had just begun secondary vitellogenesis, in that yolk globules were seen only around the inside of the oocyte membrane.

From stage C_1 to D_3 of the reproductive molt cycle, further maturation of the oocytes was observed, with the nucleus becoming condensed in appearance, and the pre-molting GSI reaching 8.73 ± 0.82 (significantly higher than that of stage C_1 at $p<0.01$). In mature oocytes at molt stage

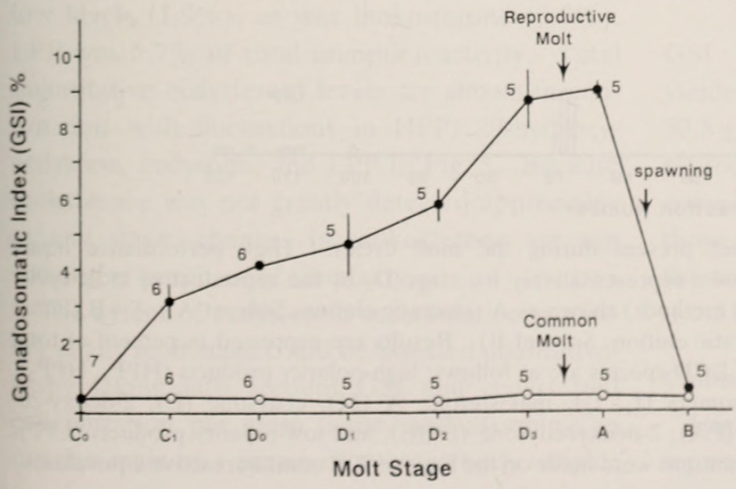

FIG. 1. Changes in gonadosomatic index during the reproductive and common molt cycles. Reproductive molt cycle (●—●); common molt cycle (○—○). Values expressed as mean±SEM. Molt type indistinguishable at stage C_0. Reproductive molt cycle post-molt stage B histologically distinguishable from common post-molt stage B by presence of some unspawned eggs. Number of individuals sampled is indicated in the figure.

D_3, while the nucleus stained basophilic and remained in its central location, the nucleoli had become no longer visible. Following molting, GSI in stage A was maintained at 9.00 ± 0.36. GVBD had apparently occurred, as chromosomes in metaphase were observed at the oocyte periphery. This was immediately followed by spawning, after which GSI values returned to nearly basal levels of 0.56 ± 0.06 in post-reproductive molt stage B. However, histological observation revealed the presence of some unspawned eggs.

Ovarian ecdysteroid species present during the molt cycle

Ecdysteroid extracts from stages D_0, D_1, D_2, D_3 and A of the reproductive molt cycle and stage D_3 of the common molt cycle were separated on two solvent phases consisting of methanol/acetonitrile/water and analysed by RIA. The elution pattern corresponding to stage D_3 of the reproductive molt cycle is shown in Fig. 2 for representative purposes. Detected ecdysteroids were 20-hydroxyecdysone, ecdysone, and inokosterone, as confirmed by identical retention times with authentic standards. Additionally, high-polarity products (HPP), and low-polarity products (LPP) eluting prior to 20-hydroxyecdysone and subsequent to ecdysone, were also present. Regarding the nature of LPP, immunoreactive material exhibiting retention times similar to those of ponasterone A and 2-deoxyecdysone was detected, but was not further characterized. HPP was detected at all stages examined, eluting as a main peak at a retention time of 5 min, and a minor peak at a retention time of 10 min. The nature of HPP has been further examined; these results are provided later in this section. Immunoreactive material eluting at retention times corresponding to those of makisterone A, or 2-deoxy-20-hydroxyecdysone, was not detected.

Ecdysteroid levels during the molt cycles: quantitative and qualitative changes

Quantitative changes in total ecdysteroid con-

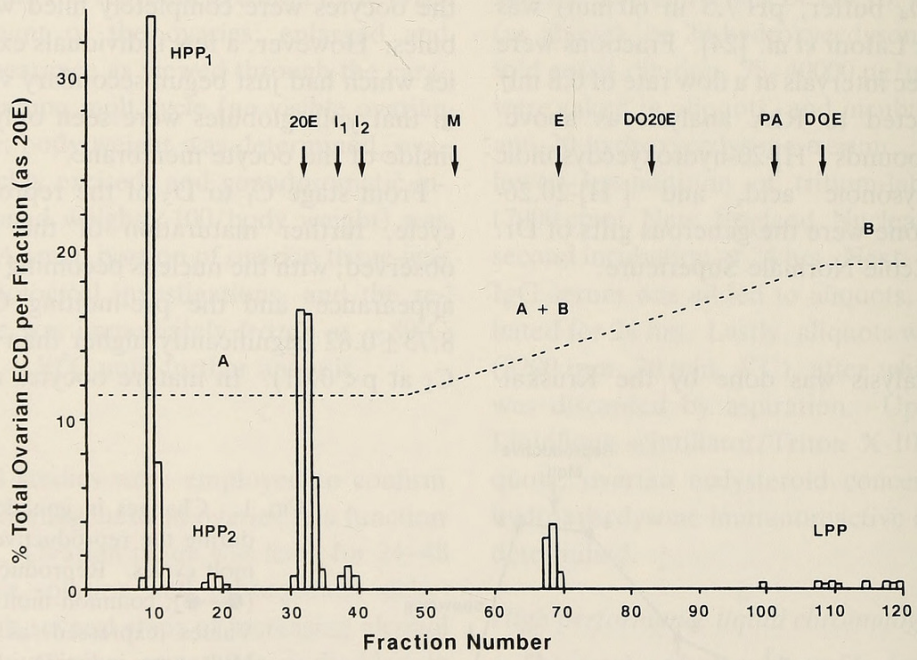

Fig. 2. Detection of ecdysteroid (ECD) species present during the molt cycles. High performance liquid chromatography (HPLC) elution pattern shown representatively for stage D_3 of the reproductive molt cycle. HPLC solvent system (refer to Materials and methods) shown as A (isocratic elution, Solvent A); A+B (linear gradient from Solvent A to B), and B (isocratic elution, Solvent B). Results are expressed as percent of total ovarian ECD per fraction. Abbreviations of ECD species are as follows: high-polarity products (HPP_1, HPP_2), 20-hydroxyecdysone (20E), inokosterone isomers (I_1, I_2), makisterone A (M), ecdysone (E), 2-deoxy-20-hydroxyecdysone (DO20E), ponasterone A (PA), 2-deoxyecdysone (DOE), and low-polarity products (LPP). These and all subsequent ecdysteroid determinations were made on the basis of 20E immunoreactive equivalents.

centration per gram ovarian tissue was determined by RIA for both molt cycles. During the period between stages C_0 and D_0, ecdysteroid levels were similar in both the reproductive and common molt cycles, decreasing slightly from about 2 ng/g to 1 ng/g. During the reproductive molt cycle, from stage D_1 (3.1 ± 0.6 ng/g), ovarian ecdysteroid concentrations began to rise, reaching peak levels (significant at $p < 0.01$) in stage D_3 (22.9 ± 2.2 ng/g), which were sustained after molting through stage A (22.9 ± 2.9). Following spawning in stage B, concentrations fell to values one-third those of stage A; the incomplete return to basal levels was attributed to the presence of unspawned eggs in the ovaries as revealed by histological observation. Although ecdysteroid concentrations were also observed to rise during the common molt cycle, values attained only half those of the reproductive molt cycle. From the D_1 stage (2.7 ng/g) to the D_3 (11.4 ng/g), a peak was reached, but in contrast to the reproductive molt cycle, these concentrations returned completely to basal levels following molting in stage A.

During the reproductive molt cycle, the principal ecdysteroids were HPP and 20-hydroxyecdysone, present in nearly equal amounts, increasing in concentration from stage D_1. Also during the reproductive molt cycle, ecdysone reached relative levels of 10.0% and 7.8% at stages D_2 and D_3. Low polarity products were detected at stages D_2 and D_3 (9.0% and 3.1%, respectively). Inokosterone was detected slightly at most stages (1.8–4.0%). In common molt stage D_3 ovaries, 20-hydroxyecdysone was somewhat prominent over HPP. Ecdysone was detected at low levels (1.9%), as was inokosterone (2.7%). LPP was 5.7% of total immunoreactivity. Total quantitative ecdysteroid levels are shown in conjunction with fluctuations in HPP, 20-hydroxyecdysone, ecdysone, and LPP in Fig. 3. Because inoksterone was not greatly detected appreciably at any stage, changes in inokosterone are not shown for purposes of simplicity.

The extent of ecdysteroid accumulation per ovary at any molt stage could be assessed qualitatively by taking into account GSI and ecdysteroid concentration per gram tissue, and assuming an ovarian mass of a prawn of average body weight:

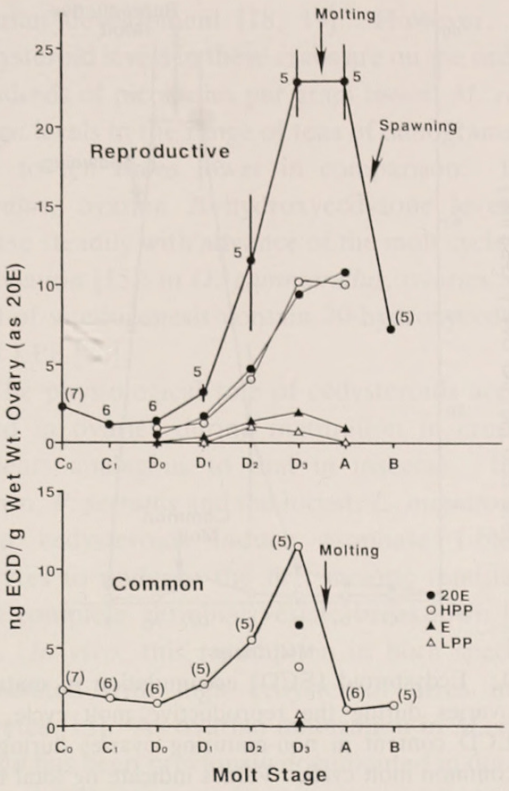

FIG. 3. Changes in total ovarian ecdysteroid (ECD) concentrations and fluctuations in high-polarity products (HPP), 20-hydroxyecdysone (20E), ecdysone (E), and low-polarity products (LPP) during the molt cycles. Fluctuations in inokosterone are not shown. Values indicate ng ECD per g wet weight ovary (total ECD concentrations during the reproductive molt cycle expressed as mean ± SEM). Heavy lines indicate total ECD concentrations during the reproductive molt cycle (●—●) or common molt cycle (○—○). Thin lines or symbols only indicate 20E (●—●), HPP (○—○), E (▲—▲), or LPP (△—△). Numbers indicate number of samples employed in determination of total ECD concentrations; parentheses indicate stages for which samples were combined prior to extraction.

GSI (%) × ECD (ng/g) × 25 g (Fig. 4). This yielded values of ecdysteroid content per ovary at 50.8 ± 7.5 ng and 51.1 ± 6.0 ng for ovaries of the reproductive molt cycle stage D_3 and A (significant compared to stage C_1 at $p < 0.01$); in contrast, those values corresponding to stages D_3 and A of the common molt cycle were estimated at only 1.5 ± 0.3 ng and 0.12 ± 0.02 ng.

Characterization of high polarity products (HPP)

High-polarity immunoreactive fractions were separated on an HPLC solvent system employing a

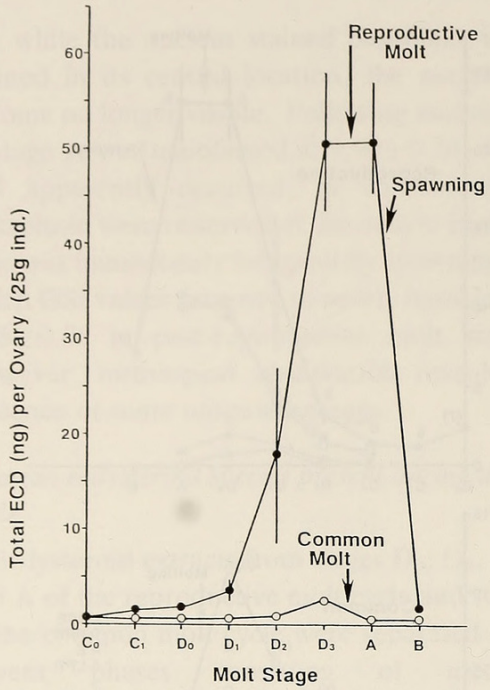

FIG. 4. Ecdysteroid (ECD) accumulation in maturing ovaries during the reproductive molt cycle, and ECD content in non-maturing ovaries during the common molt cycle. Values indicate ng total ECD per ovary per 25 g individual (total ECD per ovary during the reproductive molt cycle expressed as mean ± SEM). Reproductive molt cycle (●—●); common molt cycle (○—○).

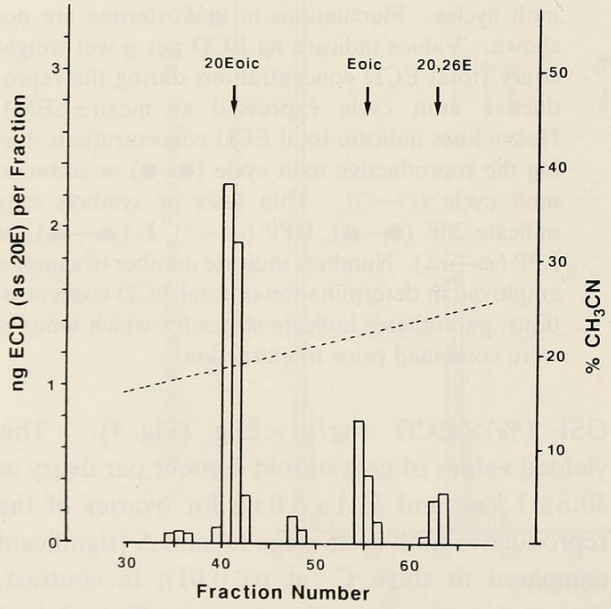

FIG. 5. Tentative identification of high-polarity products (HPP). Results are expressed as ng ecdysteroid (ECD) per fraction. Abbreviations are as follows: 20-hydroxyecdysononic acid (20Eoic), ecdysonoic acid (Eoic), and 20,26-dihydroxyecdysone (20, 26E). The HPLC elution pattern shown was obtained for HPP from ovarian tissue from stage D_3 of the reproductive molt cycle.

linear gradient of acetonitrile in Tris/$HClO_4$ buffer. Three major peaks of immunoreactivity were detected; these exhibited retention times identical to those of 20-hydroxyecdysonoic acid, ecdysonoic acid, and 20,26-dihydroxyecdysone (Fig. 5). Two minor peaks eluting prior to 20-hydroxyecdysonoic acid and between 20-hydroxyecdysonoic acid and ecdysonoic acid were also detected; these were not further characterized. The three peaks tentatively identified as 20-hydroxyecdysonoic acid, ecdysonoic acid, and 20,26-dihydroxyecdysone constituted approximately 65%, 20% and 10% of the total immunoreactivity of HPP.

DISCUSSION

In this investigation, ecdysteroids were present in ovaries at low concentrations during the early stages of the molt cycle of *M. rosenbergii.* However, during the reproductive molt cycle, ovarian ecdysteroid concentrations were seen to reach levels twice that of the common molt cycle and were sustained even after molting in stage A. In contrast, by stage A of the common molt cycle, ecdysteroid concentrations had dropped rapidly to basal levels with the completion of molting. This indicates that ecdysteroids were accumulated in maturing ovaries to the extent of over thirty-fold greater than that of ecdysteroid content in ovaries of any stage of the common molt cycle.

A premolt ecdysteroid peak on the order of tens of nanograms per milliliter has been demonstrated in *M. rosenbergii,* by Meusy and Payen [4]. In this investigation, changes in total ovarian ecdysteroid levels during the common molt cycle paralleled those normally observed in the hemolymph of adult crustacea. These results suggest that ecdysteroids detected in ovaries during the common molt cycle were simply present as the result of hemolymph flow-through. Regarding the physiological role of ovarian ecdysteroid accumulation during gonadal maturation, a possible function in early embryonic events [16] and involvement in GVBD are speculated.

According to HPLC-RIA analysis of *M. rosenbergii* ovarian ecdysteroids, 20-hydroxyecdysone, ecdysone, and inokosterone, as well as high (HPP) and low (LPP) polarity immunoreactive substances

were detected during both molt cycles. HPP has been determined to consist mostly of 20-hydroxyecdysonoic acid, followed by ecdysonic acid and lesser amounts of 20,26-dihydroxyecdysone. LPP could not be positively identified, but possibly contained ponastarone A and 2-deoxyecdysone. Regarding relative amounts of ecdysteroid groups, HPP and 20-hydroxyecdysone predominated, with less significant amounts of ecdysone, inokosterone, and LPP. 20-Hydroxyecdysone is generally thought to be the active ecdysteroid in insects and crustaceans. However, other ecdysteroid species have also been determined to have roles in molting and reproduction, for example, ponasterone A in crab [25]. Related ecdysteroids are considered to have complimentary roles in molting and reproduction [26].

The 26-oic acids and 20,26-dihydroxyecdysone have been reported in crustacea to a very limited extent [27, 28]. Cross-reactivities to the antisera employed in this study for 20-hydroxyecdysone, ecdysone, inokosterone, ponasterone A, and 2-deoxyecdsone (100%, 93%, 127–128%, 130%, 4.9%) have been previously reported in Okumura *et al.* [23]; however, cross-reactivities are unknown for these HPP compounds. The physiological significance of the presence of these compounds in the ovaries of *M. rosenbergii* can not yet be speculated upon. In insects, 26-hydroxylation of 20-hydroxyecdysone to 20,26-dihydroxyecdysone, and subsequent conversion to 20-hydroxy-ecdysonoic acid, or likewise conversion of ecdysone to ecdysonoic acid, has been reported as a metabolic pathway of active ecdysteroids [24, 29, 30]. On the other hand, ecdysteroid conjugates have been extensively reported in the ovaries of insects [31–33]; these substances serve as precursors to or storage molecules of active ecdysteroids.

Although relative composition of ecdysteroid species present in ovaries differs according to species, *M. rosenbergii* exhibited similarities to other crustacea in that ecdysteroid titres were high at particular stages of reproductive importance. In *A. lunulatus*, ecdysteroid concentrations are high at the beginning of vitellogenesis and at the end of oocyte maturation [17], and in *C. maenas*, ecdysteroid levels increase 4-fold, towards the end of ovarian development [18, 19]. However, peak ecdysteroid levels in these crabs are on the order of hundreds of picomoles per gram tissue; *M. rosenbergii* levels in the range of tens of nanograms, are five to ten times lower in comparison. In *P. serratus*, ovarian 20-hydroxyecdysone levels increase steadily with advance of the molt cycle until exuviation [15]; in *O. gammarellus*, ovaries at the end of vitellogenesis contain 20-hydroxyecdysone and LPP [20].

The physiological role of ecdysteroids accumulated in ovaries during maturation in crustacea appears analagous to that in insectae. In the prawn, *P. serratus* and the locust, *L. migratoria, in vitro* ecdysteroids induce prophase I-blocked oocytes to undergo the first meiotic reinitiation, and complete germinal vesicle breakdown [8, 9, 11]. *In vivo*, this resumption in both species is associated with high ecdysteroid titres in the oocytes [15]. As ovarian maturation in *M. rosenbergii* has been previously documented in detail by Fauvel [22], our examination of ovarian development with molt stage has suggested that during the reproductive cycle of *M. rosenbergii*, nuclear sequences progress similarly as in *P. serratus* [12]. Further investigation is needed in *M. rosenbergii* regarding ovarian ecdysteroids, their involvement in GVBD, and their site of production (whether secreted by the Y-organ or synthesized by the follicle cells).

Through this investigation, it has been shown that ecdysteroids are accumulated in the ovary during vitellognesis in the prawn, *M. rosenbergii*. The advancement of vitellogenesis is in synchrony with the reproductive molt cycle, culminating in high ecdysteroid titres, GVBD, molting, mating, and spawning. However, in diecdysic crustacean species such as *M. rosenbergii*, many questions yet remain concerning the endocrinological mechanisms which ensure the synchronization of vitellogenesis and the molt cycle.

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dihydroxyecdysone. We also thank Dr. Nobuhiro Fusetani, the University of Tokyo, for helpful discussion. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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